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INVENTARIO

**Industria y Comercio**
SUPERINTENDENCIA

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# SOLICITUD
# PATENTE DE INVENCIÓN

21. EXPEDIENTE No. 08-137618

54. TÍTULO **"INHIBIDORES DE ALDH-2 EN EL TRATAMIENTO DE LA ADICCIÓN"**

51. CLASIFICACIÓN INTERNACIONAL **C07D 311/36**

71. SOLICITANTE **CV THERAPEUTICS, INC.**

DOMICILIO : PALO ALTO, ESTADO DE CALIFORNIA, ESTADOS UNIDOS DE AMÉRICA.

74. REPRESENTANTE LEGAL ALICIA LLOREDA RICAURTE
T.P. 53.215

22. BOGOTÁ, D.C. 29 DIC. 2008

(FORMA P 10)

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Versión 2-2009

# PETITORIO

Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 08-137618- -00000-0000
Fecha: 2008-12-29 16:42:37 Dep: 2020 NUECREACION
Tra: 11 PATENTEIYII Eve: 379 FASENACIONALII
Act: 411 PRESENTACION Folios: 498

## FORMULARIO UNICO DE SOLICITUD DE PATENTE
## PCT
## ENTRADA EN FASE NACIONAL

**① SOLICITUD DE:**

☒ Patente de Invención ☐ Patente de Modelo de Utilidad

☐ Capítulo I. ☒ Capítulo II.

| | | |
|---|---|---|
| **② SOLICITANTE (71)** | Nombre: **CV THERAPEUTICS, INC.** (Ver folio 504 Nuevo solicitante)<br>Dirección: 3172 Porter Drive, Palo Alto, CA 94304, Estados Unidos de América.<br>Nacionalidad o Domicilio: Palo Alto, Estado de California, Estados Unidos de América.<br>Lugar de Constitución:<br>Teléfono: 3264270 Fax: 6069701<br>E-mail: jllcco@lloredacamacho.com | **IDENTIFICACION**<br>C.C. ☐ NIT ☐<br>C.E. ☐ Otro ☐<br>Cual _____<br>Número _____ |
| **③ REPRESENTANTE O APODERADO** | Nombre: **ALICIA LLOREDA RICAURTE**<br>Dirección: Calle 72 No. 5-83 Piso 5º, Bogotá, Colombia<br>Teléfono: 3264270 Fax: 6069701<br>E-mail: jllcco@lloredacamacho.com | C.C. ☒ NIT ☐<br>C.E. ☐ Otro ☐<br>Cual _____<br>Número 39.690.713 TP 53.215 |
| **④ INVENTOR(ES) (72)** | Nombre: ZABLOCKI, Jeff; ABELMAN, Matthew; ORGAN, Michael; DIAMOND, Ivan; AROLFO, Maria, Pia; YAO, Lina; FAN, Peidong; ELZEIN, Elfatih; KALLA, Rao; PERRY, Thao; KOBAYASHI, Tetsuya; LI, Xiaofen; KEUNG, Wing, Ming; BILOKIN, Yaroslav y TAO, Guoxin.<br>Dirección: 580 Sleeper Avenue, Mountain View, CA 94040; 1040 Grant Road, Mountain View, CA 94040. 291 Northshore Blvd. West, Burlington, Ontario L7T 1A6; 761 San Diego Road, Berkeley, CA 94707; 3024 Badger Drive, Pleasanton, CA 94566; 2106 26th Avenue, San Francisco CA 94116; 2375 24th Avenue, San Francisco CA 94116; 4644 Creekwood Drive, Fremont, CA 94555; 125 Connemara Way #9, Sunnyvale, CA 94087; 5109 Adair Way, San Jose, CA 95124; 730 East Evelyn Avenue #828, Sunnyvale, CA 94086; 161 Montelena Court, Mountain View, CA 94040; 2 Juniper Lane Wayland, MA 01778; 15 Dundonald Street, Apt. No. 1902 Toronto, Ontario M4Y7 1K4 y 319 Allston Street, Apartment 5 Brighton, MA 02135. (Ver folio 513 Nuevo inventor)<br>Nacionalidad o Domicilio: Estados Unidos de América y Canada. | |

**⑤ PCT (86)**

Solicitud Internacional No. PCT/US2007/074665 Fecha: 27/07/07

Publicación Internacional No. WO 2008/014497 A3 Fecha: 31/01/08

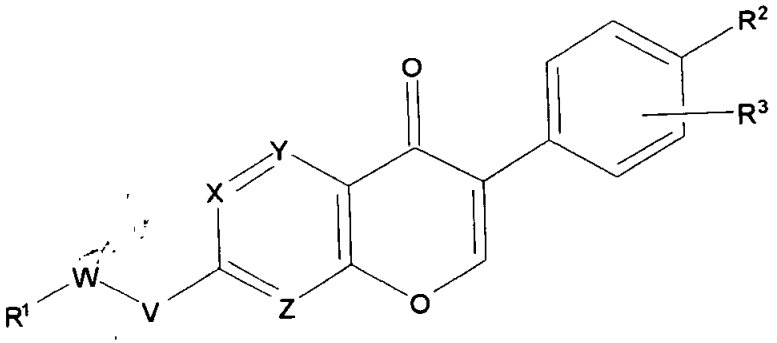
**⑥ Título (54) "INHIBIDORES DE ALDH-2 EN EL TRATAMIENTO DE LA ADICCIÓN"**

**⑦ Clasificación Internacional (51)** C07D 311/36

| **⑧ Prioridad** SI ☒ NO ☐ | (33) País de Origen | (32) Fecha | (31) Número de Solicitud |
|---|---|---|---|
| | US<br>US | 27/07/06<br>21/09/06 | 60/834,083<br>60/846,428 |

**⑨ Comprobante de Pago No.** 08-91,878 **Fecha:** Noviembre 25 de 2008

***** CONSIGNACION *****




Formula 1

$R^3$ es hidrógeno, ciano, amino opcionalmente sustituido, alquilo inferior, alcoxi inferior, o halo;

X, Y y Z se eligen de $-CR^7-$ y -N-, en el cual $R^7$ es hidrógeno, alquilo inferior, alcoxi inferior, o halo;

V es oxígeno, azufre, o -NH-; y

W es $-Q^1-T-Q^2-$, en donde

$Q^1$ es un enlace covalente o alquileno $C_{1-6}$ lineal o ramificado opcionalmente sustituido con hidroxi alcoxi inferior, amino, ciano, u =O;

$Q^2$ es alquileno $C_{1-6}$ lineal o ramificado opcionalmente sustituido con hidroxi, alcoxi inferior, amino ciano, u =O; y

T es un enlace covalente, -O-, o -NH-, o

T y $Q^1$ pueden juntos formar un enlace covalente,

$R^{20}$ y $R^{22}$ se seleccionan independientemente del grupo que consiste de hidrógeno, hidroxi, alquilo $C_{1-15}$ alquenilo $C_{2-15}$, alquinilo $C_{2-15}$, cicloalquilo, heterociliclo, arilo, bencilo, y heteroarilo,

en donde las mitades alquilo, alquenilo, alquinilo, cicloalquilo, heterociclilo, arilo, bencilo, y heteroarilo estár opcionalmente sustituidas con de 1 a 3 sustituyentes seleccionados independientemente de halo alquilo, mono o dialquilamino, alquilo o arilo o heteroaril amida, CN, O-alquilo $C_{1-6}$, $CF_3$, COOH, $OCF_3$, $B(OH)_2$, $Si(CH_3)_3$, arilo, y heteroarilo.

Industria y Comercio
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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**
**DIVISION DE NUEVAS CREACIONES**
**TARJETA ARCHIVO PROPIETARIOS**

PATENTE DE INVENCION ___ PCT _X_ MODELO DE UTILIDAD ___ DISEÑO INDUSTRIAL __

(21) N° de solicitud:
(22) Fecha de solicitud: 29-12-08
(51) Clasificación Internacional : C07D 311/36

(71)Solicitante(s) **CV THERAPEUTICS, INC.**

(72) Inventor(es) ZABLOCKI, Jeff; ABELMAN, Matthew; ORGAN, Michael; DIAMOND, Ivan; AROLFO, Maria, Pia; YAO, Lina; FAN, Peidong; ELZEIN, Elfatih; KALLA, Rao; PERRY, Thao; KOBAYASHI, Tetsuya; LI, Xiaofen; KEUNG, Wing, Ming; BILOKIN, Yaroslav y TAO, Guoxin.

(74) Apoderado: ALICIA LLOREDA RICAURTE

| (30) Prioridad | (31) ***No. Prioridad*** | 32) ***Fecha*** | (33) ***País*** |
|---|---|---|---|
| | **60/834,083** | **27-07-06** | **US** |
| | **60/846,428** | **21-09-06** | **US** |

(85) Fecha límite inicio fase nacional: 27-02-09
(86) Datos relativos a la presentación de la solicitud PCT
Fecha de presentación de la solicitud: **27-07-07**
No. De solicitud ***PCT/US2007/074665***

(87) Publicación internacional
Fecha: **31-01-08**
N° publicación: ***WO 2008/014497 A3***

(54) Titulo: **"INHIBIDORES DE ALDH-2 EN EL TRATAMIENTO DE LA ADICCIÓN"**

ıdustria y Comercio
ıPERINTENDENCIA

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**
**DIVISION DE NUEVAS CREACIONES**
**TARJETA ARCHIVO TEMATICO**

PATENTE DE INVENCION ___ PCT X MODELO DE UTILIDAD ___ DISEÑO INDUSTRIAL

) N° de solicitud: (22) Fecha de solicitud: 29-12-08
) Clasificación Internacional : C07D 311/36

) Solicitante(s) **CV THERAPEUTICS, INC.**

) Inventor(es) ZABLOCKI, Jeff; ABELMAN, Matthew; ORGAN, Michael; DIAMOND, Ivan; AROLFO, Maria, ; YAO, Lina; FAN, Peidong; ELZEIN, Elfatih; KALLA, Rao; PERRY, Thao; KOBAYASHI, Tetsuya; LI, Xiaofen; JNG, Wing, Ming; BILOKIN, Yaroslav y TAO, Guoxin.

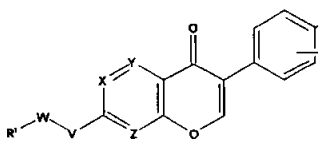
) Apoderado: ALICIA LLOREDA RICAURTE

) Prioridad

| (31) *No. Prioridad* | (32) *Fecha* | (33) *País* |
|---|---|---|
| **60/834,083** | **27-07-06** | **US** |
| **60/846,428** | **21-09-06** | **US** |

) Fecha límite inicio fase nacional: 27-02-09
) Datos relativos a la presentación de la solic. PCT
Fecha de presentación de la solicitud: 27-07-07
No. de solicitud ***PCT/US2007/074665***

(87) Publicación internacional:
Fecha: 31-01-08
No. Publicación : **WO 2008/014497 A3**

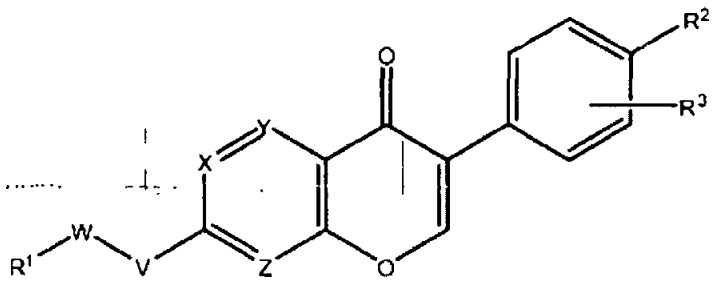


Formula I

) Titulo: **"INHIBIDORES DE ALDH-2 EN EL TRATAMIENTO DE LA ADICCIÓN"**

) Resumen:
ivindicación número 1:

Un compuesto de la Fórmula I:



Formula I

en donde:

$R^1$ es fenilo opcionalmente sustituido, heteroarilo opcionalmente sustituido, o heterociclilo opcionalmente sustituido;

$R^2$ es hidrógeno, hidroxi, halógeno, alcoxi inferior opcionalmente sustituido, alquilo inferior opcionalmente sustituido, ciano, heteroarilo opcionalmente sustituido, $C(O)OR^5$, $-C(O)R^5$, $-SO_2R^{15}$, $-B(OH)_2$, $-OP(O)(OR^5)_2$, $-C(NR^{20})NHR^{22}$, $-NHR^4$, o $C(O)NHR^5$, en el cual,

$R^4$ es hidrógeno, $-C(O)NHR^5$, o $-SO_2R^{15}$, o $-C(O)R^5$;

$R^5$ es hidrógeno, alquilo inferior opcionalmente sustituido;

$R^{15}$ es alquilo inferior opcionalmente sustituido o fenilo opcionalmente sustituido; o

$R^2$ es $-O-Q-R^6$, en el cual Q es un enlace covalente o alquileno inferior y $R^6$ es heteroarilo opcionalmente sustituido;

$R^3$ es hidrógeno, ciano, amino opcionalmente sustituido, alquilo inferior, alcoxi inferior, o halo;

X, Y y Z se eligen de $-CR^7-$ y -N-, en el cual $R^7$ es hidrógeno, alquilo inferior, alcoxi inferior, o halo;

V es oxígeno, azufre, o -NH-; y

W es $-Q^1-T-Q^2-$, en donde

$Q^1$ es un enlace covalente o alquileno $C_{1-6}$ lineal o ramificado opcionalmente sustituido con hidroxi, alcoxi inferior, amino, ciano, u =O;

$Q^2$ es alquileno $C_{1-6}$ lineal o ramificado opcionalmente sustituido con hidroxi, alcoxi inferior, amino, ciano, u =O; y

T es un enlace covalente, -O-, o -NH-, o

T y $Q^1$ pueden juntos formar un enlace covalente,

$R^{20}$ y $R^{22}$ se seleccionan independientemente del grupo que consiste de hidrógeno, hidroxi, alquilo $C_{1-15}$, alquenilo $C_{2-15}$, alquinilo $C_{2-15}$, cicloalquilo, heterociliclo, arilo, bencilo, y heteroarilo,

en donde las mitades alquilo, alquenilo, alquinilo, cicloalquilo, heterociclilo, arilo, bencilo, y heteroarilo están opcionalmente sustituidas con de 1 a 3 sustituyentes seleccionados independientemente de halo,

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference | FOR FURTHER ACTION | see Form PCT/ISA/220 as well as, where applicable, item 5 below. |
|---|---|---|
| 06-0223-WO | | |
| International application No. | International filing date *(day/month/year)* | (Earliest) Priority Date *(day/month/year)* |
| PCT/US2007/074665 | 27/07/2007 | 27/07/2006 |
| Applicant | | |
| CV THERAPEUTICS, INC. | | |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. [ ] **Certain claims were found unsearchable** (See Box No. II)
3. [ ] **Unity of invention is lacking** (see Box No III)
4. With regard to the **title**,
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority
6. With regard to the **drawings**,
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/074665

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D311/36 C07D413/10 C07D413/14 A61K31/352

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 2003/229065 A1 (LEVY STUART B [US] ET AL) 11 December 2003 (2003-12-11) page 118; compound MK | 1 |
| X | WO 2006/002422 A (CHIRON CORP [US]; VALIANTE NICHOLAS [US]; XU FENG [US]) 5 January 2006 (2006-01-05) page 112; example 6; table 2 | 1 |

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 December 2007 | 14/02/2008 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Jeanjean, Fabien |

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/074665

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| X | KINOSHITA T ET AL: "One-step conversion of flavanones into isoflavones: A new facile biomimetic synthesis of isoflavones" TETRAHEDRON LETTERS 1990 UNITED KINGDOM, vol. 31, no. 50, 1990, pages 7355-7356, XP002462448 ISSN: 0040-4039 isoflavone with 29% yield page 7355; table I | 1 |
| X | US 4 166 862 A (ANTUS SANDOR [HU] ET AL) 4 September 1979 (1979-09-04) column 9, line 1; example 7 | 1 |
| X | EP 0 031 885 A (MERCK PATENT GMBH [DE]) 15 July 1981 (1981-07-15) page 25; example 228 | 1 |
| X | DEWICK P M: "BIOSYNTHESIS OF PTEROCARPAN PHYTO ALEXINS IN TRIFOLIUM-PRATENSE" PHYTOCHEMISTRY (OXFORD), vol. 16, no. 1, 1977, pages 93-98, XP002462449 ISSN: 0031-9422 7-benzyloxy-4'-hydroxy-2'-methoxyisoflavon e page 96 | 1 |
| X | WO 2004/002470 A (ENDOWMENT FOR RES IN HUMAN BIO [US]) 8 January 2004 (2004-01-08) page 28; compound 18 page 42; compound 83 page 44; compound 89 page 46; compound 99 page 2 page 53; claim 1 | 1,8 |
| X | BARZ W ET AL: "The degradation of formononetin and daidzein in Cicer arietinum and Phaseolus aureus*1" PHYTOCHEMISTRY, vol. 9, no. 8, August 1970 (1970-08), pages 1735-1744, XP002462450 Oxford ISSN: 0031-9422 page 1736; compound V | 1,8 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

*Information on patent family members*

International application No
PCT/US2007/074665

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
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1. US 2003/0229065 A1
2. WO 2006/002422 A
3. XP002462448
4. US 4 166 862 A
5. EP 0 031 885 A
6. XP002462449
7. WO 2004/002470 A
8. XP002462450

US 20030229065A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0229065 A1**

**Levy et al.** (43) **Pub. Date: Dec. 11, 2003**

(54) **TRANSCRIPTION FACTOR MODULATING COMPOUNDS AND METHODS OF USE THEREOF**

(76) Inventors: **Stuart B. Levy**, Boston, MA (US); **Michael N. Alekshun**, Wakefield, MA (US); **Brent L. Podlogar**, Hamden, CT (US); **Kwasi Ohemeng**, Norwood, MA (US); **Atul K. Verma**, Arlington, MA (US); **Tadeusz Warchol**, Norborough, MA (US); **Beena Bhatia**, Arlington, MA (US)

Correspondence Address:
**LAHIVE & COCKFIELD**
**28 STATE STREET**
**BOSTON, MA 02109 (US)**

(21) Appl. No.: **10/139,591**

(22) Filed: **Aug. 14, 2002**

**Related U.S. Application Data**

(60) Provisional application No. 60/288,660, filed on May 4, 2001.

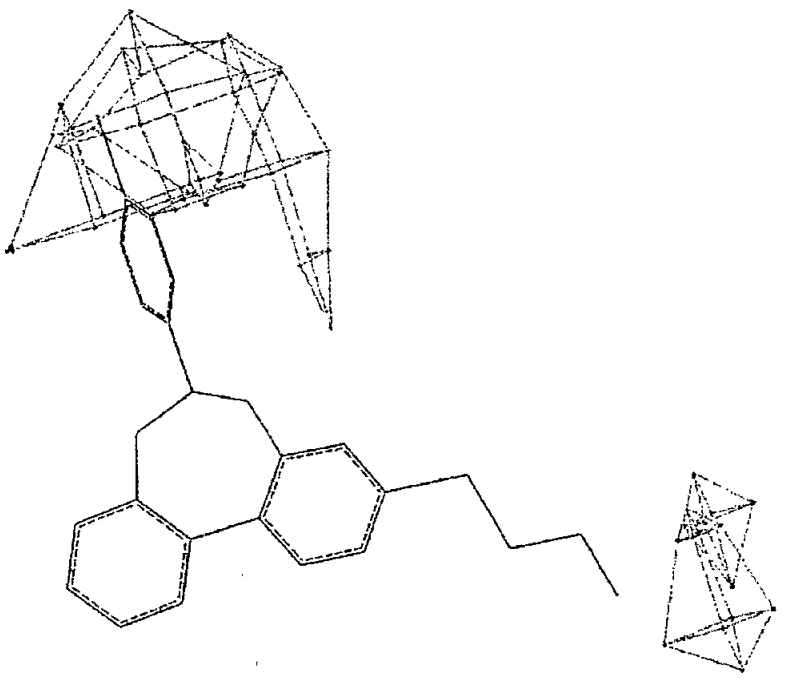
**Publication Classification**

(51) **Int. Cl.**[7] ..... **A61K 31/555**; A61K 31/505; A61K 31/4745; A61K 31/47; A61K 31/415; A61K 31/40; A61K 31/407

(52) **U.S. Cl.** ..... **514/185**; 514/256; 514/311; 514/303; 514/383; 514/381; 514/394; 514/410; 514/408

(57) **ABSTRACT**

Methods for identifying compound useful as anti-infectives that decrease resistance, virulence, or growth of microbes are provided. In one embodiment, the method comprises contacting a microbial cell comprising: 1) a selectable marker under the control of a transcription factor responsive element and 2) a transcription factor, with a compound under conditions which allow interaction of the compound with the microbial cell; and measuring the ability of the compound to affect the growth or survival of the microbial cell as an indication of whether the test compound modulates the activity of a transcription factor.



Multiple sequence alignment of AraC-XylS family members.

```
AARE_PROST/22-120       SEILVWIEGNLTNR.......LSLDDIAQHSGYTKWHLQRVFRKIVGMPLGEYIRRRRI
ADA_ECOLI/85-183        DKITHACRLLEQETp.......VTLEALADQVAMSPFHLHRLFKATTGMTPKAWQQAWRA
ADA_MYCTU/87-185        ARAMRLIADGTVDR........DGVSGLAAQLGYTIRQLERLLQAVVGAGPLALARAQRM
ADA_SALTY/94-183        ----------LEQEt......pVTLAFLAQAVAMSPFHLHRLFKASTGMTPKGWQQAWRA
ADAA_BACSU/102-200      DLITEYIDKNFTEK........LTLESLADICHGSPYHMHRTFKKIKGITLVEYIQQVRV
ADIY_ECOLI/149-246      DSVYQIIESDIHKD.....WNLSMVASCLCLSPSLLKKKLKSENT-SYSQIITCRM
AGGR_ECOLI/164-261      DKVRNTIEKDLSKR........WTLAIIADEFNVSEITIRKRLESEYI-TFNQILMQSRM
APPY_ECOLI/139-236      CKITGIISFNIERQ........WHLKDIAELIYTSESLIKKRLRDEGT-SFTEILRDTRM
ARAC_CITFR/180-279      RDACQYISDHLADSn.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAC_ECOLI/180-279      REACQYISDHLADSn.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAC_ERWCH/186-284      IEACQFITSNLAGE........LRIDEVARHVCLSPSRLAHLFREQVGINILRWREDQRV
ARAC_SALTY/180-279      RDACQYISDHLADSh.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAL_STRAT/202-300      ASALTFLHRDPAHS........WTVAELASAAAVSRSTLAARFKATVGQGPLEYLTRWRI
ARAL_STRLI/202-300      ATALTCLHRDPARS........WTVADLADTAAVSRSTLAARFKATVGQGPLEYLTRWRI
CAFR_YERPE/8-107        NSIIQYIEENLESKf.......INIDCLVLYSGFSRRYLQISFKEYVGMPIGTYIRVRRA
CELD_ECOLI/168-274      DDVPQWLKSTVEKMhdkeqfseSALENMVALSAKSQEYLTRATQRYYGKTPMQIINEIRI
CFAD_ECOLI/164-261      DKVRNVIEKDLSRK........WTLGIIADAFNVSEITIRKRLESENT-NFNQILMQLRM
CSVR_ECOLI/166-263      DKVRGVIEKDLSRK........WTLAIIADVFNVSEITIRKRLESEDT-NFNQILMQSRM
ENVY_ECOLI/149-246      DSVCRIIQSDIQHY........WNLRIVASSLCLSPSLLKKKLKNENT-SYSQIVTECRM
EUTR_ECOLI/243-344      SRAREYVLENMSEP........VTVLDLCNQLHVSRRTLQNAFHAILGIGPNAWLKRIRL
EUTR_SALTY/243-344      SRAREYVLENMSEP........LTVLDLCNQLHVSRRTLQNAFHAILGIGPNAWLKRIRL
EXSA_PSEAE/171-269      ERLQLFMEKHYLNE........WKLSDFSREFGMGLTTFKELFGSVYGVSPRAWISERRI
FAPR_ECOLI/154-251      ERIVTLLFSDLTRK........WKLSDIAEEMHISEISVRKRLEQECL-NFNQLILDVRM
FEAR_ECOLI/199-299      QKVVTLIDDNIREEi.......LRPEWIAGETGMSVRSLYRMFADKGL-VVAQYIRNRRL
GADX_ECO27/145-242      TRVCTVINNNIAHE........WTLARIASELLMSPSLLKKKLREEGT-SYSQLLTECRM
GADX_ECO57/145-242      TRVCTVINNNIAHE........WTLARIASELLMSPSLLKKKLREEET-SYSQLLTECRM
GADX_ECOLI/145-242      TRVCTVINNNIAHE........WTLARIASELLMSPSLLKKKLREEET-SYSQLLTECRM
GLXA_RHIME/223-321      LAVLEKMETAIERP........LDRTAMARLAGVSPRHLDRLFREHRGTGFLDTYREIRL
HRPB_RALSO/375-477      RRAYRYIIENIERSd.......LTTREVAAHINVTERALQLAFKSAVGMSPSSVIRRMRL
INVF_SALTY/112-210      YWLVGYLLAQSTSG........NTMRMLGEDYGVSYTHFRRLCSRALGGKAKSELRNWRM
LACR_STAXY/174-272      QHAVDFINTNYQKH........ITVEDVAKSVNITRSHLYKLFKKNLGCSPKEYLTYIRM
LCRF_YERPE/167-265      ERLQKFMEENYLQG........WKLSKFAREFGMGLTTFKELFGTVYGISPRAWISERRI
LUMQ_PHOLE/148-246      VLIDNYIEQHLQKK........ISVAELSSVAFLAQSQFYALFKSQMGITPHQYVLRKRL
MARA_ECOLI/14-112       HSILDWIEDNLESP........LSLEKVSERSGYSKWHLQRMFKKETGHSLGQYIRSRKM
MARA_SALTY/14-112       HSILDWIEDNLESP........LSLEKVSERSGYSKWHLQRMFKKETGHSLGQYIRSRKM
MELR_ECOLI/194-292      SQMLGFIAENYDQA........LTINDVAEHVKLNANYAMGIFQRVMQLTMKQYITAMRI
MMSR_PSEAE/201-299      DGLHAYMREHLHAR........LELERLAAFCNLSKFHFVSRYKAITGRTPIQHFLHLKI
MSMR_STRMU/176-274      NQVKKIIHSQYGSS........LRVNDIAKKLNLSRSYLYKIFRKSTNLSIKEYILQVRM
MXIE_SHIFL/99-199       YHLVLYLLRTIEKEk......eVRIKSLTEHYGVSEAYFRSLCRKALGAKVKEQLNTWRL
MXIE_SHISO/99-199       YHLVLYLLRTIEKEk......eVRIKSLTEHYGVSEAYFRSLCRKALGAKVKEQLNTWRL
ORUR_PSEAE/241-338      TRVRRLLLARPGDF........PDLEQAARELHTSGRSLRRHLSSLGT-TYQQVLDDVRK
PCHR_PSEAE/201-296      HAARDLLVGALQEP........PSLDTLASRVGMNPRKLTAGFRKVFGASVFGYLQEYRL
PERA_ECO27/168-265      DRVIKVIELDISKN........WKLGDVSSSMFMSDSCLRKQLNKENL-TFKKIMLDIKM
POCR_SALTY/195-293      KKALRYIDAHLSDD........LRLEDVASHVYLSPYYFSKLFKKYQGIGFNAWVNRQRM
PQRA_PROVU/7-107        NDILKWLETQLQRNe......gIKIDTIANKSGYSKWHLQRIFKDFKGCTLGEYVRKRRL
RAFR_PEDPE/176-274      NLAVSYLQENYSTG........CTIMDLCHYLNLSRSYLYTLFKTHANTSPQKLLTKLRL
RAMA_ENTCL/9-107        DTIVEWIDDNLHQP........LRIEDIARHAGYSKWHLQRLFLQYKGESLGRYIRERKL
RAMA_KLEPN/9-107        DTIVEWIDDNLHQP........LRIDDIARHAGYSKWHLQRLFLQYKGESLGRYIRERKL
RHAR_ECOLI/209-307      DKLITRLAASLKSP........FALDKFCDEASCSERVLRQQFRQQTGMTINQYLRQVRV
RHAR_SALTY/179-277      DKLITALANSLECP........FALDAFCQQEQCSERVLRQQFRAQTGMTINQYLRQVRI
RHAS_ECOLI/174-272      NLLLAWLEDHFADE........VNWDAVADQFSLSLRTLHRQLKQQTGLTPQRYLNRLRL
RHAS_SALTY/174-272      NQLMAWLEDHFAEE........VCWEAVAEQFSLSLRTLHRQLKQHTGLTPQRYLNRLRL
RHRA_RHIME/210-310      ASIKMRVEQNLANGs.......FSITDVAEAERITPRAIQKFFSREGT-TFSRYVLGRRL
RNS_ECOLI/164-261       DKVRNLIEKDLSRK........WTLGIIADAFNASEITIRKRLESENT-NFNQILMQLRM
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Figure 1A

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ROB_ECOLI/8-106          RDLLIWLEGHLDQP.....LSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRL
SOXS_ECOLI/7-105         QDLIAWIDEHIDQP.....LNIDVVAKKSGYSKWYLQRMFRTVTHQTLGDYIRQRRL
SOXS_SALTY/7-105         QTLIEWIDEHIDQP.....LNIDVVAKKSGYSKWYLQRMFRTVTHQTLGEYIRQRRL
TCPN_VIBCH/172-269       EKISCLVKSDITRN.....WRWADICGELRTNRMILKKELESRGV-KFRELINSIRI
TETD_ECOLI/31-129        KDVLLWIEHNLDQS.....LLLDDVANKAGYTKWYFQRLFKKVTGVTLASYIRARRL
THCR_RHOER/227-328       RLAVDYLEAHAQQP.....LTVAQVARNVGVSVRSLQVGFQNSLGTTPMRQLKIIRM
URER_ECOLI/171-268       QAITHLITQEPQKK.....WHLDDVAKALFTTPSTLRRHLNREGV-SFRQLLLDVRM
URER_PROMI/171-268       QAITHLITQDPQRK.....WHLEDVAKTLYTTPSTLRRHLSKEGV-SFCQLLLDVRI
VIRF_SHIDY/161-258       DQIRKIVEKNIEKR.....WRLSDISNNLNLSEIAVRKRLESEKL-TFQQILLDIRM
VIRF_YEREN/167-265       ERLQKFMEENYLQG.....WKLSKFAREFGMGLTTFKELFGTVYGISPRAWISERRI
VIRS_MYCTU/236-334       ERVVGLARRLLPTGq.....CSAEAIADQLDMHPRTLQRRLAAEGL-RCHDLIERERR
XYLR_ECOLI/288-386       IQAMHYIRNHACKG.....IKVDQVLDAVGISRSNLEKRFKEEVGETIHAMIHAEKL
XYLR_HAEIN/288-386       IQAMHYIRHRACHR.....IKVGQVLDHLETSRSNLEQRFKNEMNKTIHQVIHEEKI
XYLS_PSEPU/214-315       ERVVQFIEENLKRN.....ISLERLAELAMMSPRSLYNLFEKHAGTTPKNYIRNRKL
XYS1_PSEPU/214-315       ERVVQFIEENLKRN.....ISLERLAELAMMSPRSLYNLFEKHAGTTPKNYIRNRKL
XYS2_PSEPU/39-140        ERVVQFIEENVKRS.....ISLEQLAELALMSPRSLYTMFEKHTGTTPMNYIRNRKL
XYS3_PSEPU/214-315       ERVVQFIEDNLKQS.....ISLERLAELALMSPRSLYTLFEKHAGTTPKNYIRNRKL
XYS4_PSEPU/214-315       ERVVQFIEENLKRN.....ISLERLAELALMSPRSLYTLFEKHAGTTPKNYIRNRKL
Y4FK_RHISN/318-417       LKAEAFMRENLTNP.....VTIEDLAAAARCTPRALQRMFRTYRGGSPMSVLCNYRL
YA52_HAEIN/194-295       KRLNTALIAILQQPqn.....dWHIEQLAELATMSRANFIRIFQQHIGMSPGRFLTKVRL
YBBB_BACSU/166-264       EKTKHYIETHADTK.....ITLAQLSQMAGISAKHYSESFKKWTGQSVTEFITKTRI
YBCM_ECOLI/165-262       SRCYNLLLSEPGTK.....WTANKVARYLYISVSTLHRRLASEGV-SFQSILDDVRL
YCGK_ALTCA/67-163        QNAMLYIENNYFND.....INIDTVAFSVGVSRSYLVKQFKLATNKTINNRIIEVRI
YD95_MYCTU/242-343       RGITALVRSKLFRDsg....lfPTFTDVAGELDMHPRTLRRRLAEEGT-SFRALLGEARS
YDEO_ECOLI/137-233       GKVRNIVNMKPAHP.....WKLKDICDCLYISESLLKKKLKQEQT-TFSQILLDARM
YDIP_ECOLI/183-281       KDILFYLNNNYREK.....ITLEQLSKKFRASVSYICHEFTKEYRISPINYVIQRRM
YEAM_ECOLI/158-258       PKIRTMVEMMAKGPve.....wGALGQWAGFFAMSERNLARLIVKETGLSFRQWRQQLQL
YFIF_BACSU/192-289       TEVKLHIKDNLSQP.....LKLTDVASHFHISGRHLSRLFAAELGVSYSEFVQNEKI
YHIW_ECOLI/139-236       GKVERLISFDIAKR.....WYLRDIAERMYTSESLIKKKLQDENT-CFSKILLASRM
YIDL_ECOLI/197-295       EKLIATLHASLQQR.....WSVADMAATIPCSEAWLRRLFLRYTGKTPKEYYLDARL
YIJO_ECOLI/172-270       EAIRDYIDERYASA.....LTRESVAQAFYISPNYLSHLFQKTGAIGFNEYLNHTRL
YISR_BACSU/183-281       WEAARYLQEHYKEK.....TTIKDLSLALHYHQDYVSRCMQQVLGVTPAQYTNRVRM
YKGA_ECOLI/19-117        QQLLEWIECNLEHP.....ISIEDIAQKSGYSRRNIQLLFRNFMHVPLGEYIRKRRL
YKGD_ECOLI/177-278       PRLGAVIQQMLEMPgh.....aWTVESLASIAHMSRASFAQLFRDVSGTTPLAVLTKLRL
YMCR_STRLA/184-281       DPLLRAVVVSLEAG.....RSVTATADSVGLGARQLHRRSLAAFGYGPKTLARVLRM
YPDC_ECOLI/184-282       HSICNWVQDNYAQP.....LTRESVAQFFNITPNHLSKLFAQHGTMRFIEYVRWVRM
YQHC_ECOLI/213-311       SRVLKRIENKYTEN.....LSVEQLAAEANMSVSAFHHNFKSVTSTSPLQYLKNYRL

AARP_PROST/22-120        CEAAKELQTTNL...QVIDIALKYQFDSQQSFAKRFKAYLGISPSLYRLS
ADA_ECOLI/85-183         RRLRESLAKGE-...SVTTSILNAGFPDSSSYYRKADETLGMTAKQFRHG
ADA_MYCTU/87-185          QTARVLIETTNL...PFGDVAFAAGFSSIRQFNDTVRLACDGTPTALRAR
ADA_SALTY/94-183          RRLREALAKGE-...PITAAIYRAGFPDSSSYYRHADQTLGMTAKQFRKG
ADAA_BACSU/102-200       HAAKKYLIQTNK...AIGDIAICVGIANAPYFITLFKKKTGQTPARFRQM
ADIY_ECOLI/149-246       RYAVNELMMDGK...NISQVSQSCGYNSTSYFISVFKDFYGMTPLHYVSQ
AGGR_ECOLI/164-261       SKAALLLLDNSY...QISQISNMIGFSSTSYFIRLFVKHFGITPKQFLTY
APPY_ECOLI/139-236       RYAKKLITSNSY...SINVVAQKCGYNSTSYFICAFKDYYGVTPSHYFEK
ARAC_CITFR/180-279       SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAC_ECOLI/180-279       SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAC_ERWCH/186-284       IRAKLLLQTTQE...SIANIGRVVGYDDQLYFSRVFRKRVGVSPSDFRRR
ARAC_SALTY/180-279       SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAL_STRAT/202-300       ELTARQLREGSA...PLAAIAHSVGYGSESALSVAFKRVLGMNPGDYRKH
ARAL_STRLI/202-300       ELAARQLREGNA...TLASIAHSVGYGSESALSVAFKRVLGMPPGDYRKH
CAFR_YERPE/8-107         SRAAALLRLTRL...TIIEISAKLFYDSQQTFTREFKKIFGYTPRQYRMI
CELD_ECOLI/168-274       NFAKKQLEMTNY...SVTDIAFEAGYSSPSLFIKTFKKLTSFTPKSYRKK
CFAD_ECOLI/164-261       SKAALLLLLENSY...QISQISNMIGISSASYFIRVFNKHYGVTPKQFFTY
CSVR_ECOLI/166-263       SKAALLLLLENSY...QISQISNMIGISSASYFIRIFNKHFGVTRSSFLII
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Figure 1B

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ENVY_ECOLI/149-246      RYAVQMLLMDNK...NITQVAQLCGYSSTSYFISVFKAFYGLTPLNYLAK
EUTR_ECOLI/243-344      NAVRRELISPWSqsmTVKDAAMQWGFWHLGQFATDYQQLFSEKPSLTLHQ
EUTR_SALTY/243-344      NAVRRELISPWSqsaTVKDAAMQWGFWHLGQFATDYQQLFAEKPSLTLHQ
EXSA_PSEAE/171-269      LYAHQLLLNSDM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSRSRQG
FAPR_ECOLI/154-251      NQAAKFIIRSDH...QIGMIASLVGYTSVSYFIKTFKEYYGVTPKKFEIG
FEAR_ECOLI/199-299      DFCADAIRHAADd.eKLAGIGFHWGFSDQSHFSTVFKQRFGMTPGEYRRK
GADX_ECO27/145-242      QRALQLIVIYGV...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GADX_ECO57/145-242      QRALQLIVIHGF...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GADX_ECOLI/145-242      QRALQLIVIHGF...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GLXA_RHIME/223-321      RHARRLLQQSPL...SIPEIAYATGFSSPAHFSNAFKRLFSQTPGSLRRR
HRPB_RALSO/375-477      EGIRSDLLDSERnpsNIIDTASRWGIRSRSALVKGYRKQFNEAPSETIWR
INVF_SALTY/112-210      AQSLLNSVEGHE...NITQLAVNHGYSSPSHFSSEIKELIGVSPRKLSNI
LACR_STAXY/174-272      YHASQLLIHTST...LISDISRQVGYKDPLLFSKNFTKHFEISASEYRHH
LCRF_YERPE/167-265      LYAHQLLLNGKM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSQARLT
LUMQ_PHOLE/148-246      DLAKQLIAERQK...PLSQVAQLCGFSSQSSFSQAFRRLYGMSPTRYQFF
MARA_ECOLI/14-112       TEIAQKLKESNE...PILYLAERYGFESQQTLTRTFKNYFDVPPHKYRMT
MARA_SALTY/14-112       TEIAQKLKESNE...PILYLAERYGFESQQTLTRTFKNYFDVPPHKYRIT
MELR_ECOLI/194-292      NHVRALLSDTDK...SILDIALTAGFRSSSRFYSTFGKYVGMSPQQYRKL
MMSR_PSEAE/201-299      EYACQLLDSSDQ...SVARVGQAVGYDDSYYFSRLFSKVMGLSPSAYRQR
MSMR_STRMU/176-274      KRSQYLLENPKL...SIAEISNSVGFSDSLAFSKAFKNYFGKSPSKFRKE
MXIE_SHIFL/99-199       VNGLLDVFLHNQ...TITSAAMNNGYRSTSHFSNEIKTRLGFSARELSNI
MXIE_SHISO/99-199       VNGLLDVFLHNQ...TITSAAMNNGYASTSHFSNEIKTRLGFSARELSNI
ORUR_PSEAE/241-338      RLALQYLTTTQL...PLYEIALLLGFNDSSNFRRAFRKWTGKLPSDYREA
PCHR_PSEAE/201-296      REAHRMLCDEEA...NVSTVAYRVGYS-PAHFSIAFRKRYGISPSEIR--
PERA_ECO27/168-265      KHASLFLRTTDK...NIDEISCLVGFNSTSYFIKVFKEYYNTTPKKYNGV
POCR_SALTY/195-293      VSARELLCHSDW...SIASIARNLGFSQTSYFCKVFRQTYQVTPQAYRQQ
PQRA_PROVU/7-107        LEAAKSLQEKDM...SILDIALMYGFSSQATFTRIFKKHFNTTPAKFREN
RAFR_PEDPE/176-274      EDAKQRLSTSNN...SVQSIANMVGYKDSFTFSKAFKRYSGASPSYYRKS
RAMA_ENTCL/9-107        LLAARDLRESDE...RVYEICLRYGFESQQTFTRIFTRTFHQPPGAYRKE
RAMA_KLEPN/9-107        LLAARDLRDTDQ...RVYDICLKYGFDSQQTFTRVFTRTFNQPPGAYRKE
RHAR_ECOLI/209-307      CHAQYLLQHSRL...LISDISTECGFEDSNYFSVVFTRETGMTPSQWRHL
RHAR_SALTY/179-277      CHAQYLLQHSPL...MISEISMQCGFEDSNYFSVVFTRETGMTPSQWRHL
RHAS_ECOLI/174-272      MKARHLLRHSEA...SVTDIAYRCGFSDSNHFSTLFRREFNWSPRDIRQG
RHAS_SALTY/174-272      IKARHLLRHSDH...SVTEIAYRCGFGDSNHFSTLFRREFNWSPRDIRQG
RHRA_RHIME/210-310      SLAKSLILAEGEa.tSISQIAYNVGFNDLSYFNRTFRSRYGVRPSDLRRL
RNS_ECOLI/164-261       SKAALLLLENSY...QISQISNMIGISSASYFIRIFNKHYGVTPKQFFTY
ROB_ECOLI/8-106         SKSAVALRLTAR...PILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRS
SOXS_ECOLI/7-105        LLAAVELRTTER...PIFDIAMDLGYVSQQTFSRVFRRQFDRTPSDYRHR
SOXS_SALTY/7-105        LLAAVELRTTER...PIFDIAMDLGYVSQQTFSRVFRREFDRTPSDYRHR
TCPN_VIBCH/172-269      SYSISLMKTGEF...KIKQIAYQSGFASVSYFSTVFKSTMNVAPSEYLFM
TETD_ECOLI/31-129       TKAAVELRLTKK...TILEIALKYQFDSQQSFTRRFKYIFKVTPSYYRRN
THCR_RHOER/227-328      QKARKDLLRADPaseGVTEIAQRWGFLHVGRFAGEYKQTFGVSPSEDLRT
URER_ECOLI/171-268      GMALNYLTFSNY...SVFQISHRCGFGSNAYFCDVFKRKYNMTPSQFRLQ
URER_PROMI/171-268      PIALNYLTFSNY...SVFQISHRCGFGSNAYFCDAFKRKYGMTPSQFRTQ
VIRF_SHIDY/161-258      HHAAKLLLNSQS...YINDVSRLIGISSPSYFIRKFNEYYGITPKKFYLY
VIRF_YEREN/167-265      LYAHQLLLNGKM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSQARLT
VIRS_MYCTU/236-334      AQAARYLAQPGL...YLSQIAVLLGYSEQSALNRSCRRWFGMTPRQYRAY
XYLR_ECOLI/288-386      EKARSLLISTTL...SINEISQMCGYPSLQYFYSVFKKAYDTTPKEYRDV
XYLR_HAEIN/288-386      SRAKNLLQQTDI...SIKEITEICGYPSIQYFYSVFKKEFEMTPKEFRLN
XYLS_PSEPU/214-315      ESIRACLNDPSAnvrSITEIALDYGFLHLGRFAENYRSAFGELPSDTLRQ
XYS1_PSEPU/214-315      ESIRACLNDPSAnvrSITEIALDYGFLHLGRFAENYRSAFGELPSDTLRQ
XYS2_PSEPU/39-140       ECVRACLSNPTTnirSITEVALDYGFLHLGRFAEKYRSTFGELPSDTLSL
XYS3_PSEPU/214-315      ECIRARLSDPNAnvrSVTEMALDYGFFHTGRFAENYRSTFGELPSDTLRR
XYS4_PSEPU/214-315      ECIRARLSDPNAnvrSVTEMALDYGFFHTGRFAENYRSTFGELPSDTLRR
Y4FK_RHISN/318-417      AAAHGAIKAGRAg..SITELALNLQFSNPGRFSVLYKSAYGLSPSSALRF
YA52_HAEIN/194-295      QSAAFLLKQSQQ...SVLAIALEVGYQSEAHFCKVFKNYYQLSPSQYRKS
YBBB_BACSU/166-264      TKAKRLMAKSNC...KLKEIAHQTGYQDEFYFSRIFKKYTGCSPTSYMKK
YBCM_ECOLI/165-262      NNALSAIQTTVK...PISEIARENGYKCPSRFTERFHNRFNITPREIRKA
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Figure 1C

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YCGK_ALTCA/67-163       EQAKKVLLKK--...SVTETAYEVGFNNSNYFATVFKKRTNYTPKQFKRT
YD95_MYCTU/242-343      TVAVDLLRNVGL...TVQQVSTRLGYTEVSTFSHAFKRWYGVAPSEYSRR
YDEO_ECOLI/137-233      QHAKNLIRVEG-...SVNKIAEQCGYASTSYFIYAFRKHFGNSPKRVSKE
YDIP_ECOLI/183-281      TEAKWSLTNTEL...SQAEISWRVGYENVDHFAKLFLRHVGCSPSDYRRQ
YEAM_ECOLI/158-258      IMALQGLVKGD-...TVQKVAHTLGYDSTTAFITMFKKGLGQTPGRYIAR
YFIF_BACSU/192-289      NKAAELLKSTNL...SIKEIAEEIGFS-VHYFTRVFSAKIGSSPGLFRSL
YHIW_ECOLI/139-236      SMARRLLELRQI...PLHTIAEKCGYSSTSYFINTFRQYYGVTPHQFAQH
YIDL_ECOLI/197-295      DLALSLLKQQGN...SVGEVADTLNFFDSFHFSKAFKHKFGYAPSAVLKN
YIJO_ECOLI/172-270      EHAKTLLKGYDL...KVKEVAHACGFVDSNYFCRLFRKNTERSPSEYRRQ
YISR_BACSU/183-281      TEAKRLLSSTND...KMGVIAETVGMEDPTYFSKLFKQIEGISPIEYRKI
YKGA_ECOLI/19-117       CRAAILVRLTAK...SMLDIALSLHFDSQQSFSREFKKLFGCSPREYRHR
YKGD_ECOLI/177-278      QIAAQMFSRETL...PVVVIAESVGYASESSFHKAFVREFGCTPGEYRER
YMCR_STRLA/184-281      QRALRLARAGV-...PFAETATLAGFADQAHLARDVREMAGSSLSELVER
YPDC_ECOLI/184-282      AKARMILQKYHL...SIHEVAQRCGFPDSDYFCRVFRRQFGLTPGEYSAR
YQHC_ECOLI/213-311      HKARMMIIHDGM...KASAAAMRVGYESASQFSREFKRYFGVTPGEDAAR
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Figure 1D

Multiple sequence alignment of PROSITE PS00041, HTH_AraC family 1.

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AARP_PROST/72-114       RIceAAkeLqtt...nlqVidIAlkyQFdsqgsFakrFKaylGiSP
ADA_MYCTU/137-179       RMqtARvlIett...nlpFgdVAfaaGFssirqFndtVRlacDgTP
ADAA_BACSU/152-194      RVhaAKkyLiqt...nkaIgdIAicvGIanapyFitlFKkktGqTP
ADIY_ECOLI/198-240      RMryAVneLmmd...gknIsqVSqscGYnstsyFisvFKdfyGmTP
AGGR_ECOLI/213-255      RMskAAllLldn...syqIsqISnmiGFsstsyFirlFVkhfGiTP
APPY_ECOLI/188-230      RMryAKklItsn...sysInvVAqkcGYnstsyFicaFKdyyGvTP
ARAC_CITFR/231-273      RIsqAKllLstt...rmpIatVGrnvGFddqlyFsrvFKkctGaSP
ARAC_ECOLI/231-273      RIsqAKllLstt...rmpIatVGrnvGFddqlyFsrvFKkctGaSP
ARAC_ERWCH/236-278      RVirAKllLqtt...qesIanIGrvvGYddqlyFsrvFRkrvGvSP
ARAC_SALTY/231-273      RIsqAKllLstt...rmpIatVGrnvGFddqlyFsrvFKkctGaSP
ARAL_STRAT/252-294      RIelTArqLreg...sapLaaIAhsvGYgsesaLsvaFKrvlGmNP
ARAL_STRLI/252-294      RIelAArqLreg...natLasIAhsvGYgsesaLsvaFKrvlGmPP
CELD_ECOLI/226-268      RInfAKkqLemt...nysVtdIAfeaGYsspslFiktFKkltSfTP
CFAD_ECOLI/211-255      QLrmSKaaLlle.nsyqIsqISnmiGIssasyFirvFNkhyGvTP
CSVR_ECOLI/215-257      RMskAAllLlen...syqIsqISnmiGIssasyFiriFNkhfGvTR
ENVY_ECOLI/198-240      RMryAVqmLlmd...nknItqVAqlcGYsstsyFisvFKafyGlTP
EUTR_ECOLI/293-338      RLnaVRreLispwsqsmtVkdAAmqwGFwhlgqFatdYQqlfSeKP
EUTR_SALTY/293-338      RLnaVRreLispwsqsatVkdAAmqwGFwhlgqFatdYQqlfAeKP
EXSA_PSBAE/221-263      RIlyAHqlLlns...dmsIvdIAmeaGFssqsyFtqsYRrrfGcTP
FAPR_ECOLI/203-245      RMnqAAkfIirs...dhqIgmIAslvGYtsvsyFiktFKeyyGvTP
GADX_ECO27/194-236      RMqrALqlIviy...gvsIkrVAvscGYhsvsyFiyvFRnyyGmTP
GADX_ECO57/194-236      RMqrALqlIvih...gfsIkrVAvscGYhsvsyFiyvFRnyyGmTP
GADX_ECOLI/194-236      RMqrALqlIvih...gfsIkrVAvscGYhsvsyFiyvFRnyyGmTP
GLXA_RHIME/273-315      RLrhARrlLqqs...plsIpeIAyatGFsspahFsnaFKrlfSqTP
HRPB_RALSO/426-471      RLegIRsdLldsernpsnIidTAsrwGIrsrsaLvkgYRkqfNeAP
INVF_SALTY/162-204      RMaqSLlnSveg...henItqLAvnhGYsspshFsseIKeliGvSP
LACR_STAXY/224-266      RMyhASqlLiht...stlIsdISrqvGYkdpllFsknFTkhfEiSA
LCRF_YERPE/217-259      RIlyAHqlLlng...kmsIvdIAmeaGFssqsyFtqsYRrrfGcTP
LUMQ_PHOLE/198-240      RLdlAKqlIaer...qkpLsqVAqlcGFssqssFsqaFRrlyGmSP
MARA_ECOLI/64-106       KMteIAqkLkes...nepIlyLAeryGFesqqtLtrtFKnyfDvPP
MARA_SALTY/64-106       KMteIAqkLkes...nepIlyLAeryGFesqqtLtrtFKnyfDvPP
MELR_ECOLI/244-286      RInhVRalLsdt...dksIldIAltaGFrsssrFystFGkyvGmSP
MMSR_PSEAE/251-293      KIeyACqlLdss...dqsVarVGqavGYddsyyFsrlFSkvmGlSP
MSMR_STRMU/226-268      RMkrSQylLenp...klsIaeISnsvGFsdslaFskaFKnyfGkSP
MXIE_SHIFL/151-193      RLvnGLldVflh...nqtItsAAmnnGYrstshFsneIKtrlGfSA
MXIE_SHISO/151-193      RLvnGLldVflh...nqtItsAAmnnGYastshFsneIKtrlGfSA
PCHR_PSEAE/251-292      RLreAHrmLcde...eanVstVAyrvGYsp.ahFsiaFRkryGiSP
PERA_ECO27/217-259      KMkhASlfLrtt...dknIdeISclvGFnstsyFikvFKeyyNtTP
POCR_SALTY/245-287      RMvsARelLchs...dwsIasIArnlGFsqtsyFckvFRqtyQvTP
PQRA_PROVU/59-101       RLleAAksLqek...dmsIldIAlmyGFssqatFtriFKkhfNtTP
RAFR_PEDPE/226-268      RLedAKqrLsts...nnsVqsIAnmvGYkdsftFskaFKrysGaSP
RAMA_KLEPN/59-101       KLllAArdLrdt...dqrVydIClkyGFdsqqtFtrvFTrtfNqPP
RHAR_ECOLI/259-301      RVchAQylLqhs...rllIsdIStecGFedsnyFsvvFTretGmTP
RHAR_SALTY/229-271      RIchAQylLqhs...plmIseISmqcGFedsnyFsvvFTretGmTP
RHAS_ECOLI/224-266      RLmkARhlLrhs...easVtdIAyrcGFsdsnhFstlFRrefNwSP
RHAS_SALTY/224-266      RLikARhlLrhs...dhsVteIAyrcGFgdsnhFstlFRrefNwSP
RNS_ECOLI/211-255       QLrmSKaaLlle.nsyqIsqISnmiGIssasyFiriFNkhyGvTP
SOXS_ECOLI/57-99        RLllAAveLrtt...erpIfdIAmdlGYvsqqtFsrvFRrqfDrTP
SOXS_SALTY/57-99        RLllAAveLrtt...erpIfdIAmdlGYvsqqtFsrvFRrefDrTP
TCPN_VIBCH/221-263      RIsySIslMktg...efkIkqIAyqsGFasvsyFstvFKstmNvAP
THCR_RHOER/277-322      RMqkARkdLlradpasegVteIAqrwGFlhvgrFageYKqtfGvSP
URER_ECOLI/220-262      RMgmALnyLtfs...nysVfqIShrcGFgsnayFcdvFKrkyNmTP
```

Figure 2A

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URER_PROMI/220-262      RIpiALnyLtfs...nysVfqIShrcGFgsnayFcdaFKrkyGmTP
VIRF_SHIDY/210-252      RMhhAAklLlns...qsyIndVSrliGIsspsyFirkFNeyyGiTP
VIRF_YEREN/217-259      RIlyAHqlLlng...kmsIvdIAmeaGFssqsyFtqsYRrrfGcTP
XYLR_ECOLI/338-380      KLekARslList...tlsIneISqmcGYpslqyFysvFKkayDtTP
XYLR_HAEIN/338-380      KIsrAKnlLqqt...disIkeITeicGYpsiqyFysvFKkefEmTP
XYLS_PSEPU/264-309      KLesIRacLndpsanvrsIteIAldyGFlhlgrFaenYRsafGeLP
XYS1_PSEPU/264-309      KLesIRacLndpsanvrsIteIAldyGFlhlgrFaenYRsafGeLP
XYS2_PSEPU/89-134       KLecVRacLsnpttnirsIteVAldyGFlhlgrFaekYRstfGeLP
XYS3_PSEPU/264-309      KLecIRarLsdpnanvrsVteMAldyGFfhtgrFaenYRstfGeLP
XYS4_PSEPU/264-309      KLecIRarLsdpnanvrsVteMAldyGFfhtgrFaenYRstfGeLP
Y4FK_RHISN/368-411      RLaaAHgaIkagr..agsIteLAlnlQFsnpgrFsvlYKsayGlSP
YA52_HAEIN/247-289      RLqsAAflLkqs...qqsVlaIAlevGYqseahFckvFKnyyQlSP
YBBB_BACSU/216-258      RItkAKrlMaks...nckLkeIAhqtGYqdefyFsriFKkytGcSP
YCGK_ALTCA/117-157      RIeqAKkvLlk.....ksVteTAyevGFnnsnyFatvFKkrtNyTP
YDEO_ECOLI/186-227      RMqhAKnlIrve....gsVnkIAeqcGYastsyFiyaFRkhfGnSP
YEAM_ECOLI/211-252      QLimALqgLvkg....dtVqkVAhtlGYdsttaFitmFKkglGqTP
YFIF_BACSU/242-283      KInkAAelLkst...nlsIkeIAeeiGFsv.hyFtrvFSakiGsSP
YHIW_ECOLI/188-230      RMsmARrlLelr...qipLhtIAekcGYsstsyFintFRqyyGvTP
YIDL_ECOLI/247-289      RLdlALslLkqq...gnsVgeVAdtlNFfdsfhFskaFKhkfGyAP
YIJO_ECOLI/222-264      RLehAKtlLkgy...dlkVkeVAhacGFvdsnyFcrlFRkntErSP
YISR_BACSU/233-275      RMteAKrlLsst...ndkMgvIAetvGMedptyFsklFKqieGiSP
YMCR_STRLA/234-275      RMqrALrlArag....vpFaeTAtlaGFadqahLardVRemaGsSL
YPDC_ECOLI/234-276      RMakARmiLqky...hlsIheVAqrcGFpdsdyFcrvFRrqfGlTP
ADA_ECOLI/86-128        KIthACrlLeqet..pvtLeaLAdqvAMsp.fhLhrlFKattGmTP
ADA_ECOLI/136-177       RArrLResLakg....esVttSIlnaGFpdsssYyrkADetlGmTA
ADA_SALTY/86-128        KIacACrlLeqet..pvtLafLAqavAMsp.fhLhrlFKastGmTP
ADA_SALTY/136-177       RArrLReaLakg....epItaAIyraGFpdsssYyrhADqtlGmTA
CAFR_YERPE/56-101       RVrrASraAallrltrltIieISaklFYdsqqtFtreFKkifGyTP
ROB_ECOLI/55-100        RArrLSksAvalrltarpIldIAlqyRFdsqqtFtraFKkqfAqTP
TETD_ECOLI/78-123       RArrLTkaAvelrltkktIleIAlkyQFdsqqsFtrrFKyifKvTP
```

Figure 2B

Multiple sequence alignment PS01124, HTH_ARAC_FAMILY_2.

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AARP_PROST/22-120       SEILVWIEGNLTNR.........LSLDDIAQHSGYTKWHLQRVFRKIVGMPLGEYIRRRRI
ADA_ECOLI/85-183        DKITHACRLLEQETp.......VTLEALADQVAMSPFHLHRLFKATTGMTPKAWQQAWRA
ADA_MYCTU/87-185        ARAMRLIADGTVDR.........DGVSGLAAQLGYTIRQLERLLQAVVGAGPLALARAQRM
ADA_SALTY/94-183        ----------LEQEt.......pVTLAFLAQAVAMSPFHLHRLFKASTGMTPKGWQQAWRA
ADAA_BACSU/102-200      DLITEYIDKNFTEK.........LTLESLADICHGSPYHMHRTFKKIKGITLVEYIQQVRV
ADIY_ECOLI/149-246      DSVYQIIESDIHKD.........WNLSMVASCLCLSPSLLKKKLKSENT-SYSQIITTCRM
AGGR_ECOLI/164-261      DKVRNTIEKDLSKR.........WTLAIIADEFNVSEITIRKRLESEYI-TFNQILMQSRM
APPY_ECOLI/139-236      CKITGIISFNIERQ.........WHLKDIAELIYTSESLIKKRLRDEGT-SFTEILRDTRM
ARAC_CITFR/180-279      RDACQYISDHLADSn.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAC_ECOLI/180-279      REACQYISDHLADSn.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAC_ERWCH/186-284      IEACQFITSNLAGE.........LRIDEVARHVCLSPSRLAHLFREQVGINILRWREDQRV
ARAC_SALTY/180-279      RDACQYISDHLADSh.......FDIASVAQHVCLSPSRLSHLFRQQLGISVLSWREDQRI
ARAL_STRAT/202-300      ASALTFLHRDPAHS.........WTVAELASAAAVSRSTLAARFKATVGQGPLEYLTRWRI
ARAL_STRLI/202-300      ATALTCLHRDPARS.........WTVADLADTAAVSRSTLAARFKATVGQGPLEYLTRWRI
CAFR_YERPE/8-107        NSIIQYIEENLESKf.......INIDCLVLYSGFSRRYLQISFKEYVGMPIGTYIRVRRA
CELD_ECOLI/168-274      DDVPQWLKSTVEKMhdkeqfseSALENMVALSAKSQEYLTRATQRYYGKTPMQIINEIRI
CFAD_ECOLI/164-261      DKVRNVIEKDLSRK.........WTLGIIADAFNVSEITIRKRLESENT-NFNQILMQLRM
CSVR_ECOLI/166-263      DKVRGVIEKDLSRK.........WTLAIIADVFNVSEITIRKRLESEDT-NFNQILMQSRM
ENVY_ECOLI/149-246      DSVCRIIQSDIQHY.........WNLRIVASSLCLSPSLLKKKLKNENT-SYSQIVTECRM
EUTR_ECOLI/243-344      SRAREYVLENMSEP.........VTVLDLCNQLHVSRRTLQNAFHAILGIGPNAWLKRIRL
EUTR_SALTY/243-344      SRAREYVLENMSEP.........LTVLDLCNQLHVSRRTLQNAFHAILGIGPNAWLKRIRL
EXSA_PSEAE/171-269      ERLQLFMEKHYLNE.........WKLSDFSREFGMGLTTFKELFGSVYGVSPRAWISERRI
FAPR_ECOLI/154-251      ERIVTLLFSDLTRK.........WKLSDIAEEMHISEISVRKRLEQECL-NFNQLILDVRM
FEAR_ECOLI/199-299      QKVVTLIDDNIREEi.......LRPEWIAGETGMSVRSLYRMFADKGL-VVAQYIRNRRL
GADX_ECO27/145-242      TRVCTVINNNIAHE.........WTLARIASELLMSPSLLKKKLREEGT-SYSQLLTECRM
GADX_ECO57/145-242      TRVCTVINNNIAHE.........WTLARIASELLMSPSLLKKKLREEET-SYSQLLTECRM
GADX_ECOLI/145-242      TRVCTVINNNIAHE.........WTLARIASELLMSPSLLKKKLREEET-SYSQLLTECRM
GLXA_RHIME/223-321      LAVLEKMETAIERP.........LDRTAMARLAGVSPRHLDRLFREHRGTGFLDTYREIRL
HRPB_RALSO/375-477      RRAYRYIIENIERSd.......LTTREVAAHINVTERALQLAFKSAVGMSPSSVIRRMRL
INVF_SALTY/112-210      YWLVGYLLAQSTSG.........NTMRMLGEDYGVSYTHFRRLCSRALGGKAKSELRNWRM
LACR_STAXY/174-272      QHAVDFINTNYQKH.........ITVEDVAKSVNITRSHLYKLFKKNLGCSPKEYLTYIRM
LCRF_YERPE/167-265      ERLQKFMEENYLQG.........WKLSKFAREFGMGLTTFKELFGTVYGISPRAWISERRI
LUMQ_PHOLE/148-246      VLIDNYIEQHLQKK.........ISVAELSSVAFLAQSQFYALFKSQMGITPHQYVLRKRL
MARA_ECOLI/14-112       HSILDWIEDNLESP.........LSLEKVSERSGYSKWHLQRMFKKETGHSLGQYIRSRKM
MARA_SALTY/14-112       HSILDWIEDNLESP.........LSLEKVSERSGYSKWHLQRMFKKETGHSLGQYIRSRKM
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Figure 3A

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MELR_ECOLI/194-292      SQMLGFIAENYDQA.......LTINDVAEHVKLNANYAMGIFQRVMQLTMKQYITAMRI
MMSR_PSEAE/201-299      DGLHAYMREHLHAR.......LELERLAAFCNLSKFHFVSRYKAITGRTPIQHFLHLKI
MSMR_STRMU/176-274      NQVKKIIHSQYGSS.......LRVNDIAKKLNLSRSYLYKIFRKSTNLSIKEYILQVRM
MXIE_SHIFL/99-199       YHLVLYLLRTIEKEk......eVRIKSLTEHYGVSEAYFRSLCRKALGAKVKEQLNTWRL
MXIE_SHISO/99-199       YHLVLYLLRTIEKEk......eVRIKSLTEHYGVSEAYFRSLCRKALGAKVKEQLNTWRL
ORUR_PSEAE/241-338      TRVRRLLLARPGDF.......PDLEQAARELHTSGRSLRRHLSSLGT-TYQQVLDDVRK
PCHR_PSEAE/201-296      HAARDLLVGALQEP.......PSLDTLASRVGMNPRKLTAGFRKVFGASVFGYLQEYRL
PERA_ECO27/168-265      DRVIKVIELDISKN.......WKLGDVSSSMFMSDSCLRKQLNKENL-TFKKIMLDIKM
POCR_SALTY/195-293      KKALRYIDAHLSDD.......LRLEDVASHVYLSPYYFSKLFKKYQGIGFNAWVNRQRM
PQRA_PROVU/7-107        NDILKWLETQLQRNe......gIKIDTIANKSGYSKWHLQRIFKDFKGCTLGEYVRKRRL
RAFR_PEDPE/176-274      NLAVSYLQENYSTG.......CTIMDLCHYLNLSRSYLYTLFKTHANTSPQKLLTKLRL
RAMA_ENTCL/9-107        DTIVEWIDDNLHQP.......LRIEDIARHAGYSKWHLQRLFLQYKGESLGRYIRERKL
RAMA_KLEPN/9-107        DTIVEWIDDNLHQP.......LRIDDIARHAGYSKWHLQRLFLQYKGESLGRYIRERKL
RHAR_ECOLI/209-307      DKLITRLAASLKSP.......FALDKFCDEASCSERVLRQQFRQQTGMTINQYLRQVRV
RHAR_SALTY/179-277      DKLITALANSLECP.......FALDAFCQQEQCSERVLRQQFRAQTGMTINQYLRQVRI
RHAS_ECOLI/174-272      NLLLAWLEDHFADE.......VNWDAVADQFSLSLRTLHRQLKQQTGLTPQRYLNRLRL
RHAS_SALTY/174-272      NQLMAWLEDHFAEE.......VCWEAVAEQFSLSLRTLHRQLKQHTGLTPQRYLNRLRL
RHRA_RHIME/210-310      ASIKMRVEQNLANGs......FSITDVAEAERITPRAIQKFFSREGT-TFSRYVLGRRL
RNS_ECOLI/164-261       DKVRNLIEKDLSRK.......WTLGIIADAFNASEITIRKRLESENT-NFNQILMQLRM
ROB_ECOLI/8-106         RDLLIWLEGHLDQP.......LSLDNVAAKAGYSKWHLQRMFKDVTGHAIGAYIRARRL
SOXS_ECOLI/7-105        QDLIAWIDEHIDQP.......LNIDVVAKKSGYSKWYLQRMFRTVTHQTLGDYIRQRRL
SOXS_SALTY/7-105        QTLIEWIDEHIDQP.......LNIDVVAKKSGYSKWYLQRMFRTVTHQTLGEYIRQRRL
TCPN_VIBCH/172-269      EKISCLVKSDITRN.......WRWADICGELRTNRMILKKELESRGV-KFRELINSIRI
TETD_ECOLI/31-129       KDVLLWIEHNLDQS.......LLLDDVANKAGYTKWYFQRLFKKVTGVTLASYIRARRL
THCR_RHOER/227-328      RLAVDYLEAHAQQP.......LTVAQVARNVGVSVRSLQVGFQNSLGTTPMRQLKIIRM
URER_ECOLI/171-268      QAITHLITQEPQKK.......WHLDDVAKALFTTPSTLRRHLNREGV-SFRQLLLDVRM
URER_PROMI/171-268      QAITHLITQDPQRK.......WHLEDVAKTLYTTPSTLRRHLSKEGV-SFCQLLLDVRI
VIRF_SHIDY/161-258      DQIRKIVEKNIEKR.......WRLSDISNNLNLSEIAVRKRLESEKL-TFQQILLDIRM
VIRF_YEREN/167-265      ERLQKFMEENYLQG.......WKLSKFAREFGMGLTTFKELFGTVYGISPRAWISERRI
VIRS_MYCTU/236-334      ERVVGLARRLLPTGq......CSAEAIADQLDMHPRTLQRRLAAEGL-RCHDLIERERR
XYLR_ECOLI/288-386      IQAMHYIRNHACKG.......IKVDQVLDAVGISRSNLEKRFKEEVGETIHAMIHAEKL
XYLR_HAEIN/288-386      IQAMHYIRHRACHR.......IKVGQVLDHLETSRSNLEQRFKNEMNKTIHQVIHEEKI
XYLS_PSEPU/214-315      ERVVQFIEENLKRN.......ISLERLAELAMMSPRSLYNLFEKHAGTTPKNYIRNRKL
XYS1_PSEPU/214-315      ERVVQFIEENLKRN.......ISLERLAELAMMSPRSLYNLFEKHAGTTPKNYIRNRKL
XYS2_PSEPU/39-140       ERVVQFIEENVKRS.......ISLEQLAELALMSPRSLYTMFEKHTGTTPMNYIRNRKL
XYS3_PSEPU/214-315      ERVVQFIEDNLKQS.......ISLERLAELALMSPRSLYTLFEKHAGTTPKNYIRNRKL
XYS4_PSEPU/214-315      ERVVQFIEENLKRN.......ISLERLAELALMSPRSLYTLFEKHAGTTPKNYIRNRKL
Y4FK_RHISN/318-417      LKAEAFMRENLTNP.......VTIEDLAAAARCTPRALQRMFRTYRGGSPMSVLCNYRL
YA52_HAEIN/194-295      KRLNTALIAILQQPqn.....dWHIEQLAELATMSRANFIRIFQQHIGMSPGRFLTKVRL
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Figure 3B

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YBBB_BACSU/166-264      EKTKHYIETHADTK........ITLAQLSQMAGISAKHYSESFKKWTGQSVTEFITKTRI
YBCM_ECOLI/165-262      SRCYNLLLSEPGTK........WTANKVARYLYISVSTLHRRLASEGV-SFQSILDDVRL
YCGK_ALTCA/67-163       QNAMLYIENNYFND........INIDTVAFSVGVSRSYLVKQFKLATNKTINNRIIEVRI
YD95_MYCTU/242-343      RGITALVRSKLFRDsg....lfPTFTDVAGELDMHPRTLRRRLAEEGT-SFRALLGEARS
YDEO_ECOLI/137-233      GKVRNIVNMKPAHP........WKLKDICDCLYISESLLKKKLKQEQT-TFSQILLDARM
YDIP_ECOLI/183-281      KDILFYLNNNYREK........ITLEQLSKKFRASVSYICHEFTKEYRISPINYVIQRRM
YEAM_ECOLI/158-258      PKIRTMVEMMAKGPve.....wGALGQWAGFFAMSERNLARLIVKETGLSFRQWRQQLQL
YFIF_BACSU/192-289      TEVKLHIKDNLSQP........LKLTDVASHFHISGRHLSRLFAAELGVSYSEFVQNEKI
YHIW_ECOLI/139-236      GKVERLISFDIAKR........WYLRDIAERMYTSESLIKKKLQDENT-CFSKILLASRM
YIDL_ECOLI/197-295      EKLIATLHASLQQR........WSVADMAATIPCSEAWLRRLFLRYTGKTPKEYYLDARL
YIJO_ECOLI/172-270      EAIRDYIDERYASA........LTRESVAQAFYISPNYLSHLFQKTGAIGFNEYLNHTRL
YISR_BACSU/183-281      WEAARYLQEHYKEK........TTIKDLSLALHYHQDYVSRCMQQVLGVTPAQYTNRVRM
YKGA_ECOLI/19-117       QQLLEWIECNLEHP........ISIEDIAQKSGYSRRNIQLLFRNFMHVPLGEYIRKRRL
YKGD_ECOLI/177-278      PRLGAVIQQMLEMPgh.....aWTVESLASIAHMSRASFAQLFRDVSGTTPLAVLTKLRL
YMCR_STRLA/184-281      DPLLRAVVVSLEAG........RSVTATADSVGLGARQLHRRSLAAFGYGPKTLARVLRM
YPDC_ECOLI/184-282      HSICNWVQDNYAQP........LTRESVAQFFNITPNHLSKLFAQHGTMRFIEYVRWVRM
YQHC_ECOLI/213-311      SRVLKRIENKYTEN........LSVEQLAAEANMSVSAFHHNFKSVTSTSPLQYLKNYRL

AARP_PROST/22-120       CEAAKELQTTNL...QVIDIALKYQFDSQQSFAKRFKAYLGISPSLYRLS
ADA_ECOLI/85-183        RRLRESLAKGE-...SVTTSILNAGFPDSSSYYRKADETLGMTAKQFRHG
ADA_MYCTU/87-185        QTARVLIETTNL...PFGDVAFAAGFSSIRQFNDTVRLACDGTPTALRAR
ADA_SALTY/94-183        RRLREALAKGE-...PITAAIYRAGFPDSSSYYRHADQTLGMTAKQFRKG
ADAA_BACSU/102-200      HAAKKYLIQTNK...AIGDIAICVGIANAPYFITLFKKKTGQTPARFRQM
ADIY_ECOLI/149-246      RYAVNELMMDGK...NISQVSQSCGYNSTSYFISVFKDFYGMTPLHYVSQ
AGGR_ECOLI/164-261      SKAALLLLDNSY...QISQISNMIGFSSTSYFIRLFVKHFGITPKQFLTY
APPY_ECOLI/139-236      RYAKKLITSNSY...SINVVAQKCGYNSTSYFICAFKDYYGVTPSHYFEK
ARAC_CITFR/180-279      SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAC_ECOLI/180-279      SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAC_ERWCH/186-284      IRAKLLLQTTQE...SIANIGRVVGYDDQLYFSRVFRKRVGVSPSDFRRR
ARAC_SALTY/180-279      SQAKLLLSTTRM...PIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAG
ARAL_STRAT/202-300      ELTARQLREGSA...PLAAIAHSVGYGSESALSVAFKRVLGMNPGDYRKH
ARAL_STRLI/202-300      ELAARQLREGNA...TLASIAHSVGYGSESALSVAFKRVLGMPPGDYRKH
CAFR_YERPE/8-107        SRAAALLRLTRL...TIIEISAKLFYDSQQTFTREFKKIFGYTPRQYRMI
CELD_ECOLI/168-274      NFAKKQLEMTNY...SVTDIAFEAGYSSPSLFIKTFKKLTSFTPKSYRKK
CFAD_ECOLI/164-261      SKAALLLLENSY...QISQISNMIGISSASYFIRVFNKHYGVTPKQFFTY
CSVR_ECOLI/166-263      SKAALLLLENSY...QISQISNMIGISSASYFIRIFNKHFGVTRSSFLII
ENVY_ECOLI/149-246      RYAVQMLLMDNK...NITQVAQLCGYSSTSYFISVFKAFYGLTPLNYLAK
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Figure 3C

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EUTR_ECOLI/243-344      NAVRRELISPWSqsmTVKDAAMQWGFWHLGQFATDYQQLFSEKPSLTLHQ
EUTR_SALTY/243-344      NAVRRELISPWSqsaTVKDAAMQWGFWHLGQFATDYQQLFAEKPSLTLHQ
EXSA_PSEAE/171-269      LYAHQLLLNSDM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSRSRQG
FAPR_ECOLI/154-251      NQAAKFIIRSDH...QIGMIASLVGYTSVSYFIKTFKEYYGVTPKKFEIG
FEAR_ECOLI/199-299      DFCADAIRHAADd.eKLAGIGFHWGFSDQSHFSTVFKQRFGMTPGEYRRK
GADX_ECO27/145-242      QRALQLIVIYGV...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GADX_ECO57/145-242      QRALQLIVIHGF...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GADX_ECOLI/145-242      QRALQLIVIHGF...SIKRVAVSCGYHSVSYFIYVFRNYYGMTPTEYQER
GLXA_RHIME/223-321      RHARRLLQQSPL...SIPEIAYATGFSSPAHFSNAFKRLFSQTPGSLRRR
HRPB_RALSO/375-477      EGIRSDLLDSERnpsNIIDTASRWGIRSRSALVKGYRKQFNEAPSETIWR
INVF_SALTY/112-210      AQSLLNSVEGHE...NITQLAVNHGYSSPSHFSSEIKELIGVSPRKLSNI
LACR_STAXY/174-272      YHASQLLIHTST...LISDISRQVGYKDPLLFSKNFTKHFEISASEYRHH
LCRF_YERPE/167-265      LYAHQLLLNGKM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSQARLT
LUMQ_PHOLE/148-246      DLAKQLIAERQK...PLSQVAQLCGFSSQSSFSQAFRRLYGMSPTRYQFF
MARA_ECOLI/14-112       TEIAQKLKESNE...PILYLAERYGFESQQTLTRTFKNYFDVPPHKYRMT
MARA_SALTY/14-112       TEIAQKLKESNE...PILYLAERYGFESQQTLTRTFKNYFDVPPHKYRIT
MELR_ECOLI/194-292      NHVRALLSDTDK...SILDIALTAGFRSSSRFYSTFGKYVGMSPQQYRKL
MMSR_PSEAE/201-299      EYACQLLDSSDQ...SVARVGQAVGYDDSYYFSRLFSKVMGLSPSAYRQR
MSMR_STRMU/176-274      KRSQYLLENPKL...SIAEISNSVGFSDSLAFSKAFKNYFGKSPSKFRKE
MXIE_SHIFL/99-199       VNGLLDVFLHNQ...TITSAAMNNGYRSTSHFSNEIKTRLGFSARELSNI
MXIE_SHISO/99-199       VNGLLDVFLHNQ...TITSAAMNNGYASTSHFSNEIKTRLGFSARELSNI
ORUR_PSEAE/241-338      RLALQYLTTTQL...PLYEIALLLGFNDSSNFRRAFRKWTGKLPSDYREA
PCHR_PSEAE/201-296      REAHRMLCDEEA...NVSTVAYRVGYS-PAHFSIAFRKRYGISPSEIR--
PERA_ECO27/168-265      KHASLFLRTTDK...NIDEISCLVGFNSTSYFIKVFKEYYNTTPKKYNGV
POCR_SALTY/195-293      VSARELLCHSDW...SIASIARNLGFSQTSYFCKVFRQTYQVTPQAYRQQ
PQRA_PROVU/7-107        LEAAKSLQEKDM...SILDIALMYGFSSQATFTRIFKKHFNTTPAKFREN
RAFR_PEDPE/176-274      EDAKQRLSTSNN...SVQSIANMVGYKDSFTFSKAFKRYSGASPSYYRKS
RAMA_ENTCL/9-107        LLAARDLRESDE...RVYEICLRYGFESQQTFTRIFTRTFHQPPGAYRKE
RAMA_KLEPN/9-107        LLAARDLRDTDQ...RVYDICLKYGFDSQQTFTRVFTRTFNQPPGAYRKE
RHAR_ECOLI/209-307      CHAQYLLQHSRL...LISDISTECGFEDSNYFSVVFTRETGMTPSQWRHL
RHAR_SALTY/179-277      CHAQYLLQHSPL...MISEISMQCGFEDSNYFSVVFTRETGMTPSQWRHL
RHAS_ECOLI/174-272      MKARHLLRHSEA...SVTDIAYRCGFSDSNHFSTLFRREFNWSPRDIRQG
RHAS_SALTY/174-272      IKARHLLRHSDH...SVTEIAYRCGFGDSNHFSTLFRREFNWSPRDIRQG
RHRA_RHIME/210-310      SLAKSLILAEGEa.tSISQIAYNVGFNDLSYFNRTFRSRYGVRPSDLRRL
RNS_ECOLI/164-261       SKAALLLLENSY...QISQISNMIGISSASYFIRIFNKHYGVTPKQFFTY
ROB_ECOLI/8-106         SKSAVALRLTAR...PILDIALQYRFDSQQTFTRAFKKQFAQTPALYRRS
SOXS_ECOLI/7-105        LLAAVELRTTER...PIFDIAMDLGYVSQQTFSRVFRRQFDRTPSDYRHR
SOXS_SALTY/7-105        LLAAVELRTTER...PIFDIAMDLGYVSQQTFSRVFRREFDRTPSDYRHR
TCPN_VIBCH/172-269      SYSISLMKTGEF...KIKQIAYQSGFASVSYFSTVFKSTMNVAPSEYLFM
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Figure 3D

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TETD_ECOLI/31-129       TKAAVELRLTKK...TILEIALKYQFDSQQSFTRRFKYIFKVTPSYYRRN
THCR_RHOER/227-328      QKARKDLLRADPaseGVTEIAQRWGFLHVGRFAGEYKQTFGVSPSEDLRT
URER_ECOLI/171-268      GMALNYLTFSNY...SVFQISHRCGFGSNAYFCDVFKRKYNMTPSQFRLQ
URER_PROMI/171-268      PIALNYLTFSNY...SVFQISHRCGFGSNAYFCDAFKRKYGMTPSQFRTQ
VIRF_SHIDY/161-258      HHAAKLLLNSQS...YINDVSRLIGISSPSYFIRKFNEYYGITPKKFYLY
VIRF_YEREN/167-265      LYAHQLLLNGKM...SIVDIAMEAGFSSQSYFTQSYRRRFGCTPSQARLT
VIRS_MYCTU/236-334      AQAARYLAQPGL...YLSQIAVLLGYSEQSALNRSCRRWFGMTPRQYRAY
XYLR_ECOLI/288-386      EKARSLLISTTL...SINEISQMCGYPSLQYFYSVFKKAYDTTPKEYRDV
XYLR_HAEIN/288-386      SRAKNLLQQTDI...SIKEITEICGYPSIQYFYSVFKKEFEMTPKEFRLN
XYLS_PSEPU/214-315      ESIRACLNDPSAnvrSITEIALDYGFLHLGRFAENYRSAFGELPSDTLRQ
XYS1_PSEPU/214-315      ESIRACLNDPSAnvrSITEIALDYGFLHLGRFAENYRSAFGELPSDTLRQ
XYS2_PSEPU/39-140       ECVRACLSNPTTnirSITEVALDYGFLHLGRFAEKYRSTFGELPSDTLSL
XYS3_PSEPU/214-315      ECIRARLSDPNAnvrSVTEMALDYGFFHTGRFAENYRSTFGELPSDTLRR
XYS4_PSEPU/214-315      ECIRARLSDPNAnvrSVTEMALDYGFFHTGRFAENYRSTFGELPSDTLRR
Y4FK_RHISN/318-417      AAAHGAIKAGRAg..SITELALNLQFSNPGRFSVLYKSAYGLSPSSALRF
YA52_HAEIN/194-295      QSAAFLLKQSQQ...SVLAIALEVGYQSEAHFCKVFKNYYQLSPSQYRKS
YBBB_BACSU/166-264      TKAKRLMAKSNC...KLKEIAHQTGYQDEFYFSRIFKKYTGCSPTSYMKK
YBCM_ECOLI/165-262      NNALSAIQTTVK...PISEIARENGYKCPSRFTERFHNRFNITPREIRKA
YCGK_ALTCA/67-163       EQAKKVLLKK--...SVTETAYEVGFNNSNYFATVFKKRTNYTPKQFKRT
YD95_MYCTU/242-343      TVAVDLLRNVGL...TVQQVSTRLGYTEVSTFSHAFKRWYGVAPSEYSRR
YDEO_ECOLI/137-233      QHAKNLIRVEG-...SVNKIAEQCGYASTSYFIYAFRKHFGNSPKRVSKE
YDIP_ECOLI/183-281      TEAKWSLTNTEL...SQAEISWRVGYENVDHFAKLFLRHVGCSPSDYRRQ
YEAM_ECOLI/158-258      IMALQGLVKGD-...TVQKVAHTLGYDSTTAFITMFKKGLGQTPGRYIAR
YFIF_BACSU/192-289      NKAAELLKSTNL...SIKEIAEEIGFS-VHYFTRVFSAKIGSSPGLFRSL
YHIW_ECOLI/139-236      SMARRLLELRQI...PLHTIAEKCGYSSTSYFINTFRQYYGVTPHQFAQH
YIDL_ECOLI/197-295      DLALSLLKQQGN...SVGEVADTLNFFDSFHFSKAFKHKFGYAPSAVLKN
YIJO_ECOLI/172-270      EHAKTLLKGYDL...KVKEVAHACGFVDSNYFCRLFRKNTERSPSEYRRQ
YISR_BACSU/183-281      TEAKRLLSSTND...KMGVIAETVGMEDPTYFSKLFKQIEGISPIEYRKI
YKGA_ECOLI/19-117       CRAAILVRLTAK...SMLDIALSLHFDSQQSFSREFKKLFGCSPREYRHR
YKGD_ECOLI/177-278      QIAAQMFSRETL...PVVVIAESVGYASESSFHKAFVREFGCTPGEYRER
YMCR_STRLA/184-281      QRALRLARAGV-...PFAETATLAGFADQAHLARDVREMAGSSLSELVER
YPDC_ECOLI/184-282      AKARMILQKYHL...SIHEVAQRCGFPDSDYFCRVFRRQFGLTPGEYSAR
YQHC_ECOLI/213-311      HKARMMIIHDGM...KASAAAMRVGYESASQFSREFKRYFGVTPGEDAAR
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Figure 3E

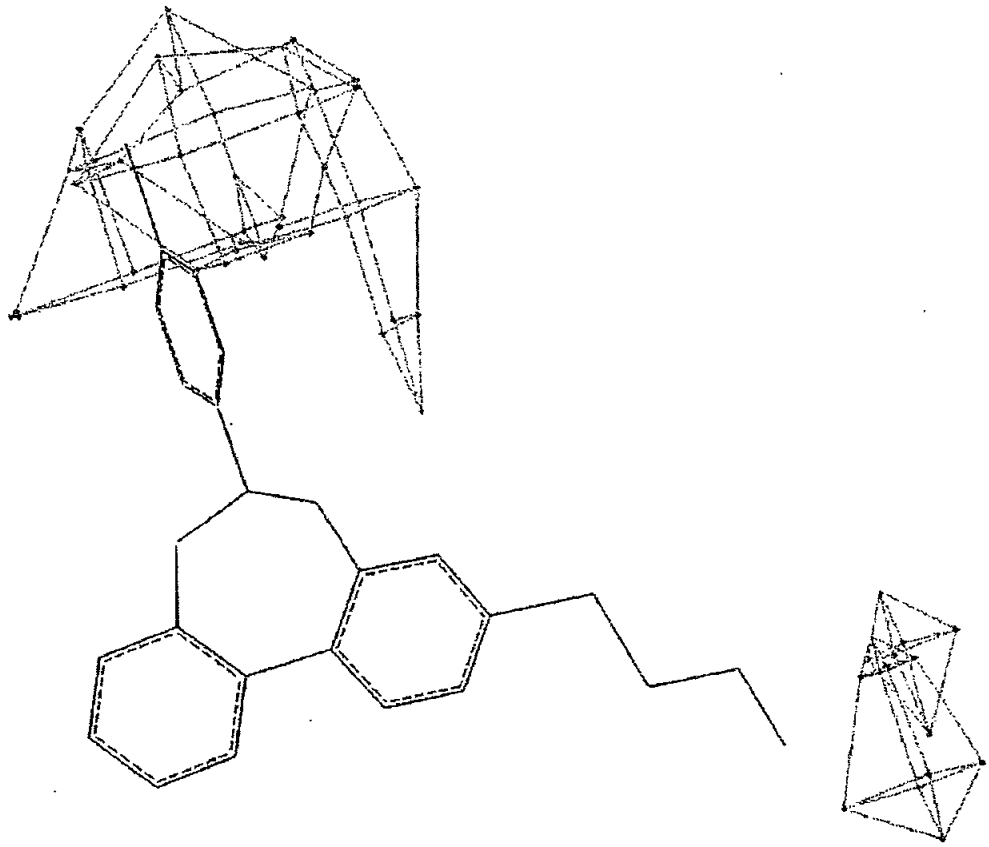


Figure 4

# TRANSCRIPTION FACTOR MODULATING COMPOUNDS AND METHODS OF USE THEREOF

## RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 60/288,660, entitled "Helix-Turn-Helix Protein Modulating Compounds and Methods of Use Thereof," filed on May 4, 2001, the entire contents of which are hereby incorporated herein by reference.

## BACKGROUND OF THE INVENTION

[0002] Multidrug resistance in bacteria is generally attributed to the acquisition of multiple transposons and plasmids bearing genetic determinants for different mechanisms of resistance (Gold et al. 1996. *N. Engl. J. Med.* 335:1445). However, descriptions of intrinsic mechanisms that confer multidrug resistance have begun to emerge. The first of these was a chromosomally encoded multiple antibiotic resistance (mar) locus in *Escherichia coli* (George and Levy, 1983. *J. Bacteriol.* 155:531; George and Levy 1983 *J. Bacteriol.* 155:541). Mar mutants of *E. coli* arose at a frequency of $10^{-6}$ to $10^{-7}$ and were selected by growth on subinhibitory levels of tetracycline or chloramphenicol (George and Levy, supra). These mutants exhibited resistance to tetracyclines, chloramphenicol, penicillins, cephalosporins, puromycin, nalidixic acid, and rifampin (George and Levy, supra). Later, the resistance phenotype was extended to include fluoroquinolones (Cohen et al. 1989. *Antimicrob. Agents Chemother.* 33:1318), oxidative stress agents (Ariza et al. 1994. *J. Bacteriol.* 176:143; Greenberg et al. 1991. *J. Bacteriol.* 73:4433), and more recently, organic solvents (White et al. 1997. *J. of Bacteriology* 179:6122; Asako, et al. 1997. *J. Bacteriol.* 176:143) and household disinfectants, e.g., pine oil and/or TRICLOSAN® (McMurry et al. 1998. *FEMS Microbiology Letters* 166:305; Moken et al. 1997. *Antimicrobial Agents and Chemotherapy* 41:2770).

[0003] The mar locus consists of two divergently positioned transcriptional units that flank a common promoter/operator region in *E. coli, Salmonella typhimurium*, and other Entrobacteriacae (Alekshun and Levy. 1997, *Antimicrobial Agents and Chemother.* 41: 2067). One operon encodes MarC, a putative integral inner membrane protein without any yet apparent function, but which appears to contribute to the Mar phenotype in some strains. The other operon comprises marRAB, encoding the Mar repressor (MarR), which binds marO and negatively regulates expression of marRAB (Cohen et al. 1994. *J. Bacteriol.* 175:1484; Martin and Rosner 1995. *PNAS* 92:5456; Seoane and Levy. 1995 *J. Bacteriol.* 177:530), an activator (MarA), which controls expression of other genes on the chromosome, e.g., the mar regulon (Cohen et al. 1994 *J. Bacteriol.* 175:1484; Gambino et. al. 1993. *J. Bacteriol.* 175:2888; Seoane and Levy, 1995 *J. Bacteriol.* 177:530), and a putative small protein (MarB) of unknown function.

[0004] Exposure of *E. coli* to several chemicals, including tetracycline and chloramphenicol (Hachler et al. 1991 *J. Bacteriol* 173(17):5532-8; Ariza, 1994, J Bacteriol; 176(1):143-8), sodium salicylate and its derivatives (Cohen, 1993, *J Bacteriol;* 175(24):7856-62) and oxidative stress agents (Seoane et al. 1995. *J Bacteriol;* 177(12):3414-9) induces the Mar phenotype. Some of these chemicals act directly at the level of MarR by interacting with the repressor and inactivating its function (Alekshun. 1999. *J. Bacteriol.* 181:3303-3306) while others (antibiotics such as tetracycline and chloramphenicol) appear to induce mar expression by an alternative mechanism (Alekshun. 1999. *J. Bacteriol.* 181:3303-3306) e.g., through a signal transduction pathway.

[0005] Once expressed, MarA activates the transcription of several genes that constitute the *E. coli* mar regulon (Alekshun, 1997, *Antimicrob. Agents Chemother.* 41:2067-2075; Alekshun, 1999, *J. Bacteriol.* 181:3303-3306). With respect to decreased antibiotic susceptibility, the increased expression of the AcrAB/TolC multidrug efflux system (Fralick, 1996, *J Bacteriol.* 178(19):5803-5; Okusu, 1996 *J Bacteriol;*178(1):306-8) and decreased synthesis of OmpF (Cohen, 1988, *J Bacteriol.;* 170(12):5416-22) an outer membrane protein, play major roles. Organic solvent tolerance, however, is attributed to MarA mediating increased expression of AcrAB, TolC, OmpX, and a 77 kDa protein (Aono, 1998, *Extremophiles;* 2(3):239-48; Aono, 1998 *J Bacteriol;* 180(4):938-44.) but is independent of OmpF levels (Asako, 1999, *Appl Environ Microbiol;* 65(1):294-6).

[0006] MarA is a member of the XylS/AraC family of transcriptional activators (Gallegos et al. 1993. *Nucleic Acids Res.* 21:807). There are more than 100 proteins within the XylS/AraC family and a defining characteristic of this group of proteins is the presence of two helix-turn-helix (HTH) DNA binding motifs. Proteins within this family activate many different genes, some of which produce antibiotic and oxidative stress resistance or control microbial metabolism and virulence (Gallegos et al. supra).

## SUMMARY OF THE INVENTION

[0007] The present invention represents an advance over the prior art by identifying transcription factor modulating compounds, such as, but not limited to helix-turn-helix protein modulating compounds, and providing novel assays that can be used to identify compounds which modulate microbial transcription factors, such as MarA family polypeptides and AraC family polypeptides. Modulation of gene transcription brought about by the modulation of transcription factors, such as helix-turn-helix domain containing proteins, can control a wide variety of cellular processes. For example, in prokaryotic cells processes such as metabolism, resistance, and virulence can be controlled.

[0008] Assays to identify compounds that are capable of modulating bacterial transcription factors would be of great benefit in the identification of agonists and antagonists that can be used to control gene transcription in both prokaryotic and eukaryotic cells.

[0009] In one embodiment, the invention pertains to a method for reducing antibiotic resistance of a cell, e.g., a eukaryotic or prokaryotic cell. In a preferred embodiment, the cell is a microbial cell. In one embodiment, the invention pertains to a method for reducing antibiotic resistance in a microbial cell, by contacting a cell with a transcription factor modulating compound, such that the antibiotic resistance of the cell is reduced. In an embodiment, the transcription factor modulating compound is of the formula (I):

A-E (I)

[0010] wherein A is a polar moiety; E is a hydrophobic moiety, and pharmaceutically acceptable salts thereof.

[0011] In another embodiment, the invention pertains to a method for modulating transcription. The method includes contacting a transcription factor with a transcription factor modulating compound, such that the transcription factor is modulated. The transcription factor modulating compound is of the formula (I):

A-E (I)

[0012] wherein A is a polar moiety; and E is a hydrophobic moiety, and pharmaceutically acceptable salts thereof.

[0013] In another embodiment, the invention also includes methods for identifying transcription factor modulating compounds. The method includes contacting a microbial cell with a test compound under conditions which allow interaction of the compound with the microbial cell and measuring the ability of the test compound to affect the cell. The microbial cell includes a selective marker under the direct control of a transcription factor responsive element and a transcription factor.

[0014] In yet another embodiment, the invention includes methods for identifying a transcription factor modulating compound. The method includes contacting a microbial cell comprising: 1) a selective marker under the control of a transcription factor responsive element and 2) a transcription factor, with a test compound under conditions which allow interaction of the compound with the microbial cell, and measuring the ability of the test compound to affect the growth (e.g., in vitro or in vivo) or survival of the microbial cell, wherein the inactivation of the transcription factor leads to a decrease in in vitro or in vivo cell survival. The invention also pertains to similar methods where the inactivation of the transcription factor leads to an increase in cell survival, as well as methods wherein the activation of the transcription factor leads to increased or, alternatively, decreased cell survival.

[0015] In another embodiment, the invention also pertains to methods for identifying a transcription factor modulating compound, by contacting a microbial cell comprising: 1) a chromosomal deletion in a guaB or purA gene, 2) heterologous guaB or purA gene under the control of its transcription factor responsive promoter, and 3) a transcription factor, with a test compound under conditions which allow interaction of the compound with the microbial cell. The method further includes the steps of measuring the ability of the compound to affect gene expression of the reporter or the growth or survival of the microbial cell as an indication of whether the compound modulates the activity of a transcription factor. The ability of the compound to modulate the activity of a transcription factor leads to an alteration in gene expression may effect cell growth or survival.

[0016] The invention pertains to transcription factor modulating compounds, HTH protein modulating compounds, and MarA family modulating compounds identified by the methods of the invention, methods of using these compounds and pharmaceutical compositions comprising these compounds. The transcription factor modulating compounds of the invention include, but are not limited to, compounds of formulae (I)-(X) and Tables 4 and 5.

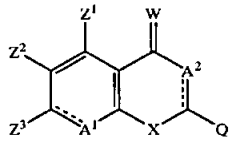
[0017] The invention also pertains to methods using computer modeling programs to identify transcription factor modulating compounds. For example, the invention pertains to a method of identifying transcription factor modulating compounds. The method includes obtaining the structure of the transcription factor modulating compound, and using or identifying a scaffold which has an interaction energy score of −20 or less with a portion of the transcription factor, thus identifying potential transcription factor modulating scaffolds.

[0018] The invention also pertains, at least in part, to a kit for identifying a transcription factor modulating compound which modulates the activity of a transcription factor polypeptide comprising a microbial cell. The kit includes 1) a selective marker under the control of a transcription factor responsive element and 2) a transcription factor.

[0019] The invention also pertains, at least in part, to pharmaceutical compositions which contain an effective amount of a transcription factor modulating compound, and, optionally, a pharmaceutically acceptable carrier. The invention also pertains to a method of inhibiting a biofilm, by administering a composition comprising a transcription factor modulating compound such that the biofilm is inhibited.

[0020] In a further embodiment, the invention pertains to a pharmaceutical composition comprising an effective amount of a transcription factor modulating compound, and a pharmaceutically acceptable carrier. The transcription factor modulating compound is of the formula (II):

(II)




[0021] wherein

[0022] W is O or S;

[0023] X is O, S, or C, optionally linked to Q;

[0024] Al is C-$Z^4$, O, or S;

[0025] $A^2$ is C-$Z^5$, or N-$Z^5$;

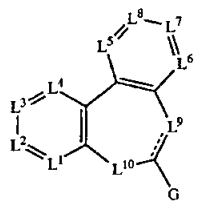
[0026] $Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

[0027] $Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

[0028] Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

[0029] In another further embodiment, the invention pertains to a pharmaceutical composition comprising an effective amount of a transcription factor modulating compound, and a pharmaceutically acceptable carrier. The compound is of the formula (III):

(III)



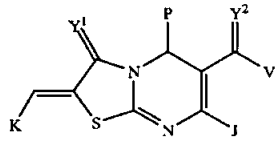
[0030] wherein

[0031] G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

[0032] $L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$, $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

[0033] In yet another embodiment, the invention pertains to a pharmaceutical composition comprising an effective amount of a transcription factor modulating compound and a pharmaceutically acceptable carrier. The transcription factor modulating compound is of the formula (IV):

(IV)



[0034] wherein

[0035] $Y^1$ and $Y^2$ are each oxygen or sulfur;

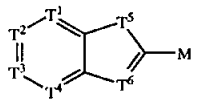
[0036] J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

[0037] V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

[0038] P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

[0039] In another embodiment, the invention pertains to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a transcription factor modulating compound. The transcription factor modulating compound is of the formula (V):

(V)



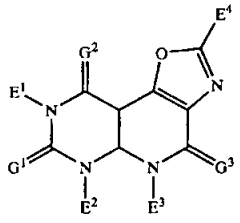
[0040] wherein

[0041] $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ are each independently substituted or unsubstituted carbon, oxygen, substituted or unsubstituted nitrogen, or sulfur;

[0042] M is hydrogen, alkyl, alkenyl, heterocyclic, alkynyl, or aryl, or pharmaceutically acceptable salts thereof.

[0043] In another embodiment, the invention pertains to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a transcription factor modulating compound. The transcription factor modulating compound may be of the formula (VI):

(VI)



[0044] wherein

[0045] $G^1$, $G^2$, and $G^3$ are each independently O, S, substituted or unsubstituted nitrogen, or substituted or unsubstituted carbon;

[0046] $E^1$, $E^2$, and $E^3$ are each independently hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, or acyl; and

[0047] $E^4$ is alkyl, alkenyl, alkynyl, aryl, halogen, cyano, amino, nitro, or acyl, and pharmaceutically acceptable salts thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0048] **FIG. 1** is a multiple sequence alignment of AraC-XylS family polypeptides.

[0049] **FIG. 2** is a multiple sequence alignment of PROSITE PS00041 and AraC family polypeptides.

[0050] **FIG. 3** is a multiple sequence alignment of PROSITE PS01124 and AraC family polypeptides.

[0051] **FIG. 4** is a CoMFA contour map for a representative triazinoxazepine.

## DETAILED DESCRIPTION OF THE INVENTION

[0052] The invention pertains, at least in part, to compounds which modulate transcription factors (e.g., helix-

turn-helix (HTH) proteins, AraC family polypeptides, MarA family polypeptides, etc.), methods of identifying the transcription factor modulating compounds (e.g., HTH protein modulating compounds, AraC family polypeptide modulating compounds, MarA family polypeptide modulating compounds, etc.), and methods of using the compounds.

[0053] 1. Transcription Factors

[0054] The term "transcription factor" includes proteins that are involved in gene regulation in both prokaryotic and eukaryotic organisms. In one embodiment, transcription factors can have a positive effect on gene expression and, thus, may be referred to as an "activator" or a "transcriptional activation factor." In another embodiment, a transcription factor can negatively effect gene expression and, thus, may be referred to as "repressors" or a "transcription repression factor." Activators and repressors are generally used terms and their functions are discerned by those skilled in the art.

[0055] The term "AraC family polypeptide,""AraC-XylS family polypeptide" or "AraC-XylS family peptide" include an art recognized group of prokaryotic transcription factors which contains more than 100 different proteins (Gallegos et al., (1997) *Micro. Mol. Biol. Rev.* 61: 393; Martin and Rosner, (2001) *Curr. Opin. Microbiol.* 4:132). AraC family polypeptides include proteins defined in the PROSITE (PS) database (http://www.expasy.ch/prosite/) as profile PS01124. The AraC family polypeptides also include polypeptides described in PS0041, HTH AraC Family 1, and PS01124, and HTH AraC Family 2. Multiple sequence alignments for the AraC-XylS family polypeptides, HTH AraC family 1, and HTH AraC family 2 are shown in FIGS. **1-3**, respectively. In an embodiment, the AraC family polypeptides are generally comprised of, at the level of primary sequence, by a conserved stretch of about 100 amino acids, which are believed to be responsible for the DNA binding activity of this protein (Gallegos et al., (1997) *Micro. Mol. Biol. Rev.* 61: 393; Martin and Rosner, (2001) *Curr. Opin. Microbiol.* 4: 132). AraC family polypeptides also may include two helix turn helix DNA binding motifs (Martin and Rosner, (2001) *Curr. Opin. Microbiol.* 4: 132; Gallegos et al., (1997) *Micro. Mol. Biol. Rev.* 61: 393; Kwon et al., (2000) *Nat. Struct. Biol.* 7: 424; Rhee et al., (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95: 10413). The term includes MarA family polypeptides and HTH proteins. In one embodiment, the invention pertains to a method for modulating an AraC family polypeptide, by contacting the AraC family polypeptide with a test compound which interacts with a portion of the polypeptide involved in DNA binding. In a further embodiment, the test compound interacts with a conserved aminoacid residue (capitalized) of the HTH AraC family 1 protein indicated in **FIG. 2**.

[0056] The term "helix-turn-helix protein,""HTH protein, ""helix-turn-helix polypeptides," and "HTH polypeptides," includes proteins comprising one or more helix-turn-helix domains. Helix-turn-helix domains are known in the art and have been implicated in DNA binding (*Ann Rev. of Biochem.* 1984. 53:293). An example of the consensus sequence for a helix-turn domain can be found in Brunelle and Schleif (1989. *J. Mol. Biol.* 209:607). The domain has been illustrated by the sequence XXXPhoAlaXXPhoGlyPhoXXXX-PhoXXPhoXX, where X is any amino acid and Pho is a hydrophobic amino acid.

[0057] The helix-turn-helix domain was the first DNA-binding protein motif to be recognized. Although originally the HTH domain was identified in bacterial proteins, the HTH domain has since been found in hundreds of DNA-binding proteins from both eukaryotes and prokaryotes. It is constructed from two alpha helices connected by a short extended chain of amino acids, which constitutes the "turn."

[0058] In one embodiment, a helix-turn-helix domain containing protein is a Mar A family polypeptide. The language "MarA family polypeptide" includes the many naturally occurring HTH proteins, such as transcription regulation proteins which have sequence similarities to MarA and which contain the MarA family signature pattern, which can also be referred to as an XylS/AraC signature pattern. An exemplary signature pattern which defines MarA family polypeptides is shown, e.g., on PROSITE and is represented by the sequence: [KRQ]-[LIVMA]-X(2)-[GSTALIV]-{FYWPGDN}X(2)-[LIVMSA]-X(4,9)-[LIVMF]-X(2)-[LIVMSTA]-X(2)-[GSTACIL]-X(3)-[GANQRF]-[LIVMFY]-X(4,5)-[LFY]-X(3)-[FYIVA]-{FYWHCM}-X(3)-[GSADENQKR]-X-[NSTAPKL]-[PARL], where X is any amino acid. MarA family polypeptides have two "helix-turn-helix" domains. This signature pattern was derived from the region that follows the first, most amino terminal, helix-turn-helix domain (HTH1) and includes the totality of the second, most carboxy terminal helix-turn-helix domain (HTH2). (See PROSITE PS00041).

[0059] The MarA family of proteins ("MarA family polypeptides") represent one subset of AraC-XylS family polypeptides and include proteins like MarA, SoxS, Rob, Rma, AarP, PqrA, etc. The MarA family polypeptides, generally, are involved in regulating resistance to antibiotics, organic solvents, and oxidative stress agents (Alekshun and Levy, (1997) *Antimicrob. Agents. Chemother.* 41: 2067). Like other AraC-XylS family polypeptides, MarA-like proteins also generally contain two HTH motifs as exemplified by the MarA and Rob crystal structures (Kwon et al., (2000) *Nat. Struct. Biol.* 7: 424; Rhee et al., (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95: 10413). Members of the MarA family can be identified by those skilled in the art and will generally be represented by proteins with homology to amino acids 30-76 and 77-106 of MarA (SEQ ID. NO. 1).

[0060] Preferably, a MarA family polypeptide or portion thereof comprises the first MarA family HTH domain (HTH1) (Brunelle, 1989, *J Mol Biol;* 209(4):607-22). In another embodiment, a MarA polypeptide comprises the second MarA family HTH domain (HTH2) (Caswell, 1992, *Biochem J.;* 287:493-509.). In a preferred embodiment, a MarA polypeptide comprises both the first and second MarA family HTH domains.

[0061] MarA family polypeptide sequences are "structurally related" to one or more known MarA family members, preferably to MarA. This relatedness can be shown by sequence or structural similarity between two MarA family polypeptide sequences or between two MarA family nucleotide sequences that specify such polypeptides. Sequence similarity can be shown, e.g., by optimally aligning MarA family member sequences using an alignment program for purposes of comparison and comparing corresponding positions. To determine the degree of similarity between sequences, they will be aligned for optimal comparison purposes (e.g., gaps may be introduced in the sequence of

one protein for nucleic acid molecule for optimal alignment with the other protein or nucleic acid molecules). The amino acid residues or bases and corresponding amino acid positions or bases are then compared. When a position in one sequence is occupied by the same amino acid residue or by the same base as the corresponding position in the other sequence, then the molecules are identical at that position. If amino acid residues are not identical, they may be similar. As used herein, an amino acid residue is "similar" to another amino acid residue if the two amino acid residues are members of the same family of residues having similar side chains. Families of amino acid residues having similar side chains have been defined in the art (see, for example, Altschul et al. 1990. *J. Mol. Biol.* 215:403) including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan). The degree (percentage) of similarity between sequences, therefore, is a function of the number of identical or similar positions shared by two sequences (i.e., % homology=of identical or similar positions/total # of positions×100). Alignment strategies are well known in the art; see, for example, Altschul et al. supra for optimal sequence alignment.

**[0062]** MarA family polypeptides may share some amino acid sequence similarity with MarA. The nucleic acid and amino acid sequences of MarA as well as other MarA family polypeptides are available in the art. For example, the nucleic acid and amino acid sequence of MarA can be found, e.g., on GeneBank (accession number M96235 or in Cohen et al. 1993. *J. Bacteriol.* 175:1484, or in SEQ ID NO:1 and SEQ ID NO:2.

**[0063]** The nucleic acid and/or amino acid sequences of MarA can be used as "query sequences" to perform a search against databases (e.g., either public or private) to, for example, identify other MarA family members having related sequences. Such searches can be performed, e.g., using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to MarA family nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to MarA protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

**[0064]** MarA family members can also be identified as being similar based on their ability to specifically hybridize to nucleic acid sequences specifying MarA. Such stringent conditions are known to those skilled in the art and can be found e.g., in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6×sodium chloride/sodium citrate (SSC) at about 45° C., followed by one or more washes in 0.2× SSC, 0.1% SDS at 50-65° C. Conditions for hybridizations are largely dependent on the melting temperature Tm that is observed for half of the molecules of a substantially pure population of a double-stranded nucleic acid. Tm is the temperature in ° C. at which half the molecules of a given sequence are melted or single-stranded. For nucleic acids of sequence 11 to 23 bases, the Tm can be estimated in degrees C. as 2(number of A+T residues)+4(number of C+G residues). Hybridization or annealing of nucleic acid molecules should be conducted at a temperature lower than the Tm, e.g., 15° C., 20° C., 25° C. or 30° C. lower than the Tm. The effect of salt concentration (in M of NaCl) can also be calculated, see for example, Brown, A., "Hybridization" pp. 503-506, in *The Encyclopedia of Molec. Biol.*, J. Kendrew, Ed., Blackwell, Oxford (1994).

**[0065]** Preferably, the nucleic acid sequence of a MarA family member identified in this way is at least about 10%, 20%, more preferably at least about 30%, more preferably at least about 40% identical and preferably at least about 50%, or 60% identical to a MarA nucleotide sequence. In preferred embodiments, the nucleic acid sequence of a MarA family member is at least about 70%, 80%, preferably at least about 90%, more preferably at least about 95% identical with a MarA nucleotide sequence. Preferably, MarA family members have an amino acid sequence at least about 20%, preferably at least about 30%, more preferably at least about 40% identical and preferably at least about 50%, or 60% or more identical with a MarA amino acid sequence. In preferred embodiments, the nucleic acid sequence of a MarA family member is at least about 70%, 80%, more preferably at least about 90%, or more preferably at least about 95% identical with a MarA nucleotide sequence. However, it will be understood that the level of sequence similarity among microbial regulators of gene transcription, even though members of the same family, is not necessarily high. This is particularly true in the case of divergent genomes where the level of sequence identity may be low, e.g., less than 20% (e.g., *B. burgdorferi* as compared e.g., to *B. subtilis*). Accordingly, structural similarity among MarA family members can also be determined based on "three-dimensional correspondence" of amino acid residues. As used herein, the language "three-dimensional correspondence" is meant to includes residues which spatially correspond, e.g., are in the same position of a MarA family polypeptide member as determined, e.g., by x-ray crystallography, but which may not correspond when aligned using a linear alignment program. The language "three-dimensional correspondence" also includes residues which perform the same function, e.g., bind to DNA or bind the same cofactor, as determined, e.g., by mutational analysis.

**[0066]** Exemplary MarA family polypeptides are shown in Table 1, FIGS. 1-3, and at Prosite (PS00041) and include: AarP, Ada, AdaA, AdiY, AfrR, AggR, AppY, AraC, CafR, CelD, CfaD, CsvR, D90812, EnvY, ExsA, FapR, HrpB, InF, InvF, LcrF, LumQ, MarA, MelR, MixE, MmsR, MsmR, OrfR, Orf f375, PchR, PerA, PocR, PqrA, RafR, RamA, RhaR, RhaS, Rns, Rob, SoxS, S52856, TetD, TcpN, TheR, TmbS, U73857, U34257, U21191, UreR, VirF, XylR, XylS, Xys1, 2, 3, 4, Ya52, YbbB, YfiF, YisR, YzbC, and YijO. The nucleotide and amino acid sequences of the *E. coli* Rob molecule are shown in SEQ ID NO:3 and 4, respectively.

TABLE 1

Some Bacterial MarA homologs*

| Gram-negative bacteria | | Gram-positive bacteria |
|---|---|---|
| *Escherichia coli* | *Klebsiella pneumoniae* | *Lactobacillus helveticus* |
| **MarA** (1) | **RamA** (27) | **U34257** (38) |
| **OrfR** (2, 3) | | |
| **SoxS** (4, 5) | *Haemophilus influenzae* | *Azorhizobium caulinodans* |
| AfrR (6) | Ya52 (28) | S52856 (39) |
| AraC (7) | | |
| CelD (8) | *Yersinia spp.* | *Streptomyces spp.* |
| D90812 (9) | CafR (29) | **U21191** (40) |
| FapR (10, 11) | LcrF (30) or VirF (30) | AraL (41) |
| MelR (12) | | |
| ORF f375 (13, 14) | *Providencia stuartii* | *Streptococcus mutans* |
| RhaR (15, 16, 17) | **AarP** (31) | MsmR (42) |
| RhaS (18) | | |
| Rob (19) | *Pseudomonas spp.* | *Pediococcus pentosaceus* |
| U73857 (20) | MmsR (32) | RafR (43) |
| XylR(21) | TmbS (33) | |
| YijO (22) | XylS (34) | *Photobacterium leiognathi* |
| | Xysl, 2, 3, 4 (35, 36) | LumQ (44) |
| *Proteus vulgaris* | | |
| **PqrA** (23) | Cyanobacteria *Synechocystis spp.* | *Bacillus subtilis* AdaA (45) |
| *Salmonella typhimurium* | **LumQ** (37) | YbbB (46) |
| **MarA** (24) | PchR (37) | YfiF (47) |
| InvF (25) | | YisR (48) |
| PocR (26) | | YzbC (49) |

*The smaller MarA homologs, ranging in size from 87 (U34257) to 138 (OrfR) amino acid residues, are represented in boldface. References are given in parentheses and are listed below.

[0067] References for Table 1:


[0068] (1) S. P. Cohen, et al. 1993. *J. Bacteriol.* 175:1484-1492

[0069] (2) G. M. Braus, et al. 1984. *J. Bacteriol.* 160:504-509

[0070] (3) K. Schollmeier, et al., 1984. *J. Bacteriol.* 160:499-503

[0071] (4) C. F. Amabile-Cuevas, et al., 1991. *Nucleic Acids Res.* 19:4479-4484

[0072] (5) J. Wu, et al., 1991. *J. Bacteriol.* 173:2864-2871

[0073] (6) M. K. Wolf, et al., 1990. *Infect. Immun.* 58:1124-1128

[0074] (7) C. M. Stoner, et al. 1982. *J. Mol. Biol.* 153:649-652

[0075] (8) L. L. Parker, et al., 1990. *Genetics* 123:455-471

[0076] (9) H. Mori, 1996. Unpublished data taken from the NCBI databases

[0077] (10) P. Klaasen, et al., 1990. *Mol. Microbiol.* 4:1779-1783

[0078] (11) M. Ahmed, et al., 1994. *J. Biol. Chem* 269-28506-28513

[0079] (12) C. Webster, et al., 1989. *Gene* 83:207-213

[0080] (13) G. Plunkett, III. 1995. Unpublished

[0081] (14) C Garcia-Martin, et al., 1992. *J. Gen. Microbiol.* 138:1109-1116

[0082] (15) G. Plunkett, III., et al. 1993. *Nucleic Acids Res.* 21:3391-3398

[0083] (16) C. G. Tate, et al. 1992. *J. Biol. Chem.* 267:6923-6932

[0084] (17) J. F. Tobin et al., 1987. *J. Mol. Biol.* 196:789-799

[0085] (18) J. Nishitani, 1991. *Gene* 105:37-42

[0086] (19) R. E. Benz, et al., 1993. *Zentralbl Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt.*1 *Orig.* 278:187-196

[0087] (20) M. Duncan, et al., 1996. Unpublished data

[0088] (21) H. J. Sofia, et al., 1994. *Nucleic Acids Res.* 22:2576-2586

[0089] (22) F. R. Blattner, et al., 1993. *Nucleic Acids Res.* 21:5408-5417

[0090] (23) H. Ishida, et al, 1995. *Antimicrob. Agents Chemother.* 39:453-457

[0091] (24) M. C. Sulavik, et al., 1997. *J. Bacteriol.* 179:1857-1866

[0092] (25) K. Kaniga, et al., 1994. *Mol. Microbiol.* 13:555-568

[0093] (26) J. R. Roth, et al. 1993. *J. Bacteriol.* 175:3303-3316

[0094] (27) A. M. George, et al., 1983. J. Bacteriol. 155:541-548

[0095] (28) R. D. Fleischmann, et al., 1995. *Science* 269:469-512

[0096] (29) E. E. Galyov, et al., 1991. *FEBS Lett.* 286:79-82

[0097] (30) N. P. Hoe, et al., 1992. *J. Bacteriol.* 174:4275-4286

[0098] (31) G. Cornelis, et al., 1989. *J. Bacteriol.* 171:254-262

[0099] (32) D. R. Macinga, et al., 1995. *J. Bacteriol.* 177:3407-3413

[0100] (33) M. I. Steele, et al., 1992. *J. Biol. Chem.* 267:13585-13592

[0101] (34) G. Deho, et al., 1995. Unpublished data

[0102] (35) N. Mermod, et al., 1984. *EMBO J.* 3:2461-2466

[0103] (36) S. J. Assinder, et al., 1992. *Nucleic Acids Res.* 20:5476

[0104] (37) S. J. Assinder, et al., 1993. *J. Gen. Microbiol.* 139:557-568

[0105] (38) E. G. Dudley, et al., 1996. *J. Bacteriol.* 178:701-704

[0106] (39) D. Geelen, et al., 1995. Unpublished data

[0107] (40) J. Kormanec, et al., 1995. *Gene* 165:77-80

[0108] (41) C. W. Chen, et al., 1992. *J. Bacteriol.* 174:7762-7769

[0109] (42) R. R. Russell, et al., 1992. *J. Biol. Chem,* 267:4631-4637

[0110] (43) K. K. Leenhouts, et al., 1995. Unpublished data

[0111] (44) J. W. Lin, et al., 1995. *Biochem. Biophys. Res. Commun.* 217:684-695

[0112] (45) F. Morohoshi, et al. 1990. *Nucleic Acids Res.* 18:5473-5480

[0113] (46) M. Rosenberg, et al., 1979. *Annu. Rev. Genet.* 13:319-353

[0114] (47) H. Yamamoto, et al., 1996. *Microbiolog* 142:1417-1421

[0115] (48) L. B. Bussey, et al., 1993. *J. Bacteriol.* 175:6348-6353

[0116] (49) P. G. Quirk, et al., 1994. *Biochim. Biophys. Acta* 1186:27-34

[0117] The term "transcription factor modulating compound" or transcription factor modulator" includes HTH protein modulating compounds, HTH protein modulators. Transcription factor modulating compounds include compounds which interact with one or more transcription factors, such that the activity of the transcription factor is modulated, e.g., enhanced or inhibited. The term also includes both AraC family modulating compounds and MarA family modulating compounds. In one embodiment, the transcription factor modulating compound is an inhibiting compound of a transcription factor, e.g., a prokaryotic transcription factor or a eukaryotic transcription activation factor. In one embodiment, the transcription factor modulating compounds modulate the activity of a transcription factor as measured by assays known in the art or LANCE assays such as those described in Example 8. In one embodiment, the transcription factor modulating compound inhibits a particular transcription factor by about 10% or greater, about 40% or greater, about 50% or greater, about 60% or greater, about 70% or greater, about 80% or greater, about 90% or greater, about 95% or greater, or about 100% as compared to the activity of the transcription factor with out the transcription factor modulating compound. In another embodiment, the transcription factor modulating compound inhibits biofilm formation. In one embodiment, the transcription factor modulating compound inhibits biofilm formation as measured by assays known in the art or the Crystal Violet assay described in Example 7. In one embodiment, the transcription factor of the invention inhibits the formation of a biofilm by about 25% or more, 50% or more, 75% or more, 80% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more, 99.9% or more, 99.99% or more, or by 100%, as compared to the formation of a biofilm without the transcription factor modulating compound.

[0118] The term "HTH protein modulating compound" or "HTH protein modulator" includes compounds which interact with one or more HTH proteins such that the activity of the HTH protein is modulated, e.g., enhanced or, inhibited. In one embodiment, the HTH protein modulating compound is a MarA family polypeptide modulating compound. In one embodiment, the activity of the HTH protein is enhanced when it interacts with the HTH protein modulating compound. For example, the activity of the HTH protein may be increased by greater than 10%, greater the 20%, greater than 50%, greater than 75%, greater than 80%, greater than 90%, or 100% of the activity of the HTH protein in the absence of the HTH modulating compound. In another embodiment, the activity of the HTH protein is decreased upon an interaction with the HTH protein modulating compound. In an embodiment, the activity of the HTH protein is decreased by about 25% or more, 50% or more, 75% or more, 80% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more, 99.9% or more, 99.99% or more, or by 100%, as compared to the activity of the protein of a HTH protein when not contacted with an HTH modulating compound of the invention using techniques and assays described herein. Values and ranges included and/or intermediate of the values set forth herein are also intended to be within the scope of the present invention.

[0119] The term "MarA family polypeptide modulating compound" or "MarA family modulating compound" include compounds which interact with one or more MarA family polypeptides such that the activity of the MarA family peptide is enhanced or inhibited. In an embodiment, the MarA family polypeptide modulating compound is an inhibiting compound. In a further embodiment, the MarA family inhibiting compound is an inhibitor of MarA, Rob, and/or SoxS. In another embodiment, the MarA family polypeptide modulating compound modulates the expression of luciferase in the Luciferase Assay described in Example 9. In one embodiment, the MarA family polypeptide modulating compound decreases luciferase expression by greater than 10%, greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90% or about 100%.

[0120] The term "polypeptide(s)" refers to a peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" includes both short chains, commonly referred to as peptides, oligopeptides and oligomers and longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-

carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, Proteins—Structure And Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Posttranslational Covalent Modification Of Proteins, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al., Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

**[0121]** Preferred polypeptides (and the nucleic acid molecules that encode them) are "naturally occurring." As used herein, a "naturally-occurring" molecule refers to a molecule having an amino acid or a nucleotide sequence that occurs in nature (e.g., a natural polypeptide). In addition, naturally or non-naturally occurring variants of the polypeptides and nucleic acid molecules which retain the same functional activity, (such as, the ability to bind to target nucleic acid molecules (e.g., comprising a marbox) or to polypeptides (e.g RNA polymerase) with a naturally occurring polypeptide are provided for. Such immunologic cross-reactivity can be demonstrated, e.g., by the ability of a variant to bind to a MarA family polypeptide responsive element. Such variants can be made, e.g., by mutation using techniques that are known in the art. Alternatively, variants can be chemically synthesized.

**[0122]** As used herein the term "variant(s)" includes nucleic acid molecules or polypeptides that differ in sequence from a reference nucleic acid molecule or polypeptide, but retain its essential properties. Changes in the nucleotide sequence of the variant may, or may not, alter the amino acid sequence of a polypeptide encoded by the reference nucleic acid molecule. Nucleotide or amino acid changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by a naturally occurring reference sequence. A typical variant of a polypeptide differs in amino acid sequence from a reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, and/or deletions in any combination.

**[0123]** A variant of a nucleic acid molecule or polypeptide may be naturally occurring, such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acid molecules and polypeptides may be made from a reference nucleic acid molecule or polypeptide by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to skilled artisans. Alternatively, variants can be chemically synthesized. For instance, artificial or mutant forms of autologous polypeptides which are functionally equivalent, (e.g., have the ability to interact with a MarA family polypeptide responsive element) can be made using techniques which are well known in the art.

**[0124]** Mutations can include, e.g., at least one discrete point mutation which can give rise to a substitution, or by at least one deletion or insertion. For example, mutations can also be made by random mutagenesis or using cassette mutagenesis. For the former, the entire coding region of a molecule is mutagenized by one of several methods (chemical, PCR, doped oligonucleotide synthesis) and that collection of randomly mutated molecules is subjected to selection or screening procedures. In the latter, discrete regions of a polypeptide, corresponding either to defined structural or functional determinants are subjected to saturating or semi-random mutagenesis and these mutagenized cassettes are re-introduced into the context of the otherwise wild type allele. In one embodiment, PCR mutagenesis can be used. For example, Megaprimer PCR can be used (O. H. Landt, 1990. Gene 96:125-128).

**[0125]** In preferred embodiments, a MarA family polypeptide excludes one or more of XylS, AraC, and MelR. In other preferred embodiments, a MarA family polypeptide is involved in antibiotic resistance. In particularly preferred embodiments, a MarA family polypeptide is selected from the group consisting of: MarA, RamA, AarP, Rob, SoxS, and PqrA.

**[0126]** The language "activity of a transcription factor" includes the ability of a transcription factor to interact with DNA, e.g., to bind to a transcription factor responsive promoter, or to initiate transcription from such a promoter. The language expressly includes the activities of AraC family polypeptides, HTH proteins and MarA family polypeptides.

**[0127]** The language "activity of a MarA family polypeptide" includes the ability of the MarA family polypeptide to interact with DNA, e.g., to bind to a MarA family polypeptide responsive promoter, or to initiate transcription from such a promoter. MarA functions both as a transcriptional activator (e.g., upregulating genes such as inaA, galT, micF, etc.) and as a repressor (e.g., downregulating genes such as fecA, purA, guaB, etc.) (Alekshun, 1997, *Antimicrob. Agents Chemother.* 41:2067-2075; Barbosa & Levy, *J. Bact.* 2000, Vol. 182, p. 3467-3474; Pomposiello et al. *J. Bact.* 2001, Vol 183, p. 3890-3902).

**[0128]** The language "transcription factor responsive element" includes a nucleic acid sequence which can interact with promoters or enhancers which are involved in initiating transcription of an operon in a microbe. The language includes marA family polypeptide responsive elements.

**[0129]** The language "marA family polypeptide responsive element" includes a nucleic acid sequence which can interact with marA, e.g., promoters or enhancers which are involved in regulating transcription of a nucleic acid sequence in a microbe. MarA responsive elements comprise approximately 16 base pair marbox sequence, the sequence critical for the binding of MarA to its target. In addition, a secondary site, the accessory marbox, upstream of the primary marbox contributes to basal and derepressed mar transcription. A marbox may be situated in either the forward

or backward orientation. (Martin, 1999, *Mol. Microbiol.* 34:431-441). In the marRAB operon, the marbox is in the backward orientation and is thus located on the sense strand with respect to marRAB (Martin, 1999, *Mol. Microbiol.* 34:431-441). Subtle differences within the marbox sequence of particular promoters may account for differential regulation by MarA and other related, e.g., SoxS and Rob, transcription factors (Martin, 2000, *Mol Microbiol;* 35(3):623-34). In one embodiment, MarA family responsive elements are promoters that are structurally or functionally related to a marA promoter, e.g., interact with MarA or a protein related to MarA. Preferably, the marA family polypeptide responsive element is a marRAB promoter. For example, in the mar operon, several promoters are marA family polypeptide responsive promoters as defined herein, e.g., the 405-bp ThaI fragment from the marO region is a marA family responsive promoter (Cohen et al. 1993. *J. Bact.* 175:7856). In addition, MarA has been shown to bind to a 16 bp MarA binding site (referred to as the "marbox" within marO (Martin et al. 1996. *J. Bacteriol.* 178:2216). MarA also affects transcription from the acrAB; micF, mir 1,2,3; sip; nfo; inaA; fir; sodA; soi-I 7,19; zwf; fum C; or rpsF promoters (Alekshun and Levy. 1997. *Antimicrobial Agents and Chemother.* 41:2067). Other marA family responsive promoters are known in the art and include: araBAD, araE, araFGH and araC, which are activated by AraC; Pm, which is activated by XylS; melAB which is activated by MelR; and oriC which is bound by Rob.

**[0130]** The language "MarA family polypeptide responsive promoter" also includes portions of the above promoters which are sufficient to activate transcription upon interaction with a MarA family member protein. The portions of any of the MarA family polypeptide-responsive promoters which are minimally required for their activity can be easily determined by one of ordinary skill in the art, e.g., using mutagenesis. Exemplary techniques are described by Gallegos et al. (1996, *J. Bacteriol.* 178:6427). A "MarA family polypeptide responsive promoter" also includes non-naturally occurring variants of MarA family polypeptide responsive promoters which have the same function as naturally occurring MarA family promoters. Preferably such variants have at least 30% or greater, 40% or greater, or 50% or greater, nucleotide sequence identity with a naturally occurring MarA family polypeptide responsive promoter. In preferred embodiments, such variants have at least about 70% nucleotide sequence identity with a naturally occurring MarA family polypeptide responsive promoter. In more preferred embodiments, such variants have at least about 80% nucleotide sequence identity with a naturally occurring MarA family polypeptide responsive promoter. In particularly preferred embodiments, such variants have at least about 90% nucleotide sequence identity and preferably at least about 95% nucleotide sequence identity with a naturally occurring MarA family polypeptide responsive promoter. In yet other embodiments nucleic acid molecules encoding variants of MarA family polypeptide responsive promoters are capable of hybridizing under stringent conditions to nucleic acid molecules encoding naturally occurring MarA family polypeptide responsive promoters.

**[0131]** The term "interact" includes close contact between molecules that results in a measurable effect, e.g., the binding of one molecule with another. For example, a MarA family polypeptide can interact with a MarA family polypeptide responsive element and alter the level of transcription of DNA. Likewise, compounds can interact with a MarA family polypeptide and alter the activity of a MarA family polypeptide.

**[0132]** The term "inducible promoter" includes promoters that are activated to induce the synthesis of the genes they control. As used herein, the term "constitutive promoter" includes promoters that do not require the presence of an inducer, e.g., are continuously active.

**[0133]** The terms "heterologous DNA" or "heterologous nucleic acid" includes DNA that does not occur naturally in the cell (e.g., as part of the genome) in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature or which is operatively linked to DNA to which it is not normally linked in nature (i.e., a gene that has been operatively linked to a heterologous promoter). Heterologous DNA is 1) not naturally occurring in a particular position (e.g., at a particular position in the genome) or 2) is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA can be from the same species or from a different species. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by the term heterologous DNA.

**[0134]** The terms "heterologous protein", "recombinant protein", and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid molecule.

**[0135]** The term "microbe" includes microorganisms expressing or made to express a transcription factor, araC family polypeptide, HTH protein, or a marA family polypeptide. "Microbes" are of some economic importance, e.g., are environmentally important or are important as human pathogens. For example, in one embodiment microbes cause environmental problems, e.g., fouling or spoilage, or perform useful functions such as breakdown of plant matter. In another embodiment, microbes are organisms that live in or on mammals and are medically important. Preferably microbes are unicellular and include bacteria, fungi, or protozoa. In another embodiment, microbes suitable for use in the invention are multicellular, e.g., parasites or fungi. In preferred embodiments, microbes are pathogenic for humans, animals, or plants. Microbes may be used as intact cells or as sources of materials for cell-free assays. In one embodiment, the microbes include prokaryotic organisms. In other embodiments, the microbes include eukaryotic organisms.

**[0136]** The term selective marker includes polypeptides that serve as indicators, e.g., provide a selectable or screenable trait when expressed by a cell. The term "selective marker" includes both selectable markers and counterselectable markers. As used herein the term "selectable marker" includes markers that result in a growth advantage when a compound or molecule that fulfills the test parameter of the assay is present. The term "counterselectable marker" includes markers that result in a growth disadvantage unless a compound or molecule is present which disrupts a condi-

tion giving rise to expression of the counterselectable marker. Exemplary selective markers include cytotoxic gene products, gene products that confer antibiotic resistance, gene products that are essential for growth, gene products that confer a selective growth disadvantage when expressed in the presence of a particular metabolic substrate (e.g., the expression of the URA3 gene confers a growth disadvantage in the presence of 5-fluoroorotic acid).

**[0137]** The term "test compound" includes any reagent or test agent which is employed in the assays of the invention and assayed for its ability to influence the activity of a transcription factor, e.g., an AraC family polypeptide, an HTH protein, or a MarA family polypeptide, e.g., by binding to the polypeptide or to a molecule with which it interacts. More than one compound, e.g., a plurality of compounds, can be tested at the same time for their ability to modulate the activity of a transcription factor, e.g., an AraC family polypeptide, an HTH protein, or a MarA family polypeptide, activity in a screening assay. In an advantageous embodiment, the test compound is a MarA family modulating compound.

**[0138]** Test compounds that can be tested in the subject assays include antibiotic and non-antibiotic compounds. In one embodiment, test compounds include candidate detergent or disinfectant compounds. Exemplary test compounds which can be screened for activity include, but are not limited to, peptides, non-peptidic compounds, nucleic acids, carbohydrates, small organic molecules (e.g., polyketides), and natural product extract libraries. The term "non-peptidic test compound" includes compounds that are comprised, at least in part, of molecular structures different from naturally-occurring L-amino acid residues linked by natural peptide bonds. However, "non-peptidic test compounds" also include compounds composed, in whole or in part, of peptidomimetic structures, such as D-amino acids, non-naturally-occurring L-amino acids, modified peptide backbones and the like, as well as compounds that are composed, in whole or in part, of molecular structures unrelated to naturally-occurring L-amino acid residues linked by natural peptide bonds. "Non-peptidic test compounds" also are intended to include natural products. The term "antagonist" includes transcription factor modulating compounds (e.g., AraC family polypeptide modulating compounds, HTH protein modulating compounds, MarA family polypeptide modulating compounds, etc.) which inhibit the activity of a transcription factor by binding to and inactivating the transcription factor (e.g., an AraC family modulating compound, an MarA family polypeptide modulating compound, etc.), by binding to a nucleic acid target with which the transcription factor interacts (e.g., for MarA, a marbox), by disrupting a signal transduction pathway responsible for activation of a particular regulon (e.g., for Mar, the inactivation of MarR or activation of MarA synthesis), and/or by disrupting a critical protein-protein interaction (e.g., MarA-RNA polymerase interactions that are required for MarA to function as a transcription factor.) Antagonists may include, for example, naturally or chemically synthesized compounds such as small cell permeable organic molecules, nucleic acid interchelators, peptides, etc.

**[0139]** The term "agonist" includes transcription factor modulating compounds (e.g., AraC family polypeptide modulating compounds, HTH protein modulating compounds, MarA family polypeptide modulating compounds, etc.) which promote the activity of a transcription factor by binding to and activating the transcription factor (e.g., an AraC family modulating compound, an MarA family polypeptide modulating compound, etc.), by binding to a nucleic acid target with which the transcription factor interacts (e.g., for MarA, a marbox), by facilitating a signal transduction pathway responsible for activation of a particular regulon (e.g., for Mar, the inactivation of MarR or activation of MarA synthesis), and/or by facilitating a critical protein-protein interaction (e.g., MarA-RNA polymerase interactions that are required for MarA to function as a transcription factor.) Agonists may include, for example, naturally or chemically synthesized compounds such as small cell permeable organic molecules, nucleic acid interchelators, peptides, etc.

**[0140]** II. MarA Family polypeptide Helix-Turn-Helix Domains

**[0141]** Helix-turn-helix domains are known in the art and have been implicated in DNA binding (*Ann Rev. of Biochem.* 1984. 53:293). An example of the consensus sequence for a helix-turn domain can be found in Brunelle and Schleif (1989, *J. Mol. Biol.* 209:607). The domain has been illustrated by the sequence XXXPhoAlaXXPhoGlyPhoXXXX-PhoXXPhoXX, where X is any amino acid and Pho is a hydrophobic amino acid.

**[0142]** The crystal structure of MarA has been determined and the first (most amino terminal) HTH domain of MarA has been identified as comprising from about amino acid 31 to about amino acid 52 and the second HTH domain of MarA has been identified as comprising from about amino acid 79 to about amino acid 102 (Rhee et al. 1998. *Proc. Natl. Acad. Sci. USA.* 95:10413).

**[0143]** Locations of the helix-turn-helix domains in other MarA family members as well as other HTH proteins can easily be found by one of skill in the art. For example using the MarA protein sequence and an alignment program, e.g., the ProDom program or other programs known in the art, a portion of the MarA amino acid sequence e.g., comprising one or both HTH domains of MarA (such as from about amino acid 30 to about amino acid 107 of MarA) to produce an alignment. Using such an alignment, the amino acid sequences corresponding to the HTH domains of MarA can be identified in other MarA family member proteins. An exemplary consensus sequence for the first helix-turn-helix domain of a MarA family polypeptide can be illustrated as XXXXAXXXXXSXXXLXXXFX, where X is any amino acid. An exemplary consensus sequence for the second helix-turn-helix domain of a MarA family polypeptide is illustrated as XXIXXIAXXXGFXSXXXFXXX[F/Y], where X is any amino acid. Preferably, a MarA family polypeptide first helix-turn-helix domain comprises the consensus sequence E/D-X-V/L-A-D/E-X-A/S-G-X-S-X3-L-Q-X2-F-K/R/E-X2-T/I. Preferably, a MarA family polypeptide second helix-turn-helix domain comprises the consensus sequence I-X-D-1-A-X3-G-F-X-S-X2-F-X3-F-X4.

**[0144]** In an embodiment, a MarA family member HTH domain is a MarA HTH domain. The first and second helix-turn-helix domains of MarA are, respectively, EKVS-ERSGYSKWHLQRMFKKET and ILYLAERYG-FESQQTLTRTFKNYF. Other exemplary MarA family helix-turn-helix domains include: about amino acid 210 to

about amino acid 229 and about amino acid 259 to about amino acid 278 of MelR; about amino acid 196 to about amino acid 215 and about amino acid 245 to about amino acid 264 of AraC; and about amino acid 230 to about amino acid 249 (or 233-253) and about amino acid 281 to about amino acid 301 (or 282-302) of XylS (see e.g., Brunelle et al. 1989. *J. Mol. Biol.* 209:607; Niland et al. 1996. *J. Mol. Biol.* 264:667; Gallegos et al. 1997. *Microbiology and Molecular Biology Reviews.* 61:393).

**[0145]** "MarA family polypeptide helix-turn-helix domains" are derived from or are homologous to the helix-turn-helix domains found in the MarA family polypeptides as described supra. In preferred embodiments, a MarA family polypeptide excludes one or more of XylS, AraC, and MelR. In particularly preferred embodiments, a MarA family polypeptide is selected from the group consisting of: MarA, RamA, AarP, Rob, SoxS, and PqrA.

**[0146]** Both of the helix-turn-helix domains present in MarA family polypeptides are in the carboxy terminal end of the protein. Proteins or portions thereof comprising either or both of these domains can be used in the instant methods. In certain embodiments, a polypeptide which is used in screening for compounds comprises the helix-turn-helix domain most proximal to the carboxy terminus (HTH2) of the MarA family polypeptide from which it is derived. In other embodiments, such a polypeptide comprises the helix-turn-helix domain most proximal to the amino terminus (HTH1) of the MarA family polypeptide from which it is derived. In one embodiment, other polypeptide sequences may also be present, e.g., sequences that might facilitate immobilizing the domain on a support, or, alternatively, might facilitate the purification of the domain.

**[0147]** In an embodiment, such a polypeptide consists essentially of the helix-turn-helix domain most proximal to the carboxy terminus of the MarA family polypeptide from which it is derived. In other preferred embodiments, such a polypeptide consists essentially of the helix-turn-helix domain most proximal to the amino terminus of the MarA family polypeptide from which it is derived.

**[0148]** In an embodiment, such a polypeptide consists of the helix-turn-helix domain most proximal to the carboxy terminus of the AraC family polypeptide or MarA family polypeptide from which it is derived. In other preferred embodiments, such a polypeptide consists of the helix-turn-helix domain most proximal to the amino terminus of the AraC family polypeptide or MarA family polypeptide from which it is derived.

**[0149]** MarA family polypeptide or AraC family polypeptide helix-turn-helix domains can be made using techniques which are known in the art. The nucleic acid and amino acid sequences of transcription factors, such as MarA family polypeptides, are available, for example, from GenBank. Using this information, the helix-turn-helix consensus motif and mutational analysis provided herein, one of ordinary skill in the art can identify MarA family or AraC family polypeptide helix-turn-helix domains.

**[0150]** In certain embodiments of the invention it will be desirable to obtain "isolated or recombinant" nucleic acid molecules encoding transcription factors or portions thereof (e.g., HTH protein helix-turn-helix domains, AraC family helix-turn-helix domains, MarA family helix-turn-helix domains or mutant forms thereof). By "isolated or recombinant" is meant a nucleic acid molecule which has been (1) amplified in vitro by, for example, polymerase chain reaction (PCR); (2) recombinantly produced by cloning, or (3) purified, as by cleavage and gel separation; or (4) synthesized by, for example, chemical synthesis. Such a nucleic acid molecule is isolated from the sequences which naturally flank it in the genome and from cellular components.

**[0151]** The isolated or recombinant nucleic acid molecules encoding transcription factors (e.g., HTH protein helix-turn-helix domains, AraC family helix-turn-helix domains, MarA family helix-turn-helix domains or mutant forms thereof) can then, for example, be utilized in binding assays, can be expressed in a cell, or can be expressed on the surface of phage, as discussed further below.

**[0152]** In yet other embodiments of the invention, it will be desirable to obtain a substantially purified or recombinant HTH protein helix-turn-helix domains (e.g., MarA family helix-turn-helix domains or mutant forms thereof). Such polypeptides, for example, can be purified from cells which have been engineered to express an isolated or recombinant nucleic acid molecule which encodes a HTH protein helix-turn-helix domain (e.g., MarA family helix-turn-helix domain or mutant forms thereof). For example, as described in more detail below, a bacterial cell can be transformed with a plasmid which encodes a MarA family helix-turn-helix domain. The MarA family helix-turn-helix protein can then be purified from the bacterial cells and used, for example, in the cell-free assays described herein.

**[0153]** Purification of a HTH protein helix-turn-helix domain (e.g., MarA family helix-turn-helix domain) can be accomplished using techniques known in the art. For example, column chromatography could be used, or antibodies specific for the domain or for a polypeptide fused to the domain can be employed, for example on a column or in a panning assay.

**[0154]** In preferred embodiments, cells used to express HTH protein helix-turn-helix domains (e.g., MarA family helix-turn-helix domains or mutant forms thereof) for purification, e.g., host cells, comprise a mutation which renders any endogenous HTH proteins nonfunctional or causes the endogenous protein to not be expressed. In other embodiments, mutations may also be made in MarR or related genes of the host cell, such that repressor proteins which bind to the same promoter as a MarA family polypeptide are not expressed by the host cell.

**[0155]** In certain embodiments of the invention, it will be desirable to use a mutant form of a HTH protein helix-turn helix domain, e.g., a non-naturally occurring form of a MarA family helix-turn-helix domain which has altered activity, e.g., does not retain wild type MarA family polypeptide helix-turn-helix domain activity, or which has reduced activity or which is more active when compared to a wild-type MarA family polypeptide helix-turn-helix domain.

**[0156]** Such mutant forms can be made using techniques which are well known in the art. For example, random mutagenesis can be used. Using random mutagenesis one can mutagenize an entire molecule or one can proceed by cassette mutagenesis. In the former instance, the entire coding region of a molecule is mutagenized by one of several methods (chemical, PCR, doped oligonucleotide

synthesis) and that collection of randomly mutated molecules is subjected to selection or screening procedures. In the second approach, discrete regions of a protein, corresponding either to defined structural or functional determinants (e.g., the first or second alpha helix of a helix-turn-helix domain) are subjected to saturating or semi-random mutagenesis and these mutagenized cassettes are re-introduced into the context of the otherwise wild type allele.

**[0157]** In a preferred embodiment, PCR mutagenesis is used. For example, Example 2 describes the use of Megaprimer PCR(O. H. Landt, *Gene* 96:125-128) used to introduce an NheI restriction site into the centers of both the helix A (position 1989) and helix B (position 2016) regions of the mara gene.

**[0158]** In one embodiment, such mutant helix-turn-helix domains comprise one or more mutations in the helix-turn-helix domain most proximal to the carboxy terminus (HTH2) of the MarA family polypeptide molecule. In a preferred embodiment, the mutation comprises an insertion into helix A and helix B of the helix-turn-helix domain most proximal to the carboxy terminus of the MarA family polypeptide. In one embodiment, such mutant helix-turn-helix domains comprise one or more mutations in the helix-turn-helix domain most proximal to the amino terminus (HTH1) of the MarA family polypeptide molecule. In a preferred embodiment, the mutation comprises an insertion into helix A and helix B of the helix-turn-helix domain most proximal to the amino terminus of the MarA family polypeptide. In particularly preferred embodiments, the mutation is selected from the group consisting of: an insertion at an amino acid corresponding to about position 33 of MarA and an insertion at an amino acid position corresponding to about position 42 of MarA. "Corresponding" amino acids can be determined, e.g., using an alignment of the helix-turn-helix domains.

**[0159]** Such mutant forms of MarA family helix-turn-helix motifs are useful as controls to verify the specificity of antiinfective compounds for a MarA family helix-turn-helix domain or as controls for the identification of genetic loci which affect resistance to antiinfectives. For example, the mutant MarA family helix-turn-helix domains described in the appended Examples demonstrate that insertional inactivation of MarA at either helix A or helix B in the first HTH domain abolished the multidrug resistance phenotype in both *E. coli* and *M. smegmatis*. By the use of an assay system such as that described in Example 2, which demonstrates the ability of MarA family polypeptide helix-turn-helix domains to increase antibiotic resistance and that mutant forms of these domains do not have the same effect, one can clearly show that the response of any genetic loci identified is specific to a MarA family helix-turn-helix domain.

**[0160]** III. Expression of Polypeptide or Portions Thereof

**[0161]** Nucleic acids encoding transcription factors, such as AraC family polypeptides, HTH proteins, e.g., MarA family polypeptides or selectable markers (or portions thereof that retain an activity of the full-length polypeptide, e.g., are capable of binding to a transcription factor responsive element or retain their indicator function) can be expressed in cells using vectors. Almost any conventional delivery vector can be used. Such vectors are widely available commercially and it is within the knowledge and discretion of one of ordinary skill in the art to choose a vector which is appropriate for use with a given microbial cell. The sequences encoding these domains can be introduced into a cell on a self-replicating vector or may be introduced into the chromosome of a microbe using homologous recombination or by an insertion element such as a transposon.

**[0162]** These nucleic acids can be introduced into microbial cells using standard techniques, for example, by transformation using calcium chloride or electroporation. Such techniques for the introduction of DNA into microbes are well known in the art. In one embodiment, a nucleic acid molecule which has been amplified in vitro by, for example, polymerase chain reaction (PCR); recombinantly produced by cloning, or) purified, as by cleavage and gel separation; or synthesized by, for example, chemical synthesis can be used to produce MarA family polypeptides (George, A. M. & Levy, S. B. (1983)*J. Bacteriol.* 155, 541-548; Cohen, S. P. et al. (1993) *J Infect. Dis.* 168, 484-488; Cohen, S. P. et al. (1993) *J Bacteriol.* 175, 1484-1492; Sulavick, M. C. et al. (1997) *J. Bacteriol.* 179, 1857-1866).

**[0163]** Host cells can be genetically engineered to incorporate nucleic acid molecules of the invention. In one embodiment nucleic acid molecules specifying transcription factors can be placed in a vector. The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. The term "expression vector" or "expression system" includes any vector, (e.g., a plasmid, cosmid or phage chromosome) containing a gene construct in a form suitable for expression by a cell (e.g., linked to a promoter). In the present specification, "plasmid" and "vector" are used interchangeably, as a plasmid is a commonly used form of vector. Moreover, the invention is intended to include other vectors which serve equivalent functions. A great variety of expression systems can be used to produce the polypeptides of the invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids.

**[0164]** Appropriate vectors are widely available commercially and it is within the knowledge and discretion of one of ordinary skill in the art to choose a vector which is appropriate for use with a given host cell. The sequences encoding a transcription factor, such as, for example, MarA family polypeptides, can be introduced into a cell on a self-replicating vector or may be introduced into the chromosome of a microbe using homologous recombination or by an insertion element such as a transposon.

**[0165]** The expression system constructs may contain control regions that regulate expression. "Transcriptional regulatory sequence" is a generic term to refer to DNA sequences, such as initiation signals, enhancers, operators, and promoters, which induce or control transcription of polypeptide coding sequences with which they are operably linked. It will also be understood that a recombinant gene encoding a transcription factor gene, e.g., an HTH protein

gene or an AraC family polypeptide, e.g., MarA family polypeptide, can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring transcription factor gene. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). For instance, any of a wide variety of expression control sequences, that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding the polypeptide.

**[0166]** Generally, any system or vector suitable to maintain, propagate or express nucleic acid molecules and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., Molecular Cloning, A Laboratory Manual, (supra).

**[0167]** Exemplary expression vectors for expression of a gene encoding a polypeptide and capable of replication in a bacterium, e.g., a gram positive, gram negative, or in a cell of a simple eukaryotic fungus such as a Saccharomyces or, Pichia, or in a cell of a eukaryotic organism such as an insect, a bird, a mammal, or a plant, are known in the art. Such vectors may carry functional replication-specifying sequences (replicons) both for a host for expression, for example a Streptomyces, and for a host, for example, *E. coli*, for genetic manipulations and vector construction. See, e.g., U.S. Pat. No. 4,745,056. Suitable vectors for a variety of organisms are described in Ausubel, F. et al., *Short Protocols in Molecular Biology*, Wiley, New York (1995), and for example, for Pichia, can be obtained from Invitrogen (Carlsbad, Calif.).

**[0168]** Useful expression control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat polypeptide, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. A useful translational enhancer sequence is described in U.S. Pat. No. 4,820,639.

**[0169]** In one embodiment, an inducible promoter will be employed to express a polypeptide of the invention. For example, in one embodiment, trp (induced by tryptophan), tac (induced by lactose), or tet (induced by tetracycline) can be used in bacterial cells, or GAL1 (induced by galactose) can be used in yeast cell.

**[0170]** In another embodiment, a constitutive promoter can be used to express a polypeptide of the invention.

**[0171]** It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of polypeptide desired to be expressed. Representative examples of appropriate hosts include bacterial cells, such as gram positive, gram negative cells; fungal cells, such as yeast cells and Aspergillus cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

**[0172]** In one embodiment, cells used to express heterologous polypeptides of the invention, comprise a mutation which renders one or more endogenous transcription factors, such as a AraC family polypeptide or a MarA family polypeptide, nonfunctional or causes one or more endogenous polypeptide to not be expressed. Manipulation of the genetic background in this manner allows for screening for compounds that modulate specific transcription factors, such as MarA family members or AraC family members, or more than one transcription factors. In other embodiments, mutations may also be made in other related genes of the host cell, such that there will be no interference from the endogenous host loci. In yet another embodiment, a mutation may be made in a chromosomal gene to create a heterotroph.

**[0173]** Introduction of a nucleic acid molecule into the host cell ("transformation") can be effected by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology, (1986) and Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Examples include calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

**[0174]** Purification of polypeptides, e.g., recombinantly expressed polypeptides, can be accomplished using techniques known in the art. For example, if the polypeptide is expressed in a form that is secreted from cells, the medium can be collected. Alternatively, if the polypeptide is expressed in a form that is retained by cells, the host cells can be lysed to release the polypeptide. Such spent medium or cell lysate can be used to concentrate and purify the polypeptide. For example, the medium or lysate can be passed over a column, e.g., a column to which antibodies specific for the polypeptide have been bound. Alternatively, such antibodies can be specific for a second polypeptide which has been fused to the first polypeptide (e.g., as a tag) to facilitate purification of the first polypeptide. Other means of purifying polypeptides are known in the art.

**[0175]** IV. Methods of Identifying Antimicrobial/Antiinfective Compounds Which Modulate an Activity of a Transcription Factor

**[0176]** In one embodiment, the invention provides for methods of identifying a test compound which modulates the activity of a transcription factor, (e.g., an HTH protein, a MarA family polypeptide, an AraC family polypeptide, etc.) by contacting a cell expressing a transcription factor (or portion thereof) with a test compound under conditions which allow interaction of the test compound with the cell. The ability of the test compound to modulate an activity of a transcription factor can be determined in a variety of ways, as outlined in more detail below. Compounds identified using the subject methods are useful, e.g., to interfere with the ability of a microbe to grow, e.g., on surfaces or in a host or cause infection in a host.

[0177] Assays

[0178] In one embodiment, the expression of a selectable marker that confers a selective growth disadvantage or lethality is placed under the direct control of a MarA responsive element in a cell expressing marA.

[0179] In one embodiment, marA is plasmid encoded. In one embodiment, the genetic background of the host organism is manipulated, e.g., to delete one or more chromosomal marA genes or marA homolog genes.

[0180] In one embodiment, expression of marA is controlled by a highly regulated and inducible promoter. For example, in one embodiment, a promoter selected from the group consisting of trp, tac, or tet in bacterial cells or GAL1 in yeast cells can be used.

[0181] In another embodiment, expression of marA is constitutive.

[0182] In one embodiment, a selective marker is a cytotoxic gene product (e.g., ccdb). In another embodiment, a selective marker is a gene that confers antibiotic resistance (e.g., kan, cat, or bla).

[0183] In another embodiment, a selective marker is an essential gene (e.g., purA or guaB in a purine or guanine heterotroph).

[0184] In still another embodiment, a selective marker is a gene that confers a selective growth disadvantage in the presence of a particular metabolic substrate (e.g., the expression of URA3 in the presence of 5-fluoroorotic acid [5-FOA] in yeast).

[0185] In one embodiment, compounds that modulate transcription factors (e.g., HTH proteins, AraC family polypeptides, or MarA family polypeptides) are identified using a one-hybrid screening assay. As used herein, the term "one-hybrid screen" as used herein includes assays that detect the disruption of protein-nucleic acid interactions. These assays will identify agents that interfere with the binding of a transcription factor (e.g., an HTH protein, a AraC family polypeptide, or a MarA family polypeptide) to a particular target, e.g., DNA containing, for MarA, a marbox, at the level of the target itself, e.g., by binding to the target and preventing the transcriptional activation factor from interacting with or binding to this site.

[0186] In another embodiment, compounds of the invention are identified using a two-hybrid screening assay. As used herein the term "two-hybrid screen" as used herein includes assays that detect the disruption of protein-protein interactions. Such two hybrid assays can be used to interfere with crucial protein-transcription factor interactions (e.g., HTH protein interactions, AraC family polypeptide interactions, MarA family polypeptide interactions). One example would be to prevent RNA polymerase-MarA family polypeptide contacts, that are necessary for the MarA family polypeptide to function as a transcription factor (either positive acting or negative acting).

[0187] In yet another embodiment, compounds of the invention are identified using a three-hybrid screening assay. As used herein the term "three-hybrid screen" as used herein includes assays that will detect the disruption of a signal transduction pathway(s) required for the activation of a particular regulon of interest. In one embodiment, the three-hybrid screen is used to detect disruption of a signal transduction pathway(s) required for the activation of the Mar regulon, i.e., synthesis of MarA. (Li and Park. *J. Bact.* 181:4824). The assay can be used to identify compounds that may be responsible for activating transcription factor expression, e.g., Mar induction by antibiotics may proceed in this manner.

[0188] In one embodiment of the assay, the expression of a selective marker (e.g., ccdB, cat, bla, kan, guaB, URA3) is put under the direct control of an activatable MarA responsive activatable promoter (e.g., *inaA*, *galT*, *micF*). In the absence of Mar A, the expression of the selective marker would be silent. For example, in the case of regulation of the cytotoxic gene ccdB, the gene would be silent and the cells would survive. Synthesis of MarA from an inducible plasmid in a suitable host would result in the activation of the MarA responsive activatable promoter and expression of the selective marker. In the case of ccdB, the gene would be expressed and result in cell death. Compounds that inhibit MarA would be identified as those that permit cell survival under conditions of MarA expression.

[0189] In another embodiment, e.g., where the expression of the MarA responsive activatable promoter regulates a gene such as URA3, a different result could be obtained. In this case, in the absence of MarA and thus, in the absence of URA3 expression, cells would grow in the presence of a 5-FOA. Upon activation of MarA expression and thus synthesis of URA3, cells would die following the conversion of 5-FOA to a toxic metabolite by URA3.

[0190] In another embodiment, a selectable marker is put under the direct control of a repressible MarA responsive promoter (e.g., fecA). In this example, under conditions of constitutive MarA synthesis, e.g., in a constitutive mar (marc) mutant the expression of the selectable marker would be silent. In the case of ccdB, this would mean that cells would remain viable. Following inactivation of MarA, the selectable marker would be turned on, resulting in cell death.

[0191] In another embodiment, a purine or guanine heterotroph can be constructed by the inactivation of the chromosomal guaB or purA genes in *E. coli*. The guaB or purA gene would then be cloned into a suitable vector, under the control of its natural promoter. This construct would then be transformed into the heterotrophic host. The heterotroph will not grow if MarA expression is constitutive and if cells are grown on media lacking purines or guanine. This can be attributed to MarA mediated repression of guaB or purA synthesis. Candidate inhibiting compounds of MarA can be identified as compounds that restored growth, i.e., relieved MarA mediated repression of guaB and purA expression. In another embodiment, genes that are required for growth in vivo, for example in an animal model of infection.

[0192] In preferred embodiments, controls may be included to ensure that any compounds which are identified using the subject assays do not merely appear to modulate the activity of a transcription factor, because they inhibit protein synthesis. For example, if a compound appears to inhibit the synthesis of a protein being translated from RNA which is transcribed upon activation of a MarA family responsive element, it may be desirable to show that the synthesis of a control, e.g., a protein which is being translated from RNA which is not transcribed upon activation of a MarA family responsive element, is not affected by the

addition of the same compound. For example, the amount of the MarA family polypeptide being made and compared to the amount of an endogenous protein being made. In another embodiment the microbe could be transformed with another plasmid comprising a promoter which is not a MarA family responsive promoter and a protein operably linked to that promoter. The expression of the control protein could be used to normalize the amount of protein produced in the presence and absence of compound.

**[0193]** V. Microbes Suitable for Testing

**[0194]** Numerous different microbes are suitable for testing in the instant assays. As such, they may be used as intact cells or as sources of material, e.g., nucleic acid molecules or polypeptides as described herein.

**[0195]** In preferred embodiments, microbes for use in the claimed methods are bacteria, either Gram negative or Gram positive bacteria. More specifically, any bacteria that are shown to become resistant to antibiotics, e.g., to display a Mar phenotype are preferred for use in the claimed methods, or that are infectious or potentially infectious.

**[0196]** Examples of microbes suitable for testing include, but are not limited to, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas putida, Stenotrophomonas maltophilia, Burkholderia cepacia, Aeromonas hydrophilia, Escherichia coli, Citrobacter freundii, Salmonella typhimurium, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia alcalifaciens, Providencia rettgeri, Providencia stuartii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia intermedia, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus haemolyticus, Haemophilus parahaemolyticus, Haemophilus ducreyi, Pasteurella multocida, Pasteurella haemolytica, Branhamella catarrhalis, Helicobacter pylori, Campylobacter fetus, Campylobacter jejuni, Campylobacter coli, Borrelia burgdorferi, Vibrio cholerae, Yibrio parahaemolyticus, Legionella pneumophila, Listeria monocytogenes, Neisseria gonorrhoeae, Neisseria meningitidis, Gardnerella vaginalis, Bacteroides fragilis, Bacteroides distasonis*, Bacteroides 3452A homology group, *Bacteroides vulgatus, Bacteroides ovalus, Bacteroides thetaiotaomicron, Bacteroides uniformis, Bacteroides eggerthii, Bacteroides splanchnicus, Clostridium difficile, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium leprae, Corynebacterium diphtheriae, Corynebacterium ulcerans, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus intermedius, Staphylococcus hyicus* subsp. *hyicus, Staphylococcus haemolyticus, Staphylococcus hominis*, and *Staphylococcus saccharolyticus*.

**[0197]** In one embodiment, microbes suitable for testing are bacteria from the family Enterobacteriaceae. In preferred embodiments, the compound is effective against a bacteria of a genus selected from the group consisting of: Escherichia, Proteus, Salmonella, Klebsiella, Providencia, Enterobacter, Burkholderia, Pseudomonas, Aeromonas, Haemophilus, Yersinia, Neisseria, and Mycobacteria.

**[0198]** In yet other embodiments, the microbes to be tested are Gram positive bacteria and are from a genus selected from the group consisting of: Lactobacillus, Azorhizobium, Streptomyces, Pediococcus, Photobacterium, Bacillus, Enterococcus, Staphylococcus, Clostridium, and Streptococcus.

**[0199]** In other embodiments, the microbes to be tested are fungi. In a preferred embodiment the fungus is from the genus Mucor or Candida, e.g., *Mucor racmeosus* or *Candida albicans*.

**[0200]** In yet other embodiments, the microbes to be tested are protozoa. In a preferred embodiment the microbe is a malaria or cryptosporidium parasite.

**[0201]** VI. Transcription Factor Modulating Compounds and Test Compounds

**[0202]** Compounds for testing in the instant methods can be derived from a variety of different sources and can be known or can be novel. In one embodiment, libraries of compounds are tested in the instant methods to identify transcriptional activation factor modulating compounds, e.g., HTH protein modulating compounds, AraC family polypeptide modulating compounds, MarA family polypeptide modulating compounds, etc. In another embodiment, known compounds are tested in the instant methods to identify transcription factor modulating compounds (such as, for example, HTH protein modulating compounds, AraC family polypeptide modulating compounds, MarA family polypeptide modulating compounds, etc.). In an embodiment, compounds among the list of compounds generally regarded as safe (GRAS) by the Environmental Protection Agency are tested in the instant methods. In another embodiment, the transcription factors which are modulated by the modulating compounds are of prokaryotic microbes.

**[0203]** A recent trend in medicinal chemistry includes the production of mixtures of compounds, referred to as libraries. While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures of other compounds, such as benzodiazepines (Bunin et al. 1992. *J. Am. Chem. Soc.* 114:10987; DeWitt et al. 1993. Proc. Natl. Acad. Sci. USA 90:6909) peptoids (Zuckermann. 1994. *J. Med. Chem.* 37:2678) oligocarbamates (Cho et al. 1993. Science. 261:1303), and hydantoins (DeWitt et al. supra). Rebek et al. have described an approach for the synthesis of molecular libraries of small organic molecules with a diversity of 104-105 (Carell et al. 1994. *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al. *Angew. Chem. Imt. Ed. Engl.* 1994. 33:2061).

**[0204]** The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches

are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K. S. Anticancer Drug Des. 1997. 12:145).

[0205] Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries. In one embodiment, the test compound is a peptide or peptidomimetic. In another, preferred embodiment, the compounds are small, organic non-peptidic compounds.

[0206] Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: Erb et al. 1994. *Proc. Natl. Acad. Sci. USA* 91:11422; Horwell et al. 1996 *Immunopharmacology* 33:68; and in Gallop et al. 1994. *J. Med. Chem.* 37:1233.

[0207] Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner U.S. Pat. No. 5,223,409), spores (Ladner U.S. Pat. No. '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) Science 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner supra.). Other types of peptide libraries may also be expressed, see, for example, U.S. Pat. Nos. 5,270,181 and 5,292,646). In still another embodiment, combinatorial polypeptides can be produced from a cDNA library.

[0208] In other embodiments, the compounds can be nucleic acid molecules. In preferred embodiments, nucleic acid molecules for testing are small oligonucleotides. Such oligonucleotides can be randomly generated libraries of oligonucleotides or can be specifically designed to reduce the activity of a transcription factor, e.g., a HTH protein, a MarA family polypeptide, or an AraC family polypeptide. For example, in one embodiment, these oligonucleotides are sense or antisense oligonucleotides. In an embodiments, oligonucleotides for testing are sense to the binding site of a particular transcription factor, e.g., a MarA family polypeptide helix-turn-helix domain. Methods of designing such oligonucleotides given the sequences of a particular transcription factor polypeptide, such as a MarA family polypeptide, is within the skill of the art.

[0209] In yet another embodiment, computer programs can be used to identify individual compounds or classes of compounds with an increased likelihood of modulating a transcription factor activity, e.g., an HTH protein, a AraC family polypeptide, or a MarA family polypeptide activity. Such programs can screen for compounds with the proper molecular and chemical complementarities with a chosen transcription factor. In this manner, the efficiency of screening for transcription factor modulating compounds in the assays described above can be enhanced.

[0210] VII. Computer Modeling Techniques for Identifying Transcription Factor Modulating Compounds

[0211] The invention also pertains to the use of molecular design techniques to design transcription factor modulating compounds, e.g., HTH protein modulating compounds, AraC family modulating compounds, MarA family modulating compounds, or MarA modulating compounds, which are capable of binding or interacting with one or more transcription factors (e.g., of a prokaryotic or eukaryotic organism). The invention pertains to both the transcription factor modulating compounds identified by the methods as well as the modeling methods, and compositions comprising the compounds identified by the methods.

[0212] In an embodiment, the invention pertains to a method of identifying transcription factor modulating compounds. The method includes obtaining the structure of a transcription factor of interest, and using GLIDE to identify a scaffold which has an interaction energy score of −20 or less (e.g., −40 or less, e.g., −60 or less) with a portion of the transcription factor.

[0213] The invention pertains, at least in part, to a computational screening of small molecule databases for chemical entities or compounds that can bind in whole, or in part, to a transcription factor, such as a HTH protein, an AraC family polypeptide, a MarA family polypeptide, e.g., MarA. In this screening, the quality of fit of such entities or compounds to the binding site may be judged either by shape complementarity or by estimated interaction energy (Meng, E. C. et al., 1992, *J. Coma. Chem.*, 13:505-524). Such a procedure allows for the screening of a very large library of potential transcription factor modulating compounds for the proper molecular and chemical complementarities with a selected protein or class or proteins. Transcription factor modulating compounds identified through computational screening can later be passed through the in vivo assays described herein as further screens. For example, a MarA inhibiting compound identified through computational screening could be tested for its ability to promote cell survival in a cell system containing a counterselectable marker under the control a MarA activated promoter. The promotion of cell survival in the foregoing assay would be indicative of a compound that inhibits MarA's activity as a transcriptional activator. Other suitable assays are described in the Examples and through the specification.

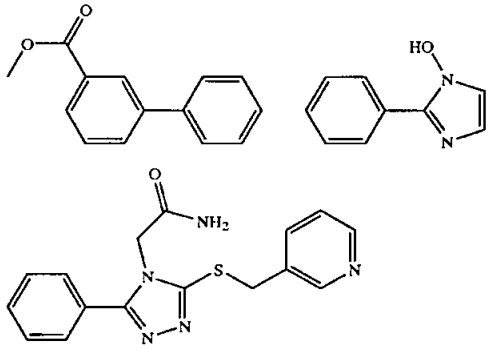
[0214] The crystal structures of both MarA (PDB ID code 1BL0) and its homolog Rob (PDB ID code 1DY5) are available in the Protein Data Bank (http://www.rcsb.org/pdb/). These structures were used to identify sites on the proteins that could be targeted by small molecule chemical inhibiting compounds. A total of at least eight potential small molecule binding sites on MarA (Table 2) and four sites on Rob (Table 3) were identified as potential small molecule binding sites. The invention pertains, at least in part, to MarA modulating compounds which interact with any one of the following sites of MarA (based on the sequence given in SEQ ID NO. 2).

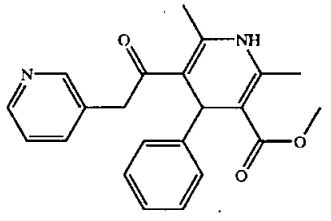
TABLE 2

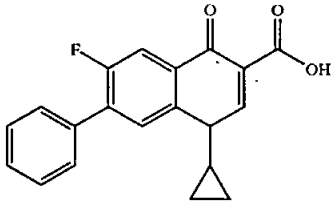
| Site Number | Residues (based on full length MarA) | Site Label |
|---|---|---|
| 1 | 42 to 50 | R46 Major Groove |
| 2 | 54 to 62 | L56 HTH core |
| 3 | 55 to 65 | R61 Minor Groove |
| 4 | 15 to 25 | W19 |
| 5 | 14 to 25 | E21 |
| 6 | 24 to 35 | L28 |
| 7 | 76 to 83 | P78 |
| 8 | 106 to 112 | R110 |

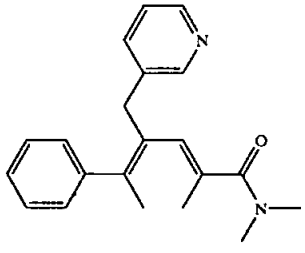
[0215] The GLIDE docking method was then used to fit combinatorial chemistry scaffolds into these sites and an

interaction energy was calculated for each. Eight scaffolds were predicted to bind to site 1, encompassing amino acids tryptophan 42 to lysine 50, with an interaction energy score of −60 or less. These scaffolds are shown below:

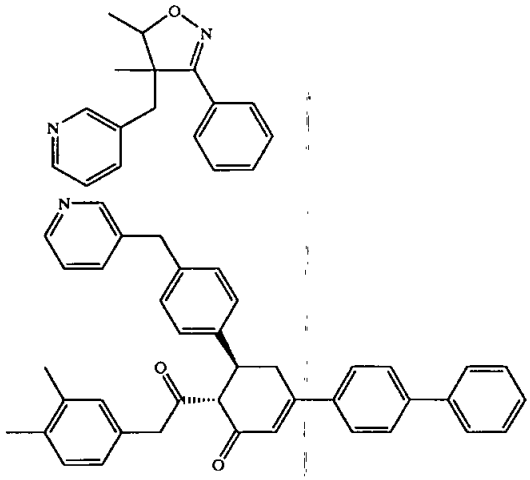




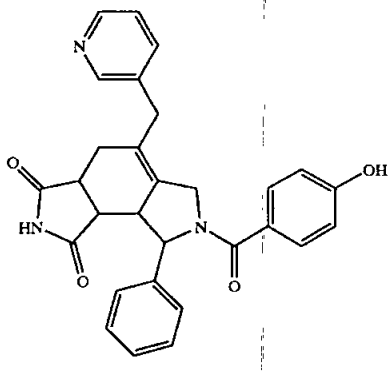


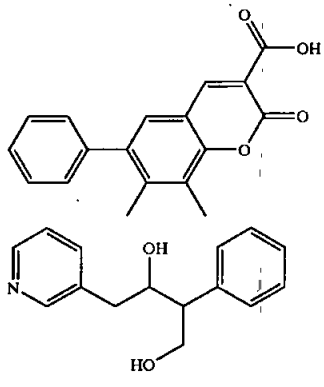


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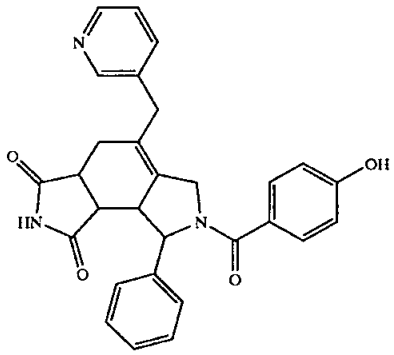


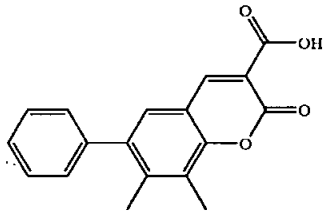
**[0216]** Three scaffolds were identified for site 2 of MarA (e.g., residues histidine 54 to serine 62).

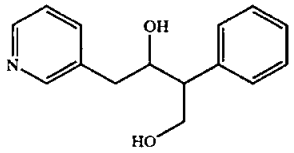


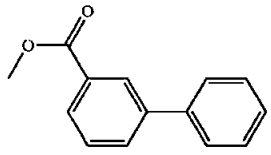


[0217] Four scaffolds were identified for MarA site 3, (e.g., residues serine 55 to methionine 65):

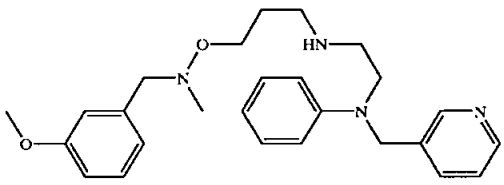


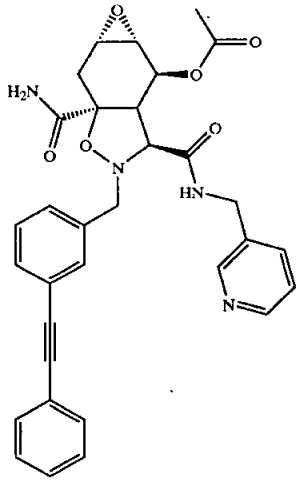




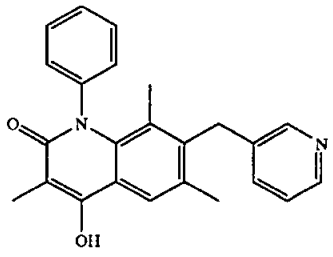


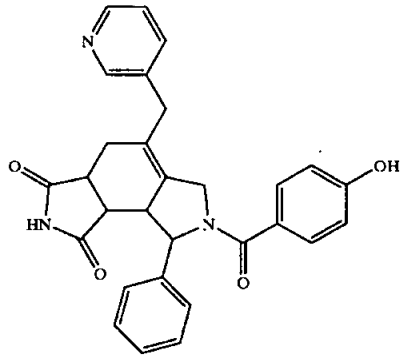
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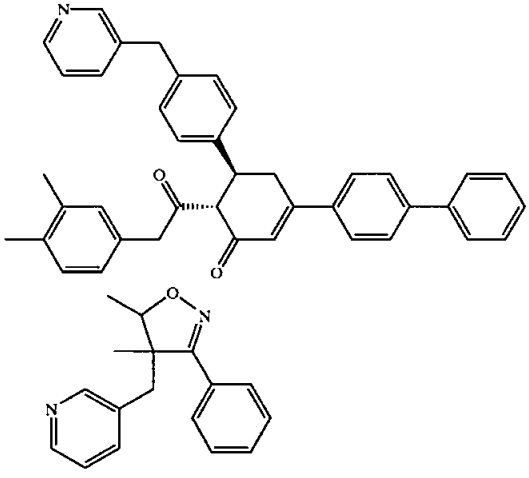


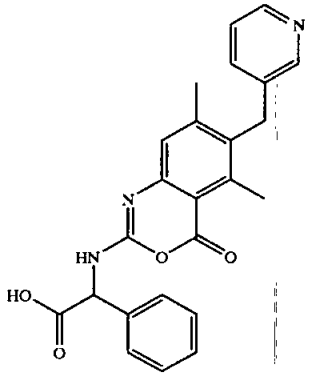
[0218] Six scaffolds were identified for site 6 (e.g., residues leucine 24 to glutamate 35).

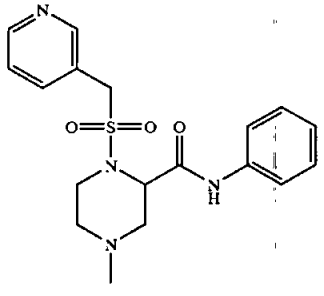


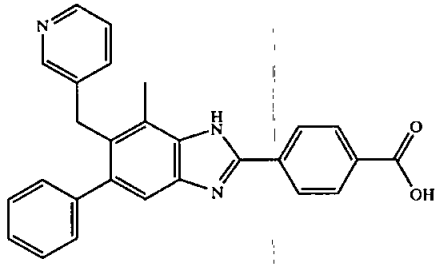


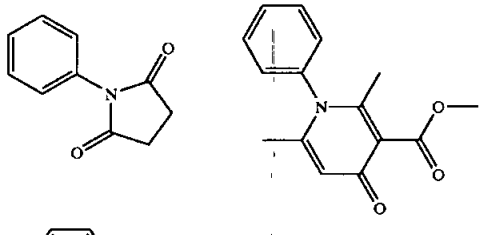
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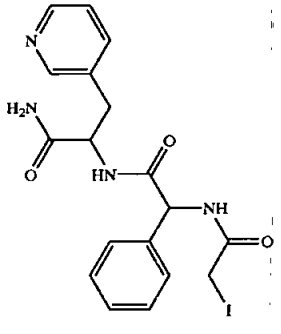












[0219] These scaffolds were then used to search the CambridgeSoft ACX—SC database of over 600,000 non-proprietary chemical structures and the number of chemicals similar to the scaffolds was determined.

[0220] The term "scaffold" includes the compounds identified by the computer modeling program. These compounds may or may not be themselves transcription factor modulating compounds. An ordinarily skilled artisan will be able to analyze a scaffold obtained from the computer modeling program and modify the scaffold such that the resulting compounds have enhanced chemical properties over the initial scaffold compound, e.g., are more stable for administration, less toxic, have enhanced affinity for a particular transcription factor, etc. The invention pertains not only to the scaffolds identified, but also the transcription factor modulating compounds which are developed using the scaffolds.

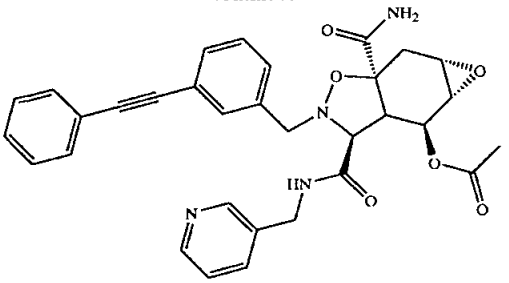
[0221] Table 3 lists portions of Rob which were identified as possible interaction sites for a modulating compound. The invention pertains, at least in part, to any compounds modeled to bind to these regions of Rob. The numbering corresponds to that given in SEQ ID NO. 4.

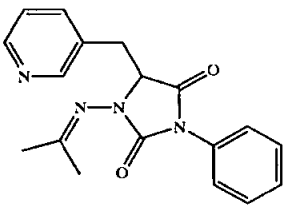
TABLE 3

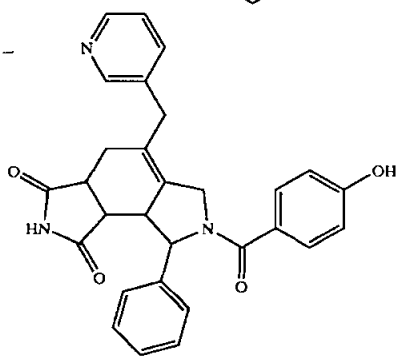
| Site Number | Residues (based on full length Rob) | Site Label |
|---|---|---|
| 1 | 37 to 45 | R40 Major Groove |
| 2 | 43 to 54 | I50 HTH Core |
| 3 | 51 to 60 | R55 Minor Groove |
| 4 | 10 to 20 | W13 |

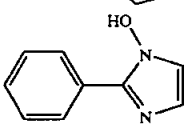
[0222] These scaffolds were identified as possible modulating compounds which with site 1 of Rob (residues 37-45), a MarA family polypeptide.

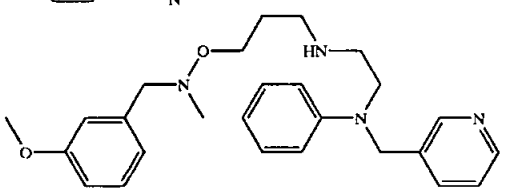
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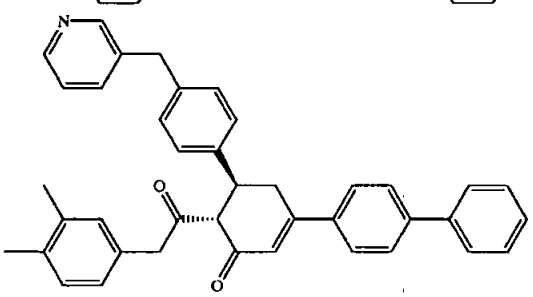




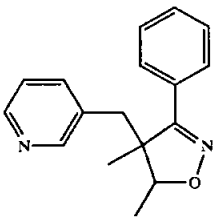


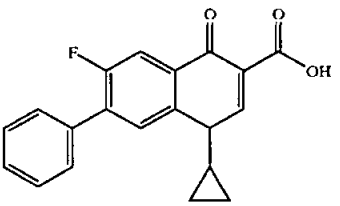


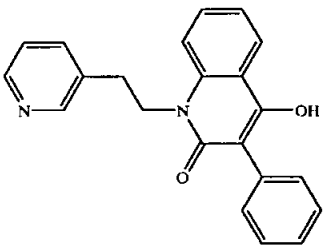


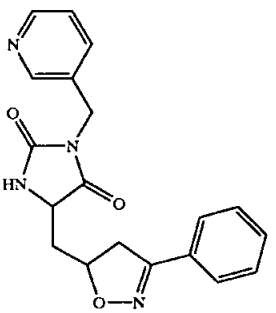


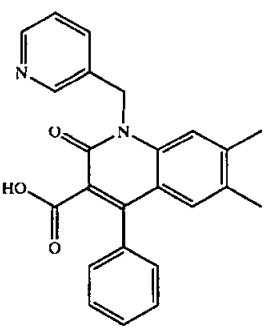
[0223] These scaffolds were identified as small molecules that may interact with site 2 of Rob (residues 43-52), a MarA family polypeptide.



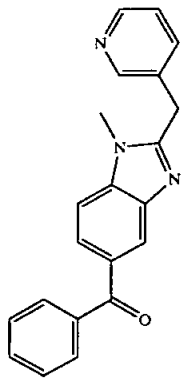


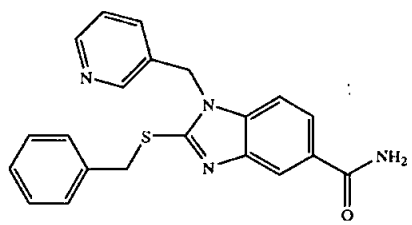


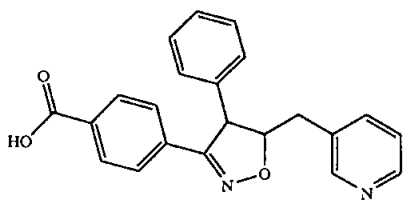


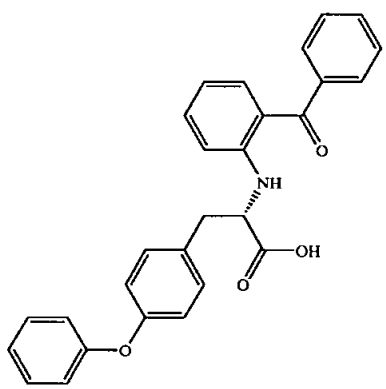


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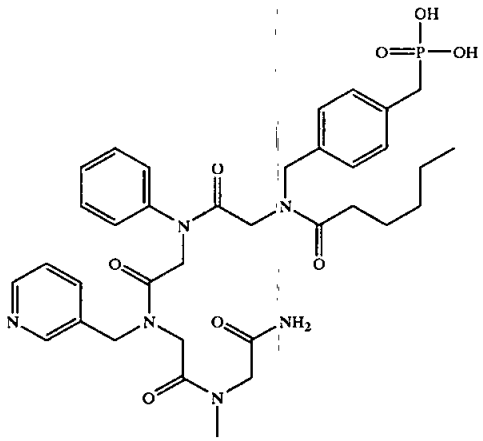


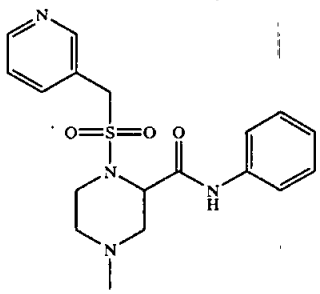


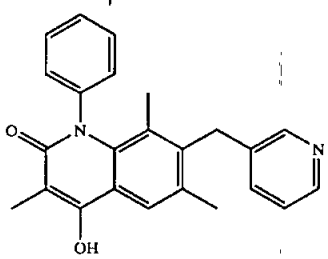


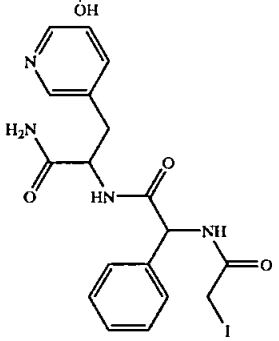


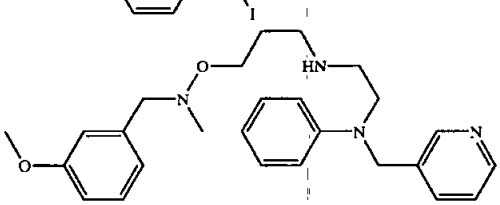
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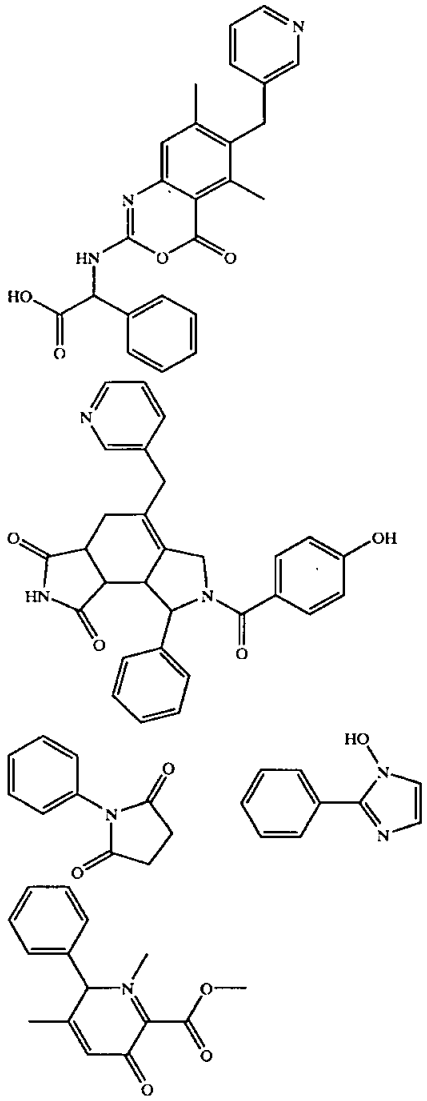








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[0224] The design of compounds that bind to, modulate, or inhibit transcription factors, generally involves consideration of two factors. First, the compound must be capable of physically and structurally associating with a particular transcription factor. Non-covalent molecular interactions important in the association of a transcription factor with a modulating compound include hydrogen bonding, van der Waals and hydrophobic interactions.

[0225] Second, the modulating compound must be able to assume a conformation that allows it to associate with the selected transcription factor. Although certain portions of the inhibiting compound will not directly participate in this association with the transcription factor, those portions may still influence the overall conformation of the molecule. This, in turn, may have a significant impact on potency. Such conformational requirements include the overall three-dimensional structure and orientation of the chemical entity or compound in relation to all or a portion of the binding site, e.g., active site or accessory binding site of a particular transcription factor such as MarA, or the spacing between functional groups of a compound comprising several chemical entities that directly interact with the particular transcription factor.

[0226] In a further embodiment, the potential modulating effect of a chemical compound on a selected transcription factor (e.g., a HTH protein, a AraC family polypeptide, a MarA'family polypeptide, e.g., MarA) is analyzed prior to its actual synthesis and testing by the use of computer modeling techniques. If the theoretical structure of the given compound suggests insufficient interaction and association between it and the selected transcription factor, synthesis and testing of the compound is avoided. However, if computer modeling indicates a strong interaction, the molecule may then be synthesized and tested for its ability to bind to the selected transcription factor and modulate the transcription factor's activity.

[0227] A transcription factor modulating compound or other binding compound (e.g., an HTH protein modulating compound, an AraC family polypeptide modulating compound, or a MarA family inhibiting compound, e.g., a MarA inhibiting compound) may be computationally evaluated and designed by screening and selecting chemical entities or fragments for their ability to associate with the individual small molecule binding sites or other areas of a transcription factor.

[0228] One skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with a selected transcription factor and more particularly with the individual small molecule binding sites of the particular transcription activation factor. This process may begin by visually inspecting the structure of the transcription factor on a computer screen based on the atomic coordinates of the transcription factor crystals. Selected chemical entities may then be positioned in a variety of orientations, or docked, within an individual binding site of the transcription factor. Docking may be performed using software such as Quanta and Sybyl, followed by energy minimization with standard molecular mechanics forcefields or dynamics with programs such as CHARMM (Brooks, B. R. et al., 1983, *J. Comp. Chem.*, 4:187-217) or AMBER (Weiner, S. J. et al., 1984, *J. Am. Chem. Soc.*, 106:765-784).

[0229] Specialized computer programs may also assist in the process of selecting molecules that bind to a selected transcription factor, (e.g., an HTH protein, an AraC family polypeptide, or a MarA family polypeptide, e.g., MarA). The programs include, but are not limited to:

[0230] 1. GRID (Goodford, P. J., 1985, "A Computational Procedure for Determining Energetically Favorable Binding Sites on Biologically Important Macromolecules"*J. Med. Chem.*, 28:849-857 GRID is available from Oxford University, Oxford, UK.

[0231] 2. AUTODOCK (Goodsell, D. S. and A. J. Olsen, 1990, "Automated Docking of Substrates to Proteins by Simulated Annealing" Proteins: Structure. *Function, and Genetics,* 8:195-202.

AUTODOCK is available from Scripps Research Institute, La Jolla, Calif. AUTODOCK helps in docking inhibiting compounds to a selected transcription factor in a flexible manner using a Monte Carlo simulated annealing approach. The procedure enables a search without bias introduced by the researcher.

[0232] 3. MCSS (Miranker, A. and M. Karplus, 1991, "Functionality Maps of Binding Sites: A Multiple Copy Simultaneous Search Method." Proteins: Structure, Function and Genetics, 11:29-34). MCSS is available from Molecular Simulations, Burlington, Mass.

[0233] 4. MACCS-3D (Martin, Y. C., 1992, J. Med. Chem., 35:2145-2154) is a 3D database system available from MDL Information Systems, San Leandro, Calif.

[0234] 5. DOCK (Kuntz, I. D. et al., 1982, "A Geometric Approach to Macromolecule-Ligand Interactions"*J. Mol. Biol.,* 161:269-288). DOCK is available from University of California, San Francisco, Calif. DOCK is based on a description of the negative image of a space-filling representation of the molecule (i.e. the selected transcription factor) that should be filled by the inhibiting compound. DOCK includes a force-field for energy evaluation, limited conformational flexibility and consideration of hydrophobicity in the energy evaluation.

[0235] 6. MCDLNG (Monte Carlo De Novo Ligand Generator) (D. K. Gehlhaar, et al. 1995. *J. Med. Chem.* 38:466-472). MCDLNG starts with a structure (i.e. an X-ray crystal structure) and fills the binding site with a close packed array of generic atoms. A Monte Carlo procedure is then used to randomly: rotate, move, change bond type, change atom type, make atoms appear, make bonds appear, make atoms disappear, make bonds disappear, etc. The energy function used by MCDLNG favors the formation of rings and certain bonding arrangements. Desolvation penalties are given for heteroatoms, but heteroatoms can benefit from hydrogen bonding with the binding site.

[0236] In an embodiment of the invention, docking is performed by using the Affinity program within InsightII (Molecular Simulations Inc., 1996, San Diego, Calif., now Accelrys Inc.). Affinity is a suite of programs for automatically docking a ligand (i.e. a transcription factor modulating compound) to a receptor (i.e. a transcription factor). Commands in Affinity automatically find the best binding structures of the ligand to the receptor based on the energy of the ligand/receptor complex. As described below, Affinity allows for the simulation of flexible-flexible docking.

[0237] Affinity consists of two commands, GridDocking and fixedDocking, under the new pulldown Affinity in the Docking module of the Insight II program. Both commands use the same, Monte Carlo type procedure to dock a guest molecule (i.e. HTH protein modulating compound) to a host (i.e., a transcription factor). They also share the feature that the "bulk" of the receptor (i.e. transcription factor), defined as atoms not in the binding (active) site specified, is held rigid during the docking process, while the binding site atoms and ligand atoms are movable. The commands differ, however, in their treatment of nonbond interactions. In GridDocking, interactions between bulk and movable atoms are approximated by the very accurate and efficient molecular mechanical/grid (MM/Grid) method developed by Luty et al. 1995. *J. Comp. Chem.* 16:454, while interactions among movable atoms are treated exactly. GridDocking also includes the solvation method of Stouten et al. 1993. Molecular Simulation 10.97. On the other hand, the fixed-Docking command computes nonbond interactions using methods in the Discover program (cutoff methods and the cell multipole method) and it does not include any solvation terms.

[0238] Affinity does not, generally, require any intervention from the user during the docking. It automatically moves the ligand (i.e. modulating compound), evaluates energies, and checks if the structure is acceptable. Moreover, the ligand and the binding site of the receptor (i.e. the selected transcription modulator) are flexible during the search.

[0239] Most of the docking methods in the literature are based on descriptors or empirical rules (for a review see Kuntz et al. 1994. *Acc. Chem. Res.* 27:117. These include DOCK (Kuntz et al. 1982. *J. Mol. Biol.* 161:269., Shoichet et al. 1992. *J. Compt. Chem.* 13:380., Oshiro et al. 1995. *J. Comp. Aided Molec. Design* 9:113.), CAVEAT (Bartlett et al. 1989. "Chemical and Biological Problems in Molecular Recognition" Royal Society of Chemistry: Cambridge, pp. 182-196., Lauri & Bartlett. 1994. *J. Comput. Aided Mol. Design* 8:51), FLOG (Miller et al. 1994. *J. Comp. Aided Molec. Design* 8:153), and PRO_LIGAND (Clark et al. 1995. *J. Comp. Aided Molec. Design* 9:13), to name a few. Affinity differs from these methods in several aspects.

[0240] First, it uses full molecular mechanics in searching for and evaluating docked structures. In contrast descriptor-based methods use empirical rules which usually take into account only hydrogen bonding, hydrophobic interactions, and steric effects. This simplified description of ligand/receptor interaction is insufficient in some cases. For example, Meng et al. 1992. *J. Compt. Chem.* 13:505 studied three scoring methods in evaluating docked structures generated by DOCK. They found that only the forcefield scores from molecular mechanics correctly identify structures closest to experimental binding geometry, while scoring functions that consider only steric factors or only electrostatic factors are less successful. Note that in the study by Meng et al. 1992. *J. Compt. Chem.* 13:505, docking was still performed using descriptors. Affinity, on the other hand, uses molecular mechanics in both docking and scoring and is therefore more consistent.

[0241] Second, in Affinity, while the bulk of the receptor is fixed, the defined binding site is free to move, thereby allowing the receptor to adjust to the binding of different ligands or different binding modes of the same ligand. By contrast, almost all of the descriptor-based methods fix the entire receptor.

[0242] Third, the ligand itself is flexible in Affinity which permits different conformations of a ligand (i.e. transcription factor modulating compound) to be docked to a receptor (i.e. transcription factor). Recently Oshiro et al. (1995 *J. Comp. Aided Molec. Design* 9;113) extended DOCK to handle flexible ligands. FLOG is also able to treat flexible ligand by

including different conformations for each structure in the database (Miller et al. 1995. J. Comp. Aided Molec. Design. 8:153). Most other methods are limited to rigid ligands.

[0243] There are also a few energy based docking methods (Kuntz et al. 1994. *Acc. Chem Res.* 27:117). These methods use either molecular dynamics (notably simulated annealing) or Monte Carlo methods. For example, Caflisch et al. 1992. *Proteins: Struct. Funct. and Genetics* 13:223) developed a two step procedure for docking flexible ligands. In their procedure, ligand is first docked using a special energy function designed to remove bad contact between the ligand and the receptor efficiently. Then Monte Carlo minimization (Li & Scheraga. 1987. *Proc. Natl. Acad. Sci. U.S.A.* 84:6611) is carried out to refine the docked structures using molecular mechanics. Hart and Read. 1992. *Proteins: Struct. Funct. and Genetics* 13:206 also employ two steps to dock ligands. They use a score function based on receptor geometry to approximately dock ligands in the first step, and then use Monte Carlo minimization similar to that of Caflisch et al. 1992. *Proteins: Struct. Funct. and Genetics* 13:223 for the second step. The method by Mizutani et al. (1994. J. Mol. Biol. 243:310) is another variation of this two step method.

[0244] Affinity uses a Monte Carlo procedure in docking ligands, but there are important distinctions over the prior art methods. First, the Monte Carlo procedure in Affinity can be used in conjunction either with energy minimization (to mimic the Monte Carlo minimization method of Li & Scheraga. 1987. *Proc. Natl. Acad. Sci. U.S.A.* 84:6611) or with molecular dynamics (to mimic the hybrid Monte Carlo method, Clamp et al. 1994. *J. Comput. Chem.* 15:838, or the smart Monte Carlo method, Senderowitz et al. 1995. *J. Am. Chem. Soc.* 117:8211). This flexibility allows Affinity to be applied to a variety of docking problems. Second, in the initial screening of docked structures, Affinity employs energy differences obtained from molecular mechanics, while the methods discussed above use empirical rules or descriptors. Therefore, Affinity is more consistent in that it uses molecular mechanics in both initial screening and final refinement of docked structures. Third, Affinity allows the binding site of the receptor to relax, while the methods discussed above fix the entire receptor. Fourth, Affinity employs two new nonbond techniques which are both accurate and efficient to make docking practical. One is the Grid/MM method of Luty et al. which represents the bulk of the receptor by grids (Luty et al. 1995. *J. Comp. Chem.* 16:454). This method is 10-20 times faster than the no-cutoff method with almost no loss in accuracy. It also incorporates the solvation method of Stouten et al. (1993. *Molecular Simulation* 10:97). The other is the cell multipole method. This method is about 50% slower than the Grid/MM method, but it does not require grid setup. Thus, a typical docking calculation takes about 1-3 hours of CPU time on an Indigo R4400 workstation.

[0245] Once suitable chemical fragments have been selected, they can be assembled into a single compound or inhibiting compound. Assembly may be proceed by visual inspection of the relationship of the fragments to each other on a three-dimensional image display on a computer screen in relation to the structure coordinates of a particular transcription factor, e.g., MarA. This may be followed by manual model building using software such as Quanta or Sybyl.

[0246] Useful programs to aid one of skill in the art in connecting the individual chemical fragments include:

[0247] 1. 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, Calif. This area is reviewed in Martin, Y. C., 1992, "3D Database Searching in Drug Design", *J. Med. Chem.*, 35, pp. 2145-2154).

[0248] 2. CAVEAT (Bartlett, P. A. et al, 1989, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules". In Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc., 78, pp. 182-196). CAVEAT is available from the University of California, Berkeley, Calif. CAVEAT suggests inhibiting compounds to MarA based on desired bond vectors.

[0249] 3. HOOK (available from Molecular Simulations, Burlington, Mass.). HOOK proposes docking sites by using multiple copies of functional groups in simultaneous searches.

[0250] In another embodiment, transcription factor modulating compounds may be designed as a whole or "de novo" using either an empty active site or optionally including some portion(s) of a known inhibiting compound(s). These methods include:

[0251] 1. LUDI (Bohm, H. -J., "The Computer Program LUDI: A New Method for the De Novo Design of Enzyme Inhibiting compounds", J. ComR. Aid.

[0252] Molec. Design, 6, pp. 61-78 (1992)). LUDI is available from Biosym Technologies, San Diego, Calif. LUDI is a program based on fragments rather than on descriptors. LUDI proposes somewhat larger fragments to match with the interaction sites of a macromolecule and scores its hits based on geometric criteria taken from the Cambridge Structural Database (CSD), the Protein Data Bank (PDB) and on criteria based on binding data. LUDI is a library based method for docking fragments onto a binding site. Fragments are aligned with 4 directional interaction sites (lipophilic-aliphatic, lipophilic-aromatic, hydrogen donor, and hydrogen acceptor) and scored for their degree of overlap. Fragments are then connected (i.e. a linker of the proper length is attached to each terminal atom in the fragments). Note that conformational flexibility can be accounted for only by including multiple conformations of a particular fragment in the library.

[0253] 2. LEGEND (Nishibata, Y. and A. Itai, Tetrahedron, 47, p. 8985 (1991)).

[0254] LEGEND is available from Molecular Simulations, Burlington, Mass.

[0255] 3. CoMFA (Conformational Molecular Field Analysis) (J. J. Kaminski. 1994. *Adv. Drug Delivery Reviews* 14:331-337.) CoMFA defines 3-dimensional molecular shape descriptors to represent properties such as hydrophobic regions, sterics, and electrostatics. Compounds from a database are then overlaid on the 3D pharmacophore model and rated for their degree of overlap. Small molecule databased that be searched include: ACD (over 1,000,000 compounds), Maybridge (about 500,000 compounds), NCI (about 500,000 compounds), and

CCSD. In measuring the goodness of the fit, molecules can either be fit to the 3D molecular shape descriptors or to the active conformation of a known inhibiting compound.

[0256] 4. LeapFrog (available from Tripos Associates, St. Louis, Mo.).

[0257] FlexX (© 1993-2002 GMD German National Research Center for Information Technology; Rarey, M. et al *J. Mol. Biol.,* 261:407-489) is a fast, flexible docking method that uses an incremental construction algorithm to place ligands into and active site of the transcription factor. Ligands (e.g., transcription factor modulating compounds) that are capable of "fitting" into the active site are then scored according to any number of available scoring schemes to determine the quality of the complimentarity between the active site and ligand.

[0258] Other molecular modeling techniques may also be employed in accordance with this invention. See, e.g., Cohen, N.C. et al., "Molecular Modeling Software and Methods for Medicinal Chemistry, J. Med. Chem., 33, pp. 883-894 (1990). See also, Navia, M. A. and M. A. Murcko, "The Use of Structural Information in Drug Design", Current Opinions in Structural Biology, 2, pp. 202-210 (1992).

[0259] Candidate transcription factor modulating compounds can be evaluated for their modulating, e.g., inhibitory, activity using conventional techniques which may involve determining the location and binding proximity of a given moiety, the occupied space of a bound inhibiting compound, the deformation energy of binding of a given compound and electrostatic interaction energies. Examples of conventional techniques useful in the above evaluations include, but are not limited to, quantum mechanics, molecular dynamics, Monte Carlo sampling, systematic searches and distance geometry methods (Marshall, G. R., 1987, *Ann. Ref Pharmacol. Toxicol.,* 27:193). Examples of computer programs for such uses include, but are not limited to, Gaussian 92, revision E2 (Gaussian, Inc. Pittsburgh, Pa.), AMBER version 4.0 (University of California, San Francisco), QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, Mass.), and Insight II/Discover (Biosym Technologies Inc., San Diego, Calif.). These programs may be implemented, for example, using a Silicon Graphics Indigo2 workstation or IBM RISC/6000 workstation model 550. Other hardware systems and software packages will be known and of evident applicability to those skilled in the art.

[0260] Once a compound has been designed and selected by the above methods, the efficiency with which that compound may bind to a particular transcription factor may be tested and optimized by computational evaluation. An effective transcription factor modulating compound should demonstrate a relatively small difference in energy between its bound and free states (i.e., a small deformation energy of binding). Transcription factor modulating compounds may interact with the selected transcription factor in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding may be taken to be the difference between the energy of the free compound and the average energy of the conformations observed when the inhibiting compound binds to the enzyme.

[0261] A compound designed or selected as interacting with a selected transcription factor, e.g., a MarA family polypeptide, e.g., MarA, may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target enzyme. Such non-complementary (e.g., electrostatic) interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all electrostatic interactions between the modulating compound and the enzyme when the modulating compound is bound to the selected transcription factor, preferably make a neutral or favorable contribution to the enthalpy of binding.

[0262] Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interaction. Examples of programs designed for such uses include: Gaussian 92, revision C [M. J. Frisch, Gaussian, Inc., Pittsburgh, Pa. ©1992]; AMBER, version 4.0 [P. A. Kollman, University of California at San Francisco, ©1994]; QUANTA/CHARMM [Molecular Simulations, Inc., Burlington, Mass. ©1994]; and Insight II/Discover (Biosysm Technologies Inc., San Diego, Calif. ©1994). These programs may be implemented, for instance, using a Silicon Graphics workstation, IRIS 4D/35 or IBM RISC/6000 workstation model 550. Other hardware systems and software packages will be known to those skilled in the art.

[0263] Once a transcription factor modulating compound has been optimally selected or designed, as described above, substitutions may then be made in some of its atoms or side groups in order to improve or modify its binding properties. Initial substitutions are preferable conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Substitutions known in the art to alter conformation should be avoided. Such substituted chemical compounds may then be analyzed for efficiency of fit to the selected transcription factor by the same computer methods described above.

[0264] Computer programs can be used to identify unoccupied (aqueous) space between the van der Waals surface of a compound and the surface defined by residues in the binding site. These gaps in atom-atom contact represent volume that could be occupied by new functional groups on a modified version of the lead compound. More efficient use of the unoccupied space in the binding site could lead to a stronger binding compound if the overall energy of such a change is favorable. A region of the binding pocket which has unoccupied volume large enough to accommodate the volume of a group equal to or larger than a covalently bonded carbon atom can be identified as a promising position for functional group substitution. Functional group substitution at this region can constitute substituting something other than a carbon atom, such as oxygen. If the volume is large enough to accommodate a group larger than a carbon atom, a different functional group which would have a high likelihood of interacting with protein residues in this region may be chosen. Features which contribute to interaction with protein residues and identification of promising substitutions include hydrophobicity, size, rigidity and polarity. The combination of docking, $K_i$ estimation, and visual representation of sterically allowed room for improvement permits prediction of potent derivatives.

[0265] Similarity Screening

[0266] Once a transcription factor modulating compound has been selected or designed, computational methods to assess its overall likeness or similarity to other molecules

can be used to search for additional compounds with similar biochemical behavior. In such a way, for instance, HTS derived hits can be tested to assure that they are bona fide ligands against a particular active site, and to eliminate the possibility that a particular hit is an artifact of the screening process. There are currently several methods and approaches to determine a particular compound's similarity to members of a virtual database of compounds. One example is the OPTISIM methodology that is distributed in the Tripos package, SYBYL (C© 1991-2002 Tripos, Inc., St. Louis, Mo.). OPTISIM exploits the fact that each 3-dimensional representation of a molecule can be broken down into a set of 2-dimensional fragments and encoded into a pre-defined binary string. The result is that each compound within a particular set is represented by a unique numerical code or fingerprint that is amenable to mathematical manipulations such as sorting and comparison. OPTISIM is automated to calculate and report the percent difference in the fingerprints of the respective compounds for instance according to the using a formalism known as the Tanimoto coefficient. For instance, a compound that is similar in structure to another will share a high coefficient. Large virtual databases of commercially available compounds or of hypothetical compounds can be quickly screened to identify compounds with high Tanimoto coefficient.

[0267] CoMFA/QSAR

[0268] Once a series of similar transcription factor modulating compounds has been identified and expanded by the methods described, their experimentally determined biological activities can be correlated with their structural features using a number of available statistical packages. In a typical project within the industry, the CoMFA (COmparative Molecular Field Analysis) and QSAR (Quantitative Structure Activity Relationship) packages within the SYBYL suite of programs (Tripos Associates, St. Louis, Mo.) are utilized. In CoMFA, a particular series of compounds with measured activities are co-aligned in a manner that is believed to emulate their arrangement as they interact with the active site. A 3-dimensional lattice, or grid is then constructed to encompass the collection of the so-aligned compounds. At each point on the lattice, an evaluation of the potential energy is determined and tabulated-typically potentials that simulate the electronic and steric fields are determined, but other potential functions are available. Using the statistical methods such as PLS (Partial Least Squares), correlation between the measured activities and the potential energy values at the grid-points can be determined and summed in a linear equation to produce the overall molecular correlation or QSAR model. A particularly useful feature in CoMFA is that the individual contribution for each grid-point is known; the importance of the grid points upon the overall correlation can be visualized graphically in what is referred to as a CoMFA field. When this field is combined with the original compound alignment, it becomes a powerful tool to rationalize the activities of the individual compounds from whence the model was derived, and to predict how chemical modification of a reference compound would be effected. As an example, a QSAR model was developed for a set of 92 benzodiazepines using the method described above. A representative CoMFA field is shown in **FIG. 4**; the region delineated by wire mesh (adjacent to the referenced triazinoxazepine) is the region where chemical modification characterized by increasing steric bulk would lead to favorable effects in transcription factor modulation.

[0269] The invention pertains, per se, to not only the methods for identifying the transcription factor modulating compounds, but to the compounds identified by the methods of the invention as well as methods for using the identified compounds.

[0270] VIII. MarA Family Modulating Compounds, and Methods of Use Thereof

[0271] In an embodiment, the invention pertains to methods for modulating a transcription factor, e.g., an HTH protein, an AraC family polypeptide, or a MarA family polypeptide. The method includes contacting the transcription factor, e.g., a MarA family polypeptide, with a transcription factor modulutating compound of the formula (I):

A-E (I)

[0272] wherein A is a polar moiety, E is a hydrophobic moiety, and pharmaceutically acceptable salts thereof. The transcription factor modulating compound, e.g., a MarA family modulating compound, may comprise one or more polar moieties and/or one or more hydrophobic moieties.

[0273] In another embodiment, the invention pertains to methods for reducing antibiotic resistance of a microbial cell. The method includes contacting the cell with a transcription factor modulating compound, e.g., a MarA family modulating compound, such that the antibiotic resistance of the cell is reduced.

[0274] In another embodiment, the invention pertains to inhibiting transcription, comprising contacting a transcription factor with a transcription factor modulating compound, such that transcription is inhibited. In a further embodiment, the transcription of a prokaryotic cell is inhibited. In another further embodiment, the transcription factor modulating compound is a compound of anyone of formulae (I)-(X).

[0275] The term "antibiotic resistance" includes resistance of a microbial cell to a antibiotic compound, especially an antibiotic compound which had been previously used to treat similar microbial organisms successfully.

[0276] The term "polar moiety" includes moieties with at least one heterocycle. It also includes moieties such as, but not limited to, hydroxyl, halogens, thioethers, carboxylic acids, metals (e.g. alkali, alkaline, Au, Hg, Ag, Mn, Co, Cu, Zn, etc.), nitro, amino, alkoxy, and other moieties which allow the compound to perform its intended function. The term "polar moiety" includes moieties which allow the transcription factor modulating compound to perform its intended function, e.g., modulate a transcription factor, e.g., an AraC family polypeptide or a MarA family polypeptide. A heterocyclic polar moiety may comprise one or more rings, one or more of which may be aromatic. In an embodiment, one or more rings of the polar moiety are fused. The heterocyclic polar moiety may also be bicyclic.

[0277] The heterocyclic polar moiety may comprise one or more nitrogen, sulfur, or oxygen atoms. Examples of heterocycles include benzodioxazole, benzofuran, benzoimidazole, benzoxazole, benzothiazole, benzothiophene, chromenone, deazapurine, furan, imidazole, imidazopyridine,

indole, indolizine, isooxazole, isothiaozole, isoquinoline, methylenedioxyphenyl, napthridine, oxazole, purine, pyrazine, pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrollidine, quinoline, tetrazole, thiazole, thiophene, triazole, and triazoletetrazole.

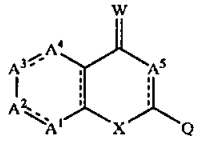
**[0278]** Furthermore, the polar moiety may be substituted when chemically feasible. For example, the polar moiety may be substituted with one or more substituents such as alkyl, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Examples of substituents also include nitro, alkoxy, aryl, amidyl, ester, thioester, alkyl (e.g., methyl, ethyl, propyl, butyl, pentyl, etc.), araalkyl (e.g., substituted or unsubstituted benzyl), hydroxy, halogen (e.g., fluorine, bromine, chlorine, iodine, etc.).

**[0279]** The term "hydrophobic moiety" includes moieties such as which allow the transcription factor modulating compound (e.g., an HTH protein modulating compound, an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) to perform its intended function, e.g., modulate a transcription factor. Examples of hydrophobic moieties include, for example, hydrogen, alkyl, alkenyl, alkynyl, and aryl moieties. The hydrophobic moieties may be unsubstituted or substituted, if chemically feasible (e.g., not hydrogen). In an embodiment, the hydrophobic moiety is substituted or unsubstituted phenyl. Examples of substituents include alkyl, alkenyl, alkynyl, alkoxy, halogen, amino, thiol, hydroxy, nitro, aryl, and heteroaryl. The substituents can be substituted or unsubstituted. In an embodiment, the phenyl hydrophobic moiety ispara-substituted, e.g., alkyl (methyl, ethyl, propyl, butyl, pentyl, etc.), halogen (e.g., fluorine, bromine, chlorine, iodine, etc.), hydroxy, substituted.

**[0280]** In another embodiment, the hydrophobic moiety is heterocyclic. Examples of heterocyclic hydrophobic moieties include imidazopyridine, quinolinyl, pyridinyl, etc.

**[0281]** In one embodiment, the transcription factor modulating compound (e.g., MarA family polypeptide modulating compound, AraC family polypeptide modulating compound, etc.) is of the formula (VII):

(VII)




**[0282]** wherein

**[0283]** W is NH, O or S;

**[0284]** X is O, S, or C, optionally linked to Q;

**[0285]** Al is C-$Z^1$, O, or S;

**[0286]** $A^2$ is C-$Z^2$, O, or S;

**[0287]** $A^3$ is C-$Z^3$, O, or S;

**[0288]** $A^4$ is C-$Z^4$, O, or S;

**[0289]** $A^5$ is C-$Z^5$, or N-$Z^5$;

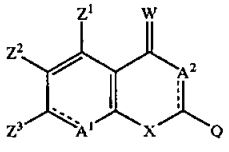
**[0290]** $Z^1$, $Z^2$, $Z^3$, and $Z^4$ are each independently selected from the group consisting of hydrogen, alkoxy, hydroxy, halogen, and alkyl;

**[0291]** $Z^5$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, or carbonyl;

**[0292]** Q is hydrogen, alkyl, alkenyl, alkynyl, halogen, hydroxy, aryl, and pharmaceutically acceptable salts thereof.

**[0293]** In yet another embodiment, the transcription factor modulating compound (e.g., the MarA family polypeptide modulating compound, AraC family polypeptide modulating compound, etc.) is of the formula (II):

(II)




**[0294]** wherein

**[0295]** W is O or S;

**[0296]** X is O, S, or C, optionally linked to Q;

**[0297]** $A^1$ is C-$Z^4$, O, or S;

**[0298]** $A^2$ is C-$Z^5$, or N-$Z^5$;

**[0299]** $Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

**[0300]** $Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

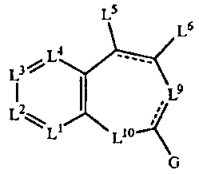
**[0301]** Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

**[0302]** In a further embodiment, W may be oxygen and X may be oxygen. Furthermore, $A^1$ and $A^2$ may be C-$Z^4$ and C-$Z^5$, respectively. Examples of $Z^4$ and $Z^5$ include hydrogen and hydroxy. Examples of $Z^1$ and $Z^2$ include hydrogen and hydroxy. Other examples of $Z^2$ also include halogen, e.g., fluorine, chlorine, bromine, and iodine. Examples of $Z^3$ include, for example, hydrogen, alkoxy and hydroxy. Examples of Q include substituted and unsubstituted phenyl. The phenyl may be para-substituted. Examples of substituents include hydroxyl, halogen (e.g., fluorine, bromine, chlorine, iodine, etc.), amino, alkyl (e.g., methyl, ethyl, propyl, butyl, pentyl, etc.), nitro, cyano, etc. In an embodiment, the transcription factor modulating compound is a

MarA modulating compound, and in a further embodiment, a MarA inhibiting compound.

[0303] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (VIII):

(VIII)



[0304] wherein:

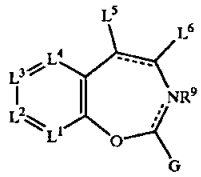
[0305] G is a substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen;

[0306] $L^1$, $L^2$, $L^3$, $L^4$, $L^9$ and $L^{10}$ are each independently oxygen, sulfur, substituted or unsubstituted nitrogen, and substituted or unsubstituted carbon; and

[0307] $L^5$ and $L^6$ are each independently hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, acyl, heterocyclic, amino, nitro, hydroxy, cyano, alkoxy, or aryl, and $L^5$ and $L^6$ may optionally be linked with a chain of one to six atoms to form a fused ring, and pharmaceutically acceptable salts thereof.

[0308] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (IX):

(IX)



[0309] wherein:

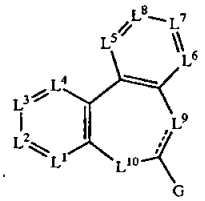
[0310] G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen;

[0311] $L^1$, $L^2$, $L^3$, and $L^4$ are each independently oxygen, sulfur, substituted or unsubstituted nitrogen, and substituted or unsubstituted carbon; and

[0312] $R^9$, $L^5$ and $L^6$ are each independently hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, acyl, heterocyclic, amino, nitro, hydroxy, cyano, alkoxy, or aryl, and L, and $L_6$ may optionally be linked with a chain of one to six atoms to form a fused ring, and pharmaceutically acceptable salts thereof.

[0313] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (III):

(III)



[0314] wherein

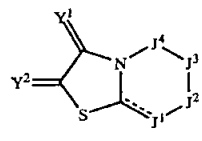
[0315] G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

[0316] $L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$, $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

[0317] In a further embodiment, $L^9$ is N—$R^9$, wherein $R^9$ is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, acyl, or aryl. In another, $L^{10}$ is oxygen. In an embodiment, $R^9$ is hydrogen. In another, G is substituted or unsubstituted phenyl or heteroaryl. In a further embodiment, G is cycloalkenyl, e.g., cyclohexenyl. In one embodiment, $L^1$, $L^2$, $L^3$, and $L^4$ are each substituted or unsubstituted carbon and $L^5$, $L^6$, and $L^8$ are each nitrogen. $L^7$ may be substituted carbon, e.g., substituted with a thioether moiety. In another embodiment, $L^9$ and $L^{10}$ are each nitrogen. In another embodiment, the invention pertains to compounds of formula (III), wherein $L^9$ is nitrogen, $L^{10}$ is oxygen, $L^1$-$L^8$ are each C—H, the dotted line represents a double bond and and where G is not hydrogen or methyl.

[0318] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (X):

(X)



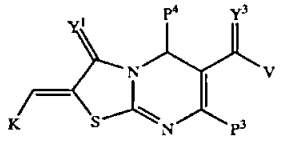
[0319] wherein

[0320] $Y^1$ and $Y^2$ are each oxygen, sulfur, or substituted or unsubstituted carbon;

[0321] $J^1$, $J^2$, $J^3$, and $J^4$ are each oxygen, nitrogen, or optionally substituted carbon, and pharmaceutically acceptable salts thereof.

[0322] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (IV):

(IV)



[0323] wherein

[0324] $Y^1$ and $Y^2$ are each oxygen or sulfur;

[0325] J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

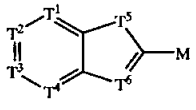
[0326] V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

[0327] P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

[0328] In a further embodiment, $Y^1$ and $Y^3$ are each oxygen, V is alkoxy and J is lower alkyl. In another embodiment, P is substituted or unsubstituted phenyl. K may be substituted or unsubstituted heteroaryl.

[0329] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (V):

(V)



[0330] wherein

[0331] $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ are each independently substituted or unsubstituted carbon, oxygen, substituted or unsubstituted nitrogen, or sulfur;

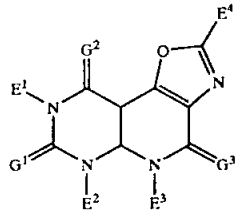
[0332] M is hydrogen, alkyl, alkenyl, alkynyl, heterocyclic or aryl, or pharmaceutically acceptable salts thereof.

[0333] In a further embodiment, $T^5$ is N-W or C-W, wherein W is alkyl, alkenyl, alkynyl, aryl, heterocyclic, acyl, hydroxy, alkoxy, alkthio, amino, nitro, halogen, or hydrogen. In another further embodiment, $T^6$ is N.

[0334] In a further embodiment, M is substituted or unsubstituted aryl. W may be substituted or unsubstituted alkyl. In another embodiment, $T^1$, $T^2$, $T^3$ and $T^4$ are each substituted or unsubstituted carbon. In a further embodiment, at least one of $T^1$, $T^2$, $T^3$, and $T^4$ is nitrogen, and the remaining T moieties are substituted or unsubstituted carbon.

[0335] In another embodiment, the transcription factor modulating compound (e.g., an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, etc.) is of the formula (VI):

(VI)



[0336] wherein

[0337] $G^1$, $G^2$, and $G^3$ are each independently O, S, substituted or unsubstituted nitrogen, or substituted or unsubstituted carbon;

[0338] $E^1$, $E^2$, and $E^3$ are each independently hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, or acyl; and

[0339] $E^4$ is alkyl, alkenyl, alkynyl, aryl, halogen, cyano, amino, nitro, or acyl, and pharmaceutically acceptable salts thereof.

[0340] In a further embodiment, $G^1$, $G^2$ and $G^3$ are each oxygen.

[0341] Other transcription factor modulating compounds are shown in Table 3. The invention pertains to each of these compounds, methods (both therapeutic and otherwise) using each of the compounds, and compositions comprising at least one of the compounds of Table 4, Table 5, or of formulae (I), (II), (III), (IV), (V), (VI), (VII), (VIII), (IX) or (X).

[0342] The invention also pertains to each of the following compounds:

[0343] 2-(4-isopropylphenyl)-4H-chromen-4-one;

[0344] 2-(3,4-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one

[0345] N-isopropyl-2-[(4-methyl-5-quinolin-6-yl-4H-1,2,4-triazol-3-yl)thio]acetamide;

[0346] 4-hydroxy-6-methyl-5,6-dihydro-2H-pyrano [3,2-c]quinoline-2,5-dione;

[0347] 5,7-Dihydroxy-2-(4-hydroxy-phenyl)-chromen-4-one;

[0348] 2-[4-(dimethylamino)phenyl]-4H-chromen-4-one;

[0349] 1-(benzyloxy)-2-phenyl-1H-imidazo[4,5-b] pyridine;

[0350] 2-(benzylthio)-4-phenyl-5-(1-phenyl-1H-1,2,3,4-tetraazol-5-yl)pyrimidine;

[0351] 6-fluoro-2-phenyl-4H-chromen-4-one;

[0352] 7-methoxy-2-phenyl-4H-chromen-4-one;

[0353] 4-(1,3-dioxo-1,3-dihydro-2H-inden-2-yliden)-2-phenyl-6-(2-pyridinyl)tetrahydropyrrolo [3,4-c]pyrrole-1,3 (2H,3aH)-dione;

[0354] 2-(2-Hydroxy-3-oxo-5-p-tolyl-2,3-dihydro-furan-2-yl)-malonamic acid ethyl ester;

[0355] 2-[(6-nitro-2-phenyl-1H-1,3-benzimidazol-1-yl)oxy] acetic acid;

[0356] 2-(4-fluorophenyl)-4H-chromen-4-one;

[0357] 1-methoxy-2-(4-methylphenyl)-1H-imidazo [4,5-b]pyridine;

[0358] 6-(5-Iodo-furan-2-yl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cyclo-heptene;

[0359] 6-(4-Ethoxy-phenyl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cyclo-heptene;

[0360] 3-Methylsulfanyl-6-(5-nitro-furan-2-yl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cyclo-heptene;

[0361] 3-Methylsulfanyl-6-[5-(4-nitro-phenyl)-furan-2-yl]-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0362] 4-(3-Ethylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cyclohepten-6-yl)-benzene-1,2-diol;

[0363] 6-(4-Benzyloxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cy-cloheptene;

[0364] 6-Benzo[1,3]dioxol-5-yl-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cy-cloheptene;

[0365] 3-Butylsulfanyl-6-(2,4-dimethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c] cycloheptene;

[0366] 6-(4-Allyloxy-phenyl)-3-butylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c]cyclo-heptene;

[0367] 3-Butylsulfanyl-6-(4-ethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cyclo-heptene;

[0368] 6-(4-Methoxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cyclo-heptene;

[0369] 6-[5-(3-Nitro-phenyl)-furan-2-yl]-3-propyl-sulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene;

[0370] 2-(3-Phenyl-1H-pyrazol-4-ylmethylene)-benzo[4,5] imidazo[2,1-b]thiazol-3 one;

[0371] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethyl-ene]-5-(2-methoxy-naphthalen-1-yl) -7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0372] 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0373] 5-Benzo[1,3]dioxol-5-yl-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0374] 5-(3,4-Dimethoxy-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0375] 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-ylmethylene]-5-(4-methyl sulfanyl-phenyl)-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0376] 2-[5-(4-Carboxy-phenyl)-furan-2-ylmethyl-ene]-5-(2-methoxy-naphthalen-1-yl) -7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0377] 5-Benzo [1,3]dioxol-5-yl-2-[5-(4-ethoxycar-bonyl-phenyl)-furan-2-ylmethylene]-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0378] 7-Methyl-3-oxo-5-phenyl-2-[5-(3-trifluorom-ethyl-phenyl)-furan-2-ylmethylene]-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0379] 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-5-phenyl-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0380] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethyl-ene]-5-(4-dimethylamino-phenyl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0381] 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(4-methyl-3-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0382] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethyl-ene]-7-methyl-5-(4-methylsulfanyl-phenyl)-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0383] [1,2]Naphthoquinone 1-[O-(6-oxo-6H-anthra [1,9-cd] isoxazol-5-yl)-oxime];

[0384] 3-Acetyl-2,5,7-triphenyl-1H-1,3a,4,8-tetraaza-7a-azonia-cyclopenta[a]inidene;

[0385] 1-Amino-3-benzo[1,3]dioxol-5-yl-benzo[4,5] imidazo[1,2-a]pyridine-2,4-dicarbonitrile;

[0386] 2-[2-(5-furan-2-yl-4-phenyl-4H-[1,2,4]tria-zol-3-yl sulfanyl)-acetylamino]-benzoic acid methyl ester;

[0387] 6,7-Dimethyl-2-(3-phenyl-1H-pyrazol-4-yl-methylene)-benzo[4,5]imidazo[2,1-b] thiazol-3-one,

[0388] 2-(5-Benzo[1,2,5]oxadiazol-5-yl-4-methyl-4H-[1,2,4] triazol-3-ylsulfanyl)-N-(3-methylsulfanyl-phenyl)-acetamide;

[0389] 4-(1,3-Dioxo-indan-2-ylidene)-2-phenyl-6-pyridin-2-yl-tetrahydro-pyrrolo[3,4-c] pyrrole-1,3-dione;

[0390] 6-Nitro-2-phenyl-1-(3-trifluoromethyl-benzyloxy)-1H-benzoimidazole;

[0391] (6-Nitro-2-phenyl-benzoimidazol-1-yloxy)-acetic acid;

[0392] 1-Benzyloxy-6-nitro-2-phenyl-1H-benzoimidazole;

[0393] 1-(4-Methyl-benzyloxy)-6-nitro-2-phenyl-1H-benzoimidazole;

[0394] 6,8-Dimethyl-2-(4-nitro-phenyl)-5-phenyl-5H,6H-1-oxa-3,5,6,8-tetraaza-cyclopenta[a]naphthalene-4,7,9-trione;

[0395] 6,8-Dimethyl-5-phenyl-2-p-tolyl-5H,6H-1-oxa-3,5,6,8-tetraaza-cyclopenta [a]naphthalene-4,7,9-trione;

[0396] 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-yl methylene]-benzo [4,5] imidazo[2,1-b]thiazol-3-one;

[0397] Cobalt 5,10,15,20-Tetra-pyridin-4-yl-porphyrine;

[0398] 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-ylmethylene]-5-methyl-6-vinyl-imidazo[2,1-b] thiazol-3-one;

[0399] Cobalt 5,10,15,20-Tetra-pyridin-3-yl-porphyrine;

[0400] Zinc 5,10,15,20-Tetra-pyridin-4-yl-porphyrine;

[0401] 2-(4-hydroxyphenyl)-4H-chromen-4-one, and pharmaceutically acceptable salts thereof.

[0402] In a further embodiment, the transcription factor modulating compound is not apigenin. In another The term "alkyl" includes saturated aliphatic groups, including straight-chain alkyl groups (e.g., methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, etc.), branched-chain alkyl groups (isopropyl, tert-butyl, isobutyl, etc.), cycloalkyl (alicyclic) groups (cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. The term alkyl further includes alkyl groups, which can further include oxygen, nitrogen, sulfur or phosphorous atoms replacing one or more carbons of the hydrocarbon backbone. In certain embodiments, a straight chain or branched chain alkyl has 6 or fewer carbon atoms in its backbone (e.g., $C_1$-$C_6$ for straight chain, $C_3$-$C_6$ for branched chain), and more preferably 4 or fewer. Likewise, preferred cycloalkyls have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term $C_1$-$C_6$ includes alkyl groups containing 1 to 6 carbon atoms.

[0403] Moreover, the term alkyl includes both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, e.g., with the substituents described above. An "alkylaryl" or an "arylalkyl" moiety is an alkyl substituted with an aryl (e.g., phenylmethyl (benzyl)). The term "alkyl" also includes the side chains of natural and unnatural amino acids.

[0404] The term "aryl" includes groups, including 5- and 6-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, phenyl, pyrrole, furan, thiophene, thiazole, isothiazole, imidazole, triazole, tetrazole, pyrazole, oxazole, isooxazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like. Furthermore, the term "aryl" includes multicyclic aryl groups, e.g., tricyclic, bicyclic, e.g., naphthalene, benzoxazole, benzodioxazole, benzothiazole, benzoimidazole, benzothiophene, methylenedioxyphenyl, quinoline, isoquinoline, napthridine, indole, benzofuran, purine, benzofuran, deazapurine, or indolizine. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles", "heterocycles,""heteroaryls" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, hydroxyl, alkoxy, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkylaminoacarbonyl, arylalkyl aminocarbonyl, alkenylaminocarbonyl, alkylcarbonyl, arylcarbonyl, arylalkylcarbonyl, alkenylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Aryl groups can also be fused or bridged with alicyclic or heterocyclic rings which are not aromatic so as to form a polycycle (e.g., tetralin). The term "aryl" also includes multicyclic aryl groups such as porphrins, phthalocyanines, etc.

[0405] The term "alkenyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double bond.

[0406] For example, the term "alkenyl" includes straight-chain alkenyl groups (e.g., ethylenyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl, etc.), branched-chain alkenyl groups, cycloalkenyl (alicyclic) groups (cyclopropenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl), alkyl or alkenyl substituted

cycloalkenyl groups, and cycloalkyl or cycloalkenyl substituted alkenyl groups. The term alkenyl further includes alkenyl groups which include oxygen, nitrogen, sulfur or phosphorous atoms replacing one or more carbons of the hydrocarbon backbone. In certain embodiments, a straight chain or branched chain alkenyl group has 6 or fewer carbon atoms in its backbone (e.g., $C_2$-$C_6$ for straight chain, $C_3$-$C_6$ for branched chain). Likewise, cycloalkenyl groups may have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term $C_2$-$C_6$ includes alkenyl groups containing 2 to 6 carbon atoms.

[0407] Moreover, the term alkenyl includes both "unsubstituted alkenyls" and "substituted alkenyls", the latter of which refers to alkenyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

[0408] The term "alkynyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but which contain at least one triple bond.

[0409] For example, the term "alkynyl" includes straight-chain alkynyl groups (e.g., ethynyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, octynyl, nonynyl, decynyl, etc.), branched-chain alkynyl groups, and cycloalkyl or cycloalkenyl substituted alkynyl groups. The term alkynyl further includes alkynyl groups which include oxygen, nitrogen, sulfur or phosphorous atoms replacing one or more carbons of the hydrocarbon backbone. In certain embodiments, a straight chain or branched chain alkynyl group has 6 or fewer carbon atoms in its backbone (e.g., $C_2$-$C_6$ for straight chain, $C_3$-$C_6$ for branched chain). The term $C_2$-$C_6$ includes alkynyl groups containing 2 to 6 carbon atoms.

[0410] Moreover, the term alkynyl includes both "unsubstituted alkynyls" and "substituted alkynyls", the latter of which refers to alkynyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

[0411] Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to five carbon atoms in its backbone structure. "Lower alkenyl" and "lower alkynyl" have chain lengths of, for example, 2-5 carbon atoms.

[0412] The term "acyl" includes compounds and moieties which contain the acyl radical ($CH_3CO$—) or a carbonyl group. The term "substituted acyl" includes acyl groups where one or more of the hydrogen atoms are replaced by for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

[0413] The term "acylamino" includes moieties wherein an acyl moiety is bonded to an amino group. For example, the term includes alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido groups.

[0414] The term "aroyl" includes compounds and moieties with an aryl or heteroaromatic moiety bound to a carbonyl group. Examples of aroyl groups include phenylcarboxy, naphthyl carboxy, etc.

[0415] The terms "alkoxyalkyl", "alkylaminoalkyl" and "thioalkoxyalkyl" include alkyl groups, as described above, which further include oxygen, nitrogen or sulfur atoms replacing one or more carbons of the hydrocarbon backbone, e.g., oxygen, nitrogen or sulfur atoms.

[0416] The term "alkoxy" includes substituted and unsubstituted alkyl, alkenyl, and alkynyl groups covalently linked to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy, isopropyloxy, propoxy, butoxy, and pentoxy groups. Examples of substituted alkoxy groups include halogenated alkoxy groups. The alkoxy groups can be substituted with groups such as alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moieties. Examples of halogen substituted alkoxy groups include, but are not limited to, fluoromethoxy, difluoromethoxy, trifluoromethoxy, chloromethoxy, dichloromethoxy, trichloromethoxy, etc.

[0417] The term "amine" or "amino" includes compounds where a nitrogen atom is covalently bonded to at least one

carbon or heteroatom. The term "alkyl amino" includes groups and compounds wherein the nitrogen is bound to at least one additional alkyl group. The term "dialkyl amino" includes groups wherein the nitrogen atom is bound to at least two additional alkyl groups. The term "arylamino" and "diarylamino" include groups wherein the nitrogen is bound to at least one or two aryl groups, respectively. The term "alkylarylamino,""alkylaminoaryl" or "arylaminoalkyl" refers to an amino group which is bound to at least one alkyl group and at least one aryl group. The term "alkaminoalkyl" refers to an alkyl, alkenyl, or alkynyl group bound to a nitrogen atom which is also bound to an alkyl group.

**[0418]** The term "amide" or "aminocarboxy" includes compounds or moieties which contain a nitrogen atom which is bound to the carbon of a carbonyl or a thiocarbonyl group. The term includes "alkaminocarboxy" groups which include alkyl, alkenyl, or alkynyl groups bound to an amino group bound to a carboxy group. It includes arylaminocarboxy groups which include aryl or heteroaryl moieties bound to an amino group which is bound to the carbon of a carbonyl or thiocarbonyl group. The terms "alkylaminocarboxy,""alkenylaminocarboxy,""alkynylaminocarboxy," and "arylaminocarboxy" include moieties wherein alkyl, alkenyl, alkynyl and aryl moieties, respectively, are bound to a nitrogen atom which is in turn bound to the carbon of a carbonyl group.

**[0419]** The term "carbonyl" or "carboxy" includes compounds and moieties which contain a carbon connected with a double bond to an oxygen atom. Examples of moieties which contain a carbonyl include aldehydes, ketones, carboxylic acids, amides, esters, anhydrides, etc.

**[0420]** The term "thiocarbonyl" or "thiocarboxy" includes compounds and moieties which contain a carbon connected with a double bond to a sulfur atom.

**[0421]** The term "ether" includes compounds or moieties which contain an oxygen bonded to two different carbon atoms or heteroatoms. For example, the term includes "alkoxyalkyl" which refers to an alkyl, alkenyl, or alkynyl group covalently bonded to an oxygen atom which is covalently bonded to another alkyl group.

**[0422]** The term "ester" includes compounds and moieties which contain a carbon or a heteroatom bound to an oxygen atom which is bonded to the carbon of a carbonyl group. The term "ester" includes alkoxycarboxy groups such as methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, butoxycarbonyl, pentoxycarbonyl, etc. The alkyl, alkenyl, or alkynyl groups are as defined above.

**[0423]** The term "thioether" includes compounds and moieties which contain a sulfur atom bonded to two different carbon or hetero atoms. Examples of thioethers include, but are not limited to alkthioalkyls, alkthioalkenyls, and alkthioalkynyls. The term "alkthioalkyls" include compounds with an alkyl, alkenyl, or alkynyl group bonded to a sulfur atom which is bonded to an alkyl group. Similarly, the term "alkthioalkenyls" and alkthioalkynyls" refer to compounds or moieties wherein an alkyl, alkenyl, or alkynyl group is bonded to a sulfur atom which is covalently bonded to an alkynyl group.

**[0424]** The term "hydroxy" or "hydroxyl" includes groups with an —OH or —O$^-$.

**[0425]** The term "halogen" includes fluorine, bromine, chlorine, iodine, etc. The term "perhalogenated" generally refers to a moiety wherein all hydrogens are replaced by halogen atoms.

**[0426]** The terms "polycyclyl" or "polycyclic radical" refer to two or more cyclic rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkoxycarbonyl, alkylaminoacarbonyl, arylalkylaminocarbonyl, alkenylaminocarbonyl, alkylcarbonyl, arylcarbonyl, arylalkyl carbonyl, alkenylcarbonyl, aminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamine, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkyl, alkylaryl, or an aromatic or heteroaromatic moiety.

**[0427]** The term "heteroatom" includes atoms of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, sulfur and phosphorus.

**[0428]** It will be noted that the structure of some of the compounds of this invention includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry (e.g., all enantiomers and diastereomers) are included within the scope of this invention, unless indicated otherwise. Such isomers can be obtained in substantially pure form by classical separation techniques and by stereochemically controlled synthesis. Furthermore, the structures and other compounds and moieties discussed in this application also include all tautomers thereof.

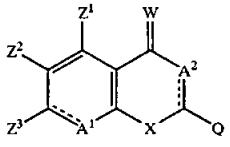
**[0429]** Bonds represented by "⋯⋯" in a structural formula mean that the bond may be either a single or a double bond.

**[0430]** IX. Formulations Comprising Transcription Factor Modulating Compounds

**[0431]** The invention provides compositions which include a therapeutically-effective amount or dose of a transcription factor modulating compound and/or a compound identified in any of the instant assays and one or more carriers (e.g., pharmaceutically acceptable additives and/or diluents). The pharmaceutical compositions of the invention may comprise any compound described in this application as a transcription factor modulating compound, an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, a MarA family inhibiting compound, a MarA inhibiting compound, compounds of formulae (I), (II), (III), (IV), (V), (VI), (VII), (VIII), (IX), (X), Table 4, Table 5, scaffold, etc. Each of these compounds may be used alone of in combination as a part of a pharmaceutical composition of the invention. Furthermore, a composition can also include a second antimicrobial agent, e.g., an antibiotic.

[0432] The invention pertains to pharmaceutical compositions comprising an effective amount of a transcription factor modulating compound (e.g., a MarA family polypeptide modulating compound or an AraC family polypeptide modulating compound), and a pharmaceutically acceptable carrier. In one embodiment, the transcription factor modulating compound is of the formula (II):

(II)



[0433] wherein

[0434] W is O or S;

[0435] X is O, S, or C, optionally linked to Q;

[0436] $A^1$ is C-$Z^4$, O, or S;

[0437] $A^2$ is C-$Z^5$, or N-$Z^5$;

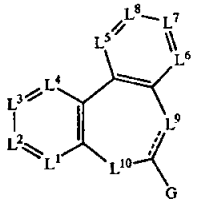
[0438] $Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

[0439] $Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

[0440] Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

[0441] In another embodiment, the pharmaceutical compositions of the invention include an effective amount of a transcription factor modulating compound of the formula (III):

(III)



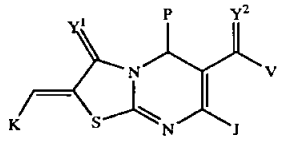
[0442] wherein

[0443] G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

[0444] $L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$; $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

[0445] In yet another embodiment, the pharmaceutical compositions of the invention include a pharmaceutically acceptable carrier (optional) and an effective amount of a transcription factor modulating compound of the formula (IV):

(IV)



[0446] wherein

[0447] $Y^1$ and $Y^2$ are each oxygen or sulfur;

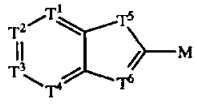
[0448] J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

[0449] V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

[0450] P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

[0451] In yet another embodiment, the pharmaceutical compositions of the invention include a pharmaceutically acceptable carrier (optional) and an effective amount of a transcription factor modulating compound of the formula (V):

(V)



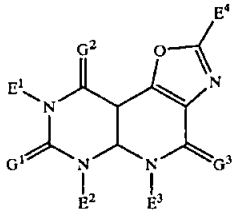
[0452] wherein

[0453] $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ are each independently substituted or unsubstituted carbon, oxygen, substituted or unsubstituted nitrogen, or sulfur;

[0454] M is hydrogen, alkyl, alkenyl, alkynyl, or aryl, or pharmaceutically acceptable salts thereof.

[0455] In yet another embodiment, the pharmaceutical compositions of the invention include a pharmaceutically acceptable carrier (optional) and an effective amount of a

transcription factor modulating compound of the formula (VI):



(VI)

[0456] wherein

[0457] $G^1$, $G^2$, and $G^3$ are each independently O, S, substituted or unsubstituted nitrogen, or substituted or unsubstituted carbon:

[0458] $E^1$, $E^2$, and $E^3$ are each independently hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, or acyl; and

[0459] $E^4$ is alkyl, alkenyl, alkynyl, aryl, halogen, cyano, amino, nitro, or acyl, and pharmaceutically acceptable salts thereof.

[0460] In yet another further embodiment, the pharmaceutical compositions of the invention comprise an effective amount of a transcription factor modulating compound listed below or found in Table 4 or Table 5:

[0461] 2-(4-isopropylphenyl)-4H-chromen-4-one;

[0462] 2-(3,4-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one N-isopropyl-2-[(4-methyl-5-quinolin-6-yl-4H-1,2,4-triazol-3-yl)thio]acetamide;

[0463] 4-hydroxy-6-methyl-5,6-dihydro-2H-pyrano [3,2-c]quinoline-2,5-dione;

[0464] 5,7-Dihydroxy-2-(4-hydroxy-phenyl)-chromen-4-one;

[0465] 2-[4-(dimethylamino)phenyl]-4H-chromen-4-one;

[0466] 1-(benzyloxy)-2-phenyl-1H-imidazo[4,5-b] pyridine;

[0467] 2-(benzylthio)-4-phenyl-5-(1-phenyl-1H-1,2,3,4-tetraazol-5-yl)pyrimidine;

[0468] 6-fluoro-2-phenyl-4H-chromen-4-one;

[0469] 7-methoxy-2-phenyl-4H-chromen-4-one;

[0470] 4-(1,3-dioxo-1,3-dihydro-2H-inden-2-yliden)-2-phenyl-6-(2-pyridinyl)tetrahydropyrrolo [3,4-c]pyrrole-1,3(2H,3aH)-dione;

[0471] 2-(2-Hydroxy-3-oxo-5-p-tolyl-2,3-dihydro-furan-2-yl)-malonamic acid ethyl ester;

[0472] 2-[(6-nitro-2-phenyl-1H-1,3-benzimidazol-1-yl)oxy]acetic acid;

[0473] 2-(4-fluorophenyl)-4H-chromen-4-one;

[0474] 1-methoxy-2-(4-methylphenyl)-1H-imidazo [4,5-b]pyridine;

[0475] 6-(5-Iodo-furan-2-yl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0476] 6-(4-Ethoxy-phenyl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0477] 3-Methylsulfanyl-6-(5-nitro-furan-2-yl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0478] 3-Methylsulfanyl-6-[5-(4-nitro-phenyl)-furan-2-yl]-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene;

[0479] 4-(3-Ethylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cyclohepten-6-yl)-benzene-1,2-diol;

[0480] 6-(4-Benzyloxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0481] 6-Benzo[1,3]dioxol-5-yl-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0482] 3-Butylsulfanyl-6-(2,4-dimethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c] cycloheptene;

[0483] 6-(4-Allyloxy-phenyl)-3-butylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c]cycloheptene;

[0484] 3-Butylsulfanyl-6-(4-ethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene;

[0485] 6-(4-Methoxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene;

[0486] 6-[5-(3-Nitro-phenyl)-furan-2-yl]-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene;

[0487] 2-(3-Phenyl-1H-pyrazol-4-ylmethylene)-benzo[4,5] imidazo[2, 1-b]thiazol-3-one;

[0488] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-5-(2-methoxy-naphthalen-1-yl) -7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0489] 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0490] 5-Benzo[1,3]dioxol-5-yl-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0491] 5-(3,4-Dimethoxy-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0492] 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-ylmethylene]-5-(4-methyl sulfanyl-phenyl)-3-

oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0493] 2-[5-(4-Carboxy-phenyl)-furan-2-ylmethylene]-5-(2-methoxy-naphthalen-1-yl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0494] 5-Benzo[1,3]dioxol-5-yl-2-[5-(4-ethoxycarbonyl-phenyl)-furan-2-yl methylene]-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0495] 7-Methyl-3-oxo-5-pheny-)-[5-(3-trifluoromethyl-phenyl)-furan-2-ylmethylene]-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0496] 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-5-phenyl-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0497] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-5-(4-dimethylamino-phenyl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0498] 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(4-methyl-3-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0499] 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-7-methyl-5-(4-methylsulfanyl-phenyl)-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester;

[0500] [1,2]Naphthoquinone 1-[O-(6-oxo-6H-anthra [1,9-cd] isoxazol-5-yl)-oxime];

[0501] 3-Acetyl-2,5,7-triphenyl-1H-1,3a,4,8-tetraaza-7a-azonia-cyclopenta[a]indene;

[0502] 1-Amino-3-benzo[1,3]dioxol-5-yl-benzo[4,5] imidazo[1,2-a]pyridine-2,4-dicarbonitrile;

[0503] 2-[2-(5-Furan-2-yl-4-phenyl-4H-[1,2,4]triazol-3-yl sulfanyl)-acetylamino]-benzoic acid methyl ester;

[0504] 6,7-Dimethyl-2-(3-phenyl-1H-pyrazol-4-yl-methylene)-benzo[4,5]imidazo[2,1-b] thiazol-3-one;

[0505] 2-(5-Benzo[1,2,5]oxadiazol-5-yl-4-methyl-4H-[1,2,4] triazol-3-ylsulfanyl)-N-(3-methylsulfanyl-phenyl)-acetamide;

[0506] 4-(1,3-Dioxo-indan-2-ylidene)-2-phenyl-6-pyridin-2-yl-tetrahydro-pyrrolo[3,4-c] pyrrole-1,3-dione;

[0507] 6-Nitro-2-phenyl-1-(3-trifluoromethyl-benzyloxy)-1H-benzoimidazole;

[0508] (6-Nitro-2-phenyl-benzoimidazol-1-yloxy)-acetic acid;

[0509] 1-Benzyloxy-6-nitro-2-phenyl-1H-benzoimidazole;

[0510] 1-(4-Methyl-benzyloxy)-6-nitro-2-phenyl-1H-benzoimidazole;

[0511] 6,8-Dimethyl-2-(4-nitro-phenyl)-5-phenyl-5H,6H-1-oxa-3,5,6,8-tetraaza-cyclopenta[a]naphthalene-4,7,9-trione;

[0512] 6,8-Dimethyl-5-phenyl-2-p-tolyl-5H,6H-1-oxa-3,5,6,8-tetraaza-cyclopenta [a]naphthalene-4,7,9-trione;

[0513] 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-yl methylene]-benzo [4,5] imidazo[2, 1-b]thiazol-3-one;

[0514] Cobalt 5,10,15,20-Tetra-pyridin-4-yl-porphyrine;

[0515] 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-ylmethylene]-5-methyl-6-vinyl-imidazo[2,1-b] thiazol-3-one;

[0516] Cobalt 5,10,15,20-Tetra-pyridin-3-yl-porphyrine;

[0517] Zinc 5,10,15,20-Tetra-pyridin-4-yl-porphyrine;

[0518] 2-(4-hydroxyphenyl)-4H-chromen-4-one, and pharmaceutically acceptable salts thereof.

[0519] In another embodiment, the method for preventing a bacterial associated state in a subject, comprising administering to the subject an effective amount of a transcription factor modulating compound, such that the bacterial associated state is prevented.

[0520] The term "subject" includes plants and animals (e.g., vertebrates, amphibians, fish, mammals, e.g., cats, dogs, horses, pigs, cows, sheep, rodents, rabbits, squirrels, bears, primates (e.g., chimpanzees, gorillas, and humans) which are capable of suffering from a bacterial associated disorder. The term "subject" also comprises immunocompromised subjects, who may be at a higher risk for infection.

[0521] The term "preventing" the administration of an effective amount of the transcription factor modulating compound to prevent a bacterial associated state from occurring.

[0522] The term "bacterial associated state" includes states characterized by the presence of bacteria which can be prevented by administering the transcription factor modulating compounds of the invention. The term includes biofilm associated states and other infections or the undesirable presence of a bacteria on or in a subject.

[0523] As described in detail below, the pharmaceutical compositions can be formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, aqueous or non-aqueous solutions or suspensions, tablets, boluses, powders, granules, pastes; (2) parental administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream, foam, or suppository; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

[0524] The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler,

diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the antiinfective agents or compounds of the invention from one organ, or portion of the body, to another organ, or portion of the body without affecting its biological effect. Each carrier should be "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical compositions. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

**[0525]** These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microbes may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin. In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

**[0526]** Pharmaceutical compositions of the present invention may be administered to epithelial surfaces of the body orally, parenterally, topically, rectally, nasally, intravaginally, intracisternally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, etc., administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal or vaginal suppositories.

**[0527]** The phrases "parenteral administration" and "administered parenterally" as used herein mean modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

**[0528]** The phrases "systemic administration,""administered systemically,""peripheral administration" and "administered peripherally" as used herein mean the administration of a sucrose octasulfate and/or an antibacterial, drug or other material other than directly into the central nervous system, such that it enters the subject's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

**[0529]** In some methods, the compositions of the invention can be topically administered to any epithelial surface. An "epithelial surface" according to this invention is defined as an area of tissue that covers external surfaces of a body, or which lines hollow structures including, but not limited to, cutaneous and mucosal surfaces. Such epithelial surfaces include oral, pharyngeal, esophageal, pulmonary, ocular, aural, nasal, buccal, lingual, vaginal, cervical, genitourinary, alimentary, and anorectal surfaces.

**[0530]** Compositions can be formulated in a variety of conventional forms employed for topical administration. These include, for example, semi-solid and liquid dosage forms, such as liquid solutions or suspensions, suppositories, douches, enemas, gels, creams, emulsions, lotions, slurries, powders, sprays, lipsticks, foams, pastes, toothpastes, ointments, salves, balms, douches, drops, troches, chewing gums, lozenges, mouthwashes, rinses.

**[0531]** Conventionally used carriers for topical applications include pectin, gelatin and derivatives thereof, polylactic acid or polyglycolic acid polymers or copolymers thereof, cellulose derivatives such as methyl cellulose, carboxymethyl cellulose, or oxidized cellulose, guar gum, acacia gum, karaya gum, tragacanth gum, bentonite, agar, carbomer, bladderwrack, ceratonia, dextran and derivatives thereof, ghatti gum, hectorite, ispaghula husk, polyvinypyrrolidone, silica and derivatives thereof, xanthan gum, kaolin, talc, starch and derivatives thereof, paraffin, water, vegetable and animal oils, polyethylene, polyethylene oxide, polyethylene glycol, polypropylene glycol, glycerol, ethanol, propanol, propylene glycol (glycols, alcohols), fixed oils, sodium, potassium, aluminum, magnesium or calcium salts (such as chloride, carbonate, bicarbonate, citrate, gluconate, lactate, acetate, glucceptate or tartrate).

**[0532]** Such compositions can be particularly useful, for example, for treatment or prevention of an unwanted cell, e.g., vaginal *Neisseria gonorrhoeae*, or infections of the oral cavity, including cold sores, infections of eye, the skin, or the lower intestinal tract. Standard composition strategies for topical agents can be applied to the antiinfective compounds or a pharmaceutically acceptable salt thereof in order to enhance the persistence and residence time of the drug, and to improve the prophylactic efficacy achieved.

**[0533]** For topical application to be used in the lower intestinal tract or vaginally, a rectal suppository, a suitable enema, a gel, an ointment, a solution, a suspension or an insert can be used. Topical transdermal patches may also be used. Transdermal patches have the added advantage of providing controlled delivery of the compositions of the invention to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium.

[0534] Compositions of the invention can be administered in the form of suppositories for rectal or vaginal administration. These can be prepared by mixing the agent with a suitable non-irritating carrier which is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum or vagina to release the drug. Such materials include cocoa butter, beeswax, polyethylene glycols, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active agent.

[0535] Compositions which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams, films, or spray compositions containing such carriers as are known in the art to be appropriate. The carrier employed in the sucrose octasulfate/contraceptive agent should be compatible with vaginal administration and/or coating of contraceptive devices. Combinations can be in solid, semi-solid and liquid dosage forms, such as diaphragm, jelly, douches, foams, films, ointments, creams, balms, gels, salves, pastes, slurries, vaginal suppositories, sexual lubricants, and coatings for devices, such as condoms, contraceptive sponges, cervical caps and diaphragms.

[0536] For ophthalmic applications, the pharmaceutical compositions can be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic uses, the compositions can be formulated in an ointment such as petrolatum. Exemplary ophthalmic compositions include eye ointments, powders, solutions and the like.

[0537] Powders and sprays can contain, in addition to sucrose octasulfate and/or antibiotic or contraceptive agent(s), carriers such as lactose, talc, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[0538] Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens. Pluronics, or polyethylene glycol), proteins like senim albumin, sorbitan, esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

[0539] Compositions of the invention can also be orally administered in any orally-acceptable dosage form including, but not limited to, capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of sucrose octasulfate and/or antibiotic or contraceptive agent(s) as an active ingredient. A compound may also be administered as a bolus, electuary or paste. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

[0540] Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

[0541] Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

[0542] Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

[0543] Suspensions, in addition to the antiinfective agent(s) may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[0544] Sterile injectable forms of the compositions of this invention can be aqueous or oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

[0545] The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as Ph. Helv or similar alcohol.

[0546] The antiinfective agent or a pharmaceutically acceptable salt thereof will represent some percentage of the total dose in other dosage forms in a material forming a combination product, including liquid solutions or suspensions, suppositories, douches, enemas, gels, creams, emulsions, lotions slurries, soaps, shampoos, detergents, powders, sprays, lipsticks, foams, pastes, toothpastes, ointments, salves, balms, douches, drops, troches, lozenges, mouthwashes, rinses and others. Creams and gels for example, are typically limited by the physical chemical properties of the delivery medium to concentrations less than 20% (e.g., 200 mg/gm). For special uses, far less concentrated preparations can be prepared, (e.g., lower percent formulations for pediatric applications). For example, the pharmaceutical composition of the invention can comprise sucrose octasulfate in an amount of 0.001-99%, typically 0.01-75%, more typically 0.1-20%, especially 1-10% by weight of the total preparation. In particular, a preferred concentration thereof in the preparation is 0.5-50%, especially 0.5-25%, such as 1-10%. It can be suitably applied 1-10 times a day, depending on the type and severity of the condition to be treated or prevented.

[0547] Given the low toxicity of an antiinfective agent or a pharmaceutically acceptable salt thereof over many decades of clinical use as an anti-ulcerant [W. R. Garnett, *Clin. Pharm.* 1:307-314 (1982); R.N. Brogden et al., *Drugs* 27:194-209 (1984); D. M. McCarthy, *New Eng J Med,* 325:1017-1025 (1991), an upper limit for the therapeutically effective dose is not a critical issue.

[0548] For prophylactic applications, the pharmaceutical composition of the invention can be applied prior to potential infection. The timing of application prior to potential infection can be optimized to maximize the prophylactic effectiveness of the compound. The timing of application will vary depending on the mode of administration, the epithelial surface to which it is applied, the surface area, doses, the stability and effectiveness of composition under the pH of the epithelial surface, the frequency of application, e.g., single application or multiple applications. One skilled in the art will be able to determine the most appropriate time interval required to maximize prophylactic effectiveness of the compound. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, genetics, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Genetics; Molecular Cloning A Laboratory Manual,* 2nd Ed., ed. by Sambrook, J. et al. (Cold Spring Harbor Laboratory Press (1989)); Short Protocols in Molecular Biology, 3rd Ed., ed. by Ausubel, F. et al. (Wiley, NY (1995)); *DNA Cloning,* Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. (1984)); Mullis et al. U.S. Pat. No. 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. (1984)); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London (1987)); *Handbook Of Experimental Immunology,* Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); and Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1972)).

[0549] X. The Role of Transcription Activation Factor Polypeptides in Biofilms

[0550] In one embodiment, the invention pertains to a method for dispersing or preventing the formation of a biofilm on a surface or in an area, by administering an effective amount of a transcription factor modulating compound, e.g., a HTH protein modulating compound, an AraC family polypeptide modulating compound, a MarA family polypeptide modulating compound, or a MarA inhibiting compound.

[0551] It has been discovered that the absence of MarA and its homologs has a negative effect on biofilm formation in *E. coli.* In order to confirm this finding genetically, plasmid encoded marA was transformed into an *E. coli* strain deleted of marA, soxS, and rob (triple knockout). The expression of MarA in this triple knockout restored biofiln formation in this host to a level that was comparable to that of the wild type host.

[0552] The term "biofilm" includes biological films that develop and persist at interfaces in aqueous and other environments. Biofilms are composed of microorganisms embedded in an organic gelatinous structure composed of one or more matrix polymers which are secreted by the resident microorganisms. The term "biofilm" also includes bacteria that are attached to a surface in sufficient numbers to be detected or communities of microorganisms attached to a surface (Costerton, J. W., et al. (1987) *Ann. Rev. Microbiol.* 41:435-464; Shapiro, J. A. (1988) *Sci Am.* 256:82-89; O'Toole, G. et al. (2000) *Annu Rev Microbiol.* 54:49-79).

[0553] In another embodiment, the invention pertains to methods of treating biofilm associated states in a subject, by administering to said subject an effective amount of a transcription factor modulating compound, e.g., a MarA family inhibiting compound, such that the biofilm associated state is treated.

[0554] The term "biofilm associated states" includes disorders which are characterized by the presence or potential presence of a bacterial biofilm. Many medically important pathogens form biofilms and biofilm formation is often one component of the infectious process (Costerton, J. W. et al. (1999) *Science* 284:1318-1322). Examples of biofilm associated states include, but are not limited to, middle ear infections, cystic fibrosis, osteomyelitis, acne, dental cavities, and prostatitis. Biofilm associated states also include infection of the subject by one or more bacteria, e.g.,

*Pseudomonas aeruginosa*. One consequence of biofilm formation is that bacteria within biofilms are generally less susceptible to a range of different antibiotics relative to their planktonic counterparts.

[0555] Furthermore, the invention also pertains to methods for preventing the formation of biofilms on surfaces or in areas, by contacting the area with an effective amount of a transcription factor modulating compound, e.g., a MarA family inhibiting compound, etc.

[0556] Industrial facilities employ many methods of preventing biofouling of industrial water systems. Many microbial organisms are involved in biofilm formation in industrial waters. Growth of slime-producing bacteria in industrial water systems causes problems including decreased heat transfer, fouling and blockage of lines and valves, and corrosion or degradation of surfaces. Control of bacterial growth in the past has been accomplished with biocides. Many biocides and biocide formulations are known in the art. However, many of these contain components which may be environmentally deleterious or toxic, and are often resistant to breakdown.

[0557] The transcription factor inhibiting compounds, such as but not limited to AraC family inhibiting compounds and MarA family inhibiting compounds, of the present invention are useful in a variety of environments including industrial, clinical, the household, and personal care. The compositions of the invention may comprise one or more compounds of the invention as an active ingredient acting alone, additively, or synergistically against the target organism.

[0558] The MarA family inhibiting compounds and modulating compounds of the invention may be formulated in a composition suitable for use in environments including industry, pharmaceutics, household, and personal care. In an embodiment, the compounds of the invention are soluble in water. The modulating compounds may be applied or delivered with an acceptable carrier system. The composition may be applied or delivered with a suitable carrier system such that the active ingredient (e.g., transcription factor modulating compound of the invention such as a MarA family modulating compound, e.g., a MarA family polypeptide inhibiting compound) may be dispersed or dissolved in a stable manner so that the active ingredient, when it is administered directly or indirectly, is present in a form in which it is available in a advantageous way.

[0559] Also, the separate components of the compositions of the invention may be preblended or each component may be added separately to the same environment according to a predetermined dosage for the purpose of achieving the desired concentration level of the treatment components and so long as the components eventually come into intimate admixture with each other. Further, the present invention may be administered or delivered on a continuous or intermittent basis.

[0560] A transcription factor modulating compound, e.g., a MarA family modulating compound of the present invention, when present in a composition will generally be present in an amount from about 0.000001% to about 100%, more preferably from about 0.001% to about 50%, and most preferably from about 0.01% to about 25%.

[0561] For compositions of the present invention comprising a carrier, the composition comprises, for example, from about 1% to about 99%, preferably from about 50% to about 99%, and most preferably from about 75% to about 99% by weight of at least one carrier.

[0562] The transcription factor modulating compound, e.g., the MarA family polypeptide inhibiting compound, of the invention may be formulated with any suitable carrier and prepared for delivery in forms, such as, solutions, microemulsions, suspensions or aerosols. Generation of the aerosol or any other means of delivery of the present invention may be accomplished by any of the methods known in the art. For example, in the case of aerosol delivery, the compound is supplied in a finely divided form along with any suitable carrier with a propellant. Liquefied propellants are typically gases at ambient conditions and are condensed under pressure. The propellant may be any acceptable and known in the art including propane and butane, or other lower alkanes, such as those of up to 5 carbons. The composition is held within a container with an appropriate propellant and valve, and maintained at elevated pressure until released by action of the valve.

[0563] The compositions of the invention may be prepared in a conventional form suitable for, but not limited to topical or local application such as an ointment, paste, gel, spray and liquid, by including stabilizers, penetrants and the carrier or diluent with the compound according to a known technique in the art. These preparations may be prepared in a conventional form suitable for enteral, parenteral, topical or inhalational applications.

[0564] The present invention may be used in compositions suitable for household use. For example, compounds of the present invention are also useful as active antimicrobial ingredients in household products such as cleansers, detergents, disinfectants, dishwashing liquids, soaps and detergents. In an embodiment, the transcription factor modulating compound of the present invention may be delivered in an amount and form effective for the prevention, removal or termination of microbes.

[0565] The compositions of the invention for household use comprise, for example, at least one transcription factor modulating compound of the invention and at least one suitable carrier. For example, the composition may comprise from about 0.00001% to about 50%, preferably from about 0.0001% to about 25%, most preferably from about 0.0005% to about 10% by weight of the modulating compound based on the weight percentage of the total composition.

[0566] The transcription factor modulating compound of the present invention may also be used in hygiene compositions for personal care. For instance, compounds of the invention can be used as an active ingredient in personal care products such as facial cleansers, astringents, body wash, shampoos, conditioners, cosmetics and other hygiene products. The hygiene composition may comprise any carrier or vehicle known in the art to obtain the desired form (such as solid, liquid, semisolid or aerosol) as long as the effects of the compound of the present invention are not impaired. Methods of preparation of hygiene compositions are not described herein in detail, but are known in the art. For its discussion of such methods, *The CTFA Cosmetic Ingredient Handbook*, Second Edition, 1992, and pages 5-484 of *A Formulary of Cosmetic Preparations* (Vol. 2. Chapters 7-16) are incorporated herein by reference.

[0567] The hygiene composition for use in personal care comprise generally at least one modulating compound of the present application and at least one suitable carrier. For example, the composition may comprise from about 0.00001% to about 50%, preferably from about 0.0001% to about 25%, more preferably from about 0.0005% to about 10% by weight of the transcription factor modulating compound of the invention based on the weight percentage of the total composition.

[0568] The transcription factor modulating compound of the present invention may be used in industry. In the industrial setting, the presence of microbes can be problematic, as microbes are often responsible for industrial contamination and biofouling. Compositions of the invention for industrial applications may comprise an effective amount of the compound of the present invention in a composition for industrial use with at least one acceptable carrier or vehicle known in the art to be useful in the treatment of such systems. Such carriers or vehicles may include diluents, deflocculating agents, penetrants, spreading agents, surfactants, suspending agents, wetting agents, stabilizing agents, compatibility agents, sticking agents, waxes, oils, co-solvents, coupling agents, foams, antifoaming agents, natural or synthetic polymers, elastomers and synergists. Methods of preparation, delivery systems and carriers for such compositions are not described here in detail, but are known in the art. For its discussion of such methods, U.S. Pat. No. 5,939,086 is herein incorporated by reference. Furthermore, the preferred amount of the composition to be used may vary according to the active ingredient(s) and situation in which the composition is being applied.

[0569] The transcription factor modulating compounds, e.g., MarA family polypeptide inhibiting compounds, and compositions of the present invention may be useful in nonaqueous environments. Such nonaqueous environments may include, but are not limited to, terrestrial environments, dry surfaces or semi-dry surfaces in which the compound or composition is applied in a manner and amount suitable for the situation.

[0570] The transcription factor modulating compounds, e.g., MarA family polypeptide modulating compounds, e.g., MarA inhibiting compounds, of the present invention may be used to form contact-killing coatings or layers on a variety of substrates including personal care products (such as toothbrushes, contact lens cases and dental equipment), healthcare products, household products, food preparation surfaces and packaging, and laboratory and scientific equipment. Further, other substrates include medical devices such as catheters, urological devices, blood collection and transfer devices, tracheotomy devices, intraocular lenses, wound dressings, sutures, surgical staples, membranes, shunts, gloves, tissue patches, prosthetic devices (e.g., heart valves) and wound drainage tubes. Still further, other substrates include textile products such as carpets and fabrics, paints and joint cement. A further use is as an antimicrobial soil fumigant.

[0571] The transcription factor modulating compounds of the invention may also be incorporated into polymers, such as polysaccharides (cellulose, cellulose derivatives, starch, pectins, alginate, chitin, guar, carrageenan), glycol polymers, polyesters, polyurethanes, polyacrylates, polyacrylonitrile, polyamides (e.g., nylons), polyolefins, polystyrenes, vinyl polymers, polypropylene, silks or biopolymers. The modulating compounds may be conjugated to any polymeric material such as those with the following specified functionality: 1) carboxy acid, 2) amino group, 3) hydroxyl group and/or 4) haloalkyl group.

[0572] The composition for treatment of nonaqueous environments may be comprise at least one transcription factor modulating compound of the present application and at least one suitable carrier. In an embodiment, the composition comprises from about 0.001% to about 75%, advantageously from about 0.01% to about 50%, and preferably from about 0.1% to about 25% by weight of a transcription factor modulating compound of the invention based on the weight percentage of the total composition.

[0573] The transcription factor modulating compounds and compositions of the invention may also be useful in aqueous environments. "Aqueous environments" include any type of system containing water, including, but not limited to, natural bodies of water such as lakes or ponds; artificial, recreational bodies of water such as swimming pools and hot tubs; and drinking reservoirs such as wells. The compositions of the present invention may be useful in treating microbial growth in these aqueous environments and may be applied, for example, at or near the surface of water.

[0574] The compositions of the invention for treatment of aqueous environments may comprise at least one transcription factor modulating compound of the present invention and at least one suitable carrier. In an embodiment, the composition comprises from about 0.001% to about 50%, advantageously from about 0.003% to about 15%, preferably from about 0.01% to about 5% by weight of the compound of the invention based on the weight percentage of the total composition.

[0575] The present invention also provides a process for the production of an anitibiofouling composition for industrial use. Such process comprises bringing at least one of any industrially acceptable carrier known in the art into intimate admixture with a transcription factor modulating compound of the present invention. The carrier may be ally suitable carrier discussed above or known in the art.

[0576] The suitable antibiofouling conmpositions may be in any acceptable form for delivery of the composition to a site potentially having, or having at least one living microbe. The antibiofouling compositions may be delivered with at least one suitably selected carrier as hereinbefore discussed using standard formulations. The mode of delivery may be such as to have a binding inhibiting effective amount of the antibiofouling composition at a site potentially having, or having at least one living microbe. The antibiofouling compositions of the present invention are useful in treating microbial growth that contributes to biofouling, such as scum or slime formation, in these aqueous environments. Examples of industrial processes in which these compounds might be effective include cooling water systems, reverse osmosis membranes, pulp and paper systems, air washer systems and the food processing industry. The antibiofouling composition may be delivered in an amount and form effective for the prevention, removal or termination of microbes.

[0577] The antibiofouling composition of the present invention generally comprise at least one compound of the

invention. The composition may comprise from about 0.001% to about 50%, more preferably from about 0.003% to about 15%, most preferably from about 0.01% to about 5% by weight of the compound of the invention based on the weight percentage of the total composition.

[0578] The amount of antibiofouling composition may be delivered in an amount of about 1 mg/l to about 1000 mg/l, advantageously from about 2 mg/l to about 500 mg/l, and preferably from about 20 mg/l to about 140 mg/l.

[0579] Antibiofouling compositions for water treatment generally comprise transcription factor modulating compounds of the invention in amounts from about 0.001% to about 50% by weight of the total composition. Other components in the antibiofouling compositions (used at 0. 1% to 50%) may include, for example, 2-bromo-2-nitropropane-1, 3-diol (BNPD), β-nitrostyrene (BNS), dodecylguanidine hydrochloride, 2,2-dibromo-3-nitrilopropionamide (DBNPA), glutaraldehyde, isothiazolin, methylene bis(thiocyanate), triazines, n-alkyl dimethylbenzylammonium chloride, trisodium phosphate-based, antimicrobials tributyltin oxide, oxazolidines, tetrakis (hydroxymethyl)phosphonium sulfate (THPS), phenols, chromated copper arsenate, zinc or copper pyrithione, carbamates, sodium or calcium hypochlorite, sodium bromide, halohydantoins (Br, Cl), or mixtures thereof.

[0580] Other possible components in the compositions of the invention include biodispersants (about 0.1% to about 15% by weight of the total composition), water, glycols (about 20-30%) or Pluronic (at approximately 7% by weight of the total composition). The concentration of antibiofouling composition for continuous or semi-continuous use is about 5 to about 70 mg/l.

[0581] Antibiofouling compositions for industrial water treatment may comprise compounds of the invention in amounts from about 0.001% to about 50% based on the weight of the total composition. The amount of compound of the invention in antibiofouling compositions for aqueous water treatment may be adjusted depending on the particular environment. Shock dose ranges are generally about 20 to about 140 mg/l; the concentration for semi-continuous use is about 0.5X of these concentrations.

[0582] The invention also pertains, at least in part, to a method of regulating biofilm development. The method includes administering a composition which contains a transcription factor modulating compound of the invention. The composition can also include other components which enhance the ability of the composition to degrade biofilms.

[0583] The composition can be formulated as a cleaning product, e.g., a household or an industrial cleaner to remove, prevent, inhibit, or modulate biofilm development. Advantageously, the biofilm is adversely affected by the administration of the compound of the invention,, e.g., biofilm development is diminished. These compositions may include compounds such as disinfectants, soaps, detergents, as well as other surfactants. Examples of surfactants include, for example, sodium dodecyl sulfate; quaternary ammonium compounds; alkyl pyridinium iodides; TWEEN 80, TWEEN 85, TRITON X-100; BRIJ 56; biological surfactants; rhamnolipid, surfactin, visconsin, and sulfonates. The composition of the invention may be applied in known areas and surfaces where disinfection is required, including but not limited to drains, shower curtains, grout, toilets and flooring. A particular application is on hospital surfaces and medical instruments. The disinfectant of the invention may be useful as a disinfectant for bacteria such as, but not limited to, Pseudomonadaceae, Azatobacteraceae, Rhizabiaceae, Mthylococcaceae, Halobacteriaceae, Acetobacteraceae, Legionellaceae, Neisseriaceae, and other genera.

[0584] The invention also pertains to a method for cleaning and disinfecting contact lenses. The method includes contacting the contact lenses with a solution of at least one compound of the invention in an acceptable carrier. The invention also pertains to the solution comprising the compound, packaged with directions for using the solution to clean contact lenses.

[0585] The invention also includes a method of treating medical indwelling devices. The method includes contacting at least one compound of the invention with a medical indwelling device, such as to prevent or substantially inhibit the formation of a biofilm. Examples of medical indwelling devices include catheters, orthopedic devices and implants.

[0586] A dentifrice or mouthwash containing the compounds of the invention may be formulated by adding the compounds of the invention to dentifrice and mouthwash formulations, e.g., as set forth in *Remington's Pharmaceutical Sciences,* 18th Ed., Mack Publishing Co., 1990, Chapter 109 (incorporated herein by reference in its entirety). The dentifrice may be formulated as a gel, paste, powder or slurry. The dentifrice may include binders, abrasives, flavoring agents, foaming agents and humectants. Mouthwash formulations are known in the art, and the compounds of the invention may be advantageously added to them.

[0587] In one embodiment, the invention pertains to each of the transcription factor modulating compounds described herein in Tables 4 and 5, and in Formulae (I)-(X).

[0588] The contents of all references, patent applications and patents, cited throughout this application are hereby expressly incorporated by reference. Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

[0589] The invention is further illustrated by the following examples, which should not be construed as further limiting.

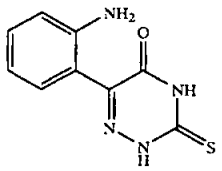
[0590] Exemplification of the Invention

## EXAMPLE 1

### Synthesis of Test Compounds

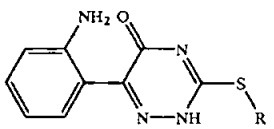
[0591] The transcriptional modulating compounds described in this application can be synthesized by art recognized techniques or using the methods described herein.

[0592] 6-(2-Amino-phenyl)-3-thioxo-3,4-dihydro-2H-[1,2,4]triazin-5-one



[0593] This was prepared by a modified literature procedure (Doleschall, G.; Lempert, K. *Tetrahedron* 1973, 29, 639-649). Isatin (10 g, 67.96 mmol) was dissolved in ca. 10% aqueous KOH (9.9 g in 100 mL of water) and then treated with thiosemicarbazide (6.28 g; 68.90 mmol). After 1 hour of heating at 115° C. (bath temperature), the reaction mixture was poured over ice and treated with glacial acetic acid drop-wise, till the pH was ca. 5. The yellow fluffy precipitate was filtered, washed copiously with water (8×50 mL) and dried first in air and then under high vacuum to afford 12.9 g of yellow solid.

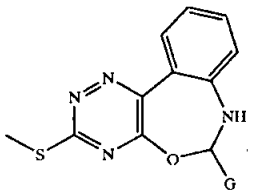
[0594] 6-(2-Aminoh-3-butylsulfanyl-2H-[1,2,4]triazin-5-one



[0595] This was prepared by a modified literature procedure (Doleschall, G.; Lempert, K. *Tetrahedron* 1973, 29, 639-649). The product from the previous experiment (8.0 g, 36.3 mmol) was dissolved in ca. 10% aq. KOH (10.3 g in 100 mL of water) and treated with nBul (7 mL). Ethanol (70 mL) was added to it and the reaction mixture was allowed to stir overnight. The reaction mixture was diluted with ether (100 mL) and water (70 mL). The ether layer was separated and the aqueous layer washed further with ether (3×100 mL) and then poured over ice. Upon careful, drop-wise addition of glacial acetic acid with vigorous stirring at 0-4° C., yellow precipitate was obtained which was filtered, washed with water (4×20 mL) and then with ether (2×10 mL) and dried. Yield: 5.12 g.

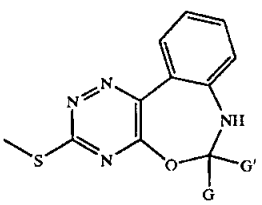
[0596] Other alkyl or substituted alkyl halides were used instead of n-butyliodide following the similar method.

[0597] 3-Methylsulfanyl-6-(G)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene



[0598] This was prepared by a modified literature procedure (Doleschall, G.; Lempert, K. *Tetrahedron* 1973, 29, 639-649). To a suspension of compound 2 (or analogs of 2) (ca. 0.384 mmol, 1 equiv) in dry ethanol (3-4 mL), 25 μL of glacial acetic acid was added, followed by ca. 1.1 equiv of the corresponding aldehyde (G-CHO, where, G=substituted or unsubstituted aliphatic, aromatic, or heterocyclic groups). The reaction mixture was refluxed for ca. 5-7 min resulting in a dark red—dark-reddish orange solution. Upon cooling to room temperature orange-orange-yellow solid crashed out of solution, which was filtered, washed with cold (ca. −30° C.) methanol (2×1 mL), and/or ether and dried. In some cases, the crude products were recrystallized from DMF/ether or methanol/ether; in most of the cases, the crude products, prepared as above, were >95% pure. Various ketones (GCOG') were reacted with 2 (or analogs of 2) in a similar way to afford compounds of structural type 4. All the final compounds were characterized by means of $^1$H NMR, LC-MS, HPLC ($C_{18}$ columns, acetonitrile/water with 0.01% triethylamine as mobile phase), and CHN analyses.

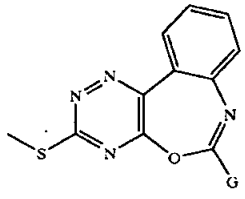
[0599] General Synthesis of Orthoesters, $GC(OR)_3$; R=Me



[0600] The syntheses of the desired orthoesters were accomplished by a modified literature procedure in multiple steps (McClelland, R. A. et al. *J. Org. Chem.* 1981, 46, 1011-1012). Several novel orthoesters were prepared by this method. To a solution of an acid chloride in dichloromethane, N-methylaniline was added slowly, followed by triethylamine and catalytic amount of 4-dimethylaminopyridine. After stirring it for ca. 12 h, the reaction mixture was diluted with ether, the precipitate was filtered, washed with ether and dried. The amide, thus prepared, was then stirred overnight with methyl triflate in dichloromethane, diluted with ether, and the precipitate was filtered, washed, and dried to obtain an imidatonium triflate salt. This salt was dissolved in dichloromethane, cooled to 0° C., and added slowly, with stirring, to a cold (0° C.) solution of sodium methoxide in dry methanol over a period of ca. 30-60 min.

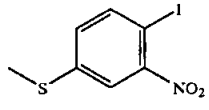
The solvent was evaporated to dryness and the residue was extracted in n-hexane. Upon evaporation of hexane, the white solid was obtained, which was dissolved in dry methanol and treated with glacial acetic acid. After 10 minutes of stirring, the excess acid was neutralized with potassium carbonate (solid), and the solvent removed under vacuum. The residue was extracted in ether, washed with water, and dried over potassium carbonate. The crude material was obtained by evaporation of ether, and further purified either by flash chromatography or fractional distillation.

[0601] 3-Methylsulfanyl-6-(G)-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c]cycloheptene



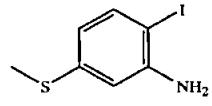
[0602] Compound of the type 2 (0.384 mmol, 1 equiv) was suspended in ethanol (2-3 mL), treated with glacial acetic acid (100 μL), followed by an orthoester (2 equiv) of the general formula G-C(OR)$_3$, where G=substituted or uinsubstituted aliphatic, aromatic, or heterocyclic group, R=H, substituted or unsubstituted aliphatic, aromatic, or heterocyclic group. The reaction mixture was refluxed for 70-180 minutes, cooled to room temperature. In some cases, the product crashed out of solution, in others, the crude reaction mixture was evaporated to dryness, re-dissolved in a minimum amount of methanol and diluted with ether. The solid was washed with ether (cold, 0-4° C.; 1×1 mL) and dried under vacuum.

[0603] 4-Iodo-3-nitrothioanisole



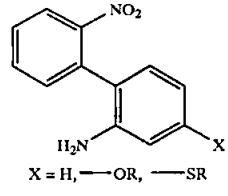
[0604] A flask was charged with 10 g of 4-iodothioanisole and 5.5 mL of dimethylsulfate and warmed to ca. 90° C. for 10 min. The resulting solution was dissolved in conc. sulfuric acid (30 mL) and cooled to ca. 0-4° C., whereupon it was treated, with extreme caution, slowly with conc. $HNO_3$ (ca. 2 mL) while maintaining the reaction temperature below 4° C. After stirring it for ca. 10 min, the reaction mixture was stirred at ca. 90° C. for ca. 3 d. The reaction was monitored with HPLC, TLC, and LC-MS, and if needed, smaller portions of nitric acid were added to the reaction mixture to force it to completion. Use of fuming sulfuric acid is also helpful. After the complete consumption of the aromatic starting material, the reaction mixture was cooled, poured over crushed ice, treated with 30% aq. perchloric acid at 4° C. The light colored precipitate was filtered, washed thoroughly with water, and dried under vacuum. The perchlorate salt was stirred with saturated aq. NaCl solution at 95° C. for 3-6 h. Upon cooling to room temperature, the precipitate was filtered, washed thoroughly with water to get rid of any inorganic salts, and dried under vacuum to obtain 4-iodo-3-nitrothioanisole in>80% yield.

[0605] 4-Iodo-3-aminothioanisole



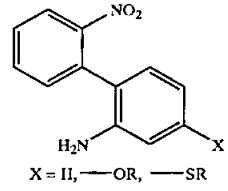
[0606] Ca. 7 g of 4-iodo-3-nitrothioanisole was dissolved in absolute ethanol and treated slowly with a solution of $SnCl_2.2H_2O$ in 12% aq. HCl. The reaction mixture was stirred at 50° C. for 25-30 min., when the HPLC monitoring indicated that the reaction was complete. The reaction mixture was poured over crushed ice, and treated with aq. NaOH solution to pH 8. The precipitate was filtered, washed with water and dried in air. The crude material was crystallized by overnight cooling (4° C.) of its ethanol (minimum amount) solution treated with 10% aq. HCl. The crystalline material was further dried under high vacuum to afford the desired amine as its hydrochloride salt in >70% yield.

[0607] 4-Methylsulfanyl-2'-nitro-biphenyl-2-ylatnine



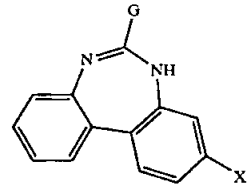
[0608] A methanol/dioxane (20 mL/5 mL of methanol/dioxane) solution/suspension of 4-iodo-3-nitrothioanisole (1 mmol), $Pd(OAc)_2$ (0.01 mmol) was purged with argon for ~5 min. To this solution $Et_3N$ (3 mmol), and 5 mL of water were added and purged with argon for another 5 min. To the above solution, 2-aminophenyl boronic acid (2 mmol, solution in 5 mL of DMF, purged with argon), was added and the reaction mixture was heated at 70° C. (oil bath temperature) for 2 h. The reaction was monitored by HPLC and LC-MS to follow the product formation. The reaction mixture was then cooled down to room temperature and filtered through diatomaceous earth. The filtrate was concentrated and purified using preparative HPLC.

[0609] 4-Methylsulfanyl-2'-amino-biphenyl-2-ylamine



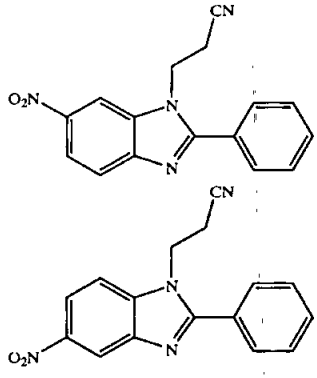

[0610] A flask was charged with ca. 1 mmol of 4-Methylsulfanyl-2'-nitro-biphenyl-2-ylamine, 15 mL of ethanol, and 0.1 mmol of $PtO_2$, and stirred under hydrogen atmosphere at 40 psi for 10 minutes. The reaction mixture was filtered through diatomaceous earth, washed with ethanol, and the combined organic layer was evaporated to dryness. The crude material was purified by preparative HPLC. The same material can also be prepared by the previous method, (Suzuki coupling conditions) starting from 4-iodo-3-aminothioanisole, and purified by preparative HPLC.

[0611] 6-(G)-3-methylsulfanyl-5H-dibenzo[d,f][1,3]diazepine



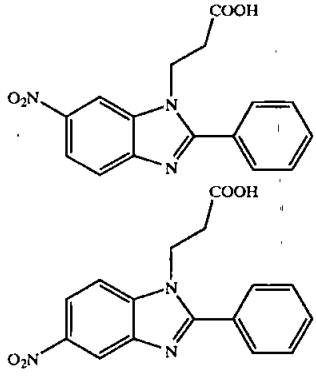

[0612] To a solution of the 2,2'-biphenyldiamine (0.093 g; 0.51 mmol) in ethanol (2 mL), were added glacial acetic acid (50 μL) and 2 equiv of an orthoester of the general formula $GC(OR)_3$. In case of TFA salt of the diamine, there was no need of adding acetic acid to the reaction mixture. The reaction mixture was refluxed for 3 h, cooled to room temperature, and evaporated to dryness. The residue was suspended in methanol saturated with dry HCl, stirred for a few minutes, filtered, washed with methanol, and finally with ether. The hydrochloride salt of the diazepine was dried under vacuum to afford a light yellow solid. In order to obtain a free base of the diazepine, the above hydrochloride salt was suspended in methanol, and treated with 10% aq. NaOH solution. After stirring at room temperature for ca. 10 min, the precipitate was filtered, washed with water, and dried under vacuum.

[0613] 3-(6-Nitro-2-phenyl-benzoimidazol-1-yl)-propionitrile and 3-(5-Nitro-2-phenyl-benzoimidazol-1-yl)-propionitrile




[0614] A mixture of 5-nitro-2-phenylbenzimidazole (1 g, 4.2 mmol), acrylonitrile (50 mL) and N,N-dimethylpiridine (25 mg) was heated at 70° C. for 4 hr. The excess of acrylonitrile was evaporated and oily residue was subjected to the flash chromatography on silica gel using hexane-ethyl acetate (75: 25 v/v) as an eluent. Structure of the regioisomers was determined using $^1H$ NOESY studies. 0.25 g (20%) of the 6-nitro isomer and 0.23 g (18.9%) of the 5-nitro isomer were obtained.

[0615] 3-(6-Nitro-2-phenyl-benzoimidazol-1-yl)-propionic acid and 3-(5-Nitro-2-phenyl-benzoimidazol-1-yl)-propionic Acid.




[0616] To the 6-nitro nitrile (0.15 g, 0.51 mmol) concentrated HCl (5 mL) was added and resulting mixture was heated at 50° C. for 0.5 hours. The acid was evaporated in vacuo and product was purified by HPLC. Yield 34 mg (21%). An identical procedure was used starting from the 5-nitro nitrile yielding the product (22 mg, 13.8

EXAMPLE 2

[0617] In this example, the expression of a selective marker (e.g., ccdB) is put under the direct control of a promoter activated by MarA (e.g., inaa, galT, or micF). In

the absence of MarA, the expression of the selective marker is silent and cells survive. Synthesis of MarA from an inducible plasmid in a bacterial or yeast cell leads to the activation of the inaA promoter, expression of ccdB, and subsequently results in cell death. Compounds that inhibit MarA are those that permit cell survival under conditions of MarA expression. The results of this assay are given in Table 4. In Table 4, * indicates good inhibition of MarA and * * indicates very good inhibition of MarA.

Example 3

**[0618]** In this example, the expression of luciferase is put under the direct control of a promoter activated by MarA (e.g., inaA, galT, or micF) in a cell constitutively expressing MarA. In the absence of MarA, cells luminesce. Upon modulating of MarA activity, the expression of the reporter is altered.

TABLE 4

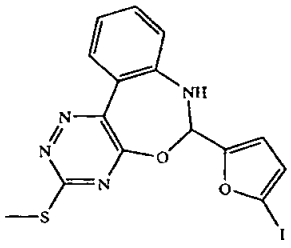
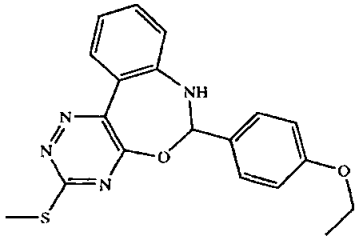
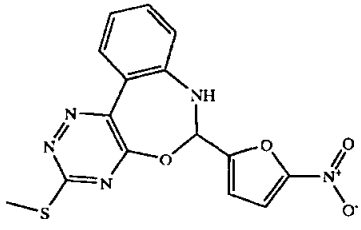
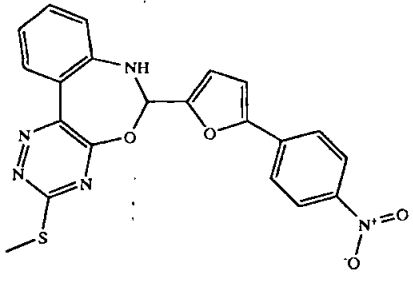
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| A |  | 6-(5-Iodo-furan-2-yl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | ** |
| B |  | 6-(4-Ethoxy-phenyl)-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | * |
| C |  | 3-Methylsulfanyl-6-(5-nitro-furan-2-yl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | * |
| D |  | 3-Methylsulfanyl-6-[5-(4-nitro-phenyl)-furan-2-yl]-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene | * |

TABLE 4-continued

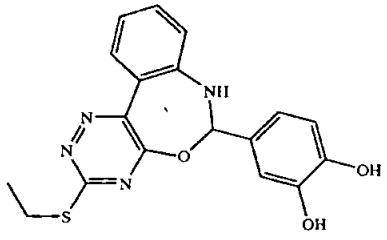
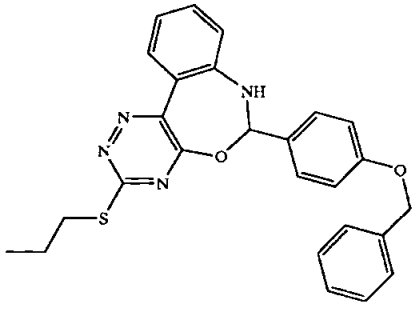
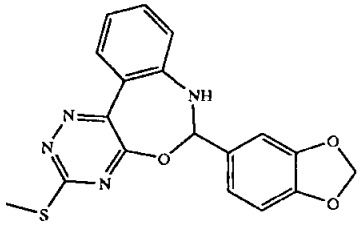
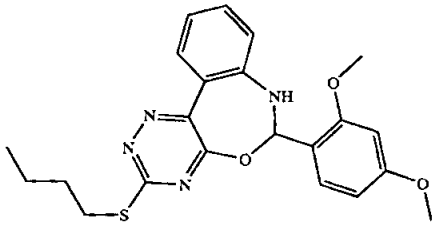
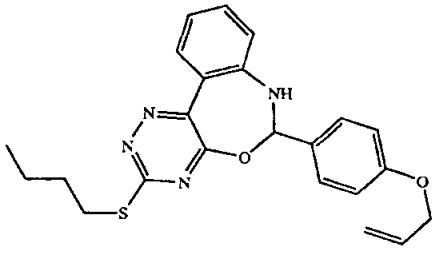
| ID | Structure | Name | Affinity |
|---|---|---|---|
| E |  | 4-(3-Ethylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cyclohepten-6-yl)-benzene-1,2-diol | * |
| F |  | 6-(4-Benzyloxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | ** |
| G |  | 6-Benzo[1,3]dioxol-5-yl-3-methylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | ??? |
| H |  | 3-Butylsulfanyl-6-(2,4-dimethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c] cycloheptene | * |
| I |  | 6-(4-Allyloxy-phenyl)-3-butylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c]cycloheptene | ** |

TABLE 4-continued

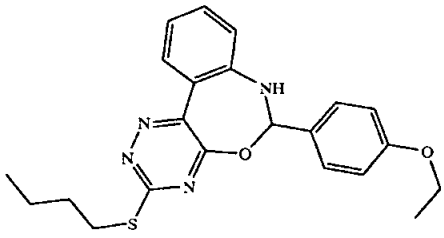
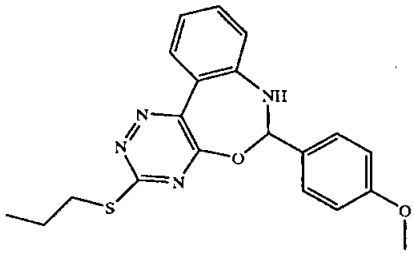
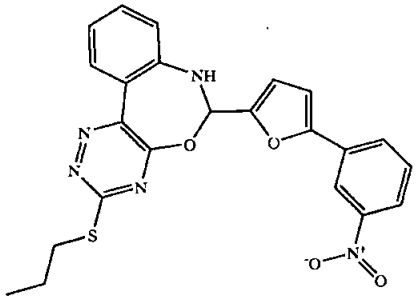
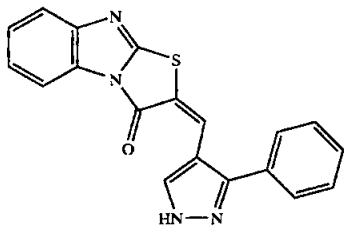
| ID | Structure | Name | Affinity |
|---|---|---|---|
| J |  | 3-Butylsulfanyl-6-(4-ethoxy-phenyl)-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c] cycloheptene | * |
| K |  | 6-(4-Methoxy-phenyl)-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo [a,c]cycloheptene | * |
| L |  | 6-[5-(3-Nitro-phenyl)-furan-2-yl]-3-propylsulfanyl-6,7-dihydro-5-oxa-1,2,4,7-tetraaza-dibenzo[a,c] cycloheptene | ** |
| M |  | 2-(3-Phenyl-1H-pyrazol-4-ylmethylene)-benzo[4,5] imidazo[2,1-b]thiazol-3-one | ** |

TABLE 4-continued

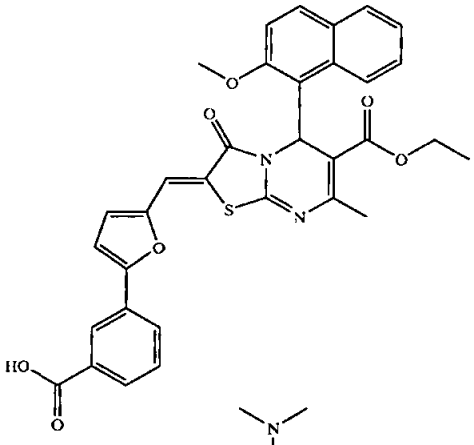
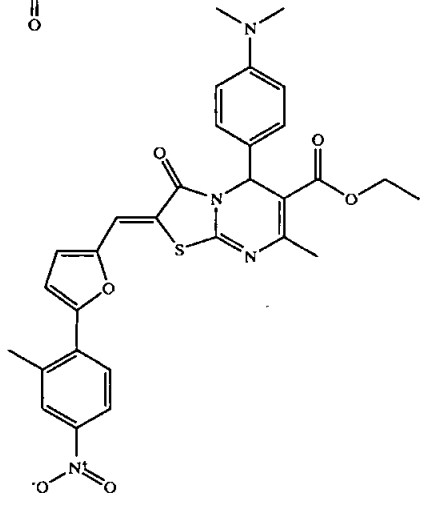
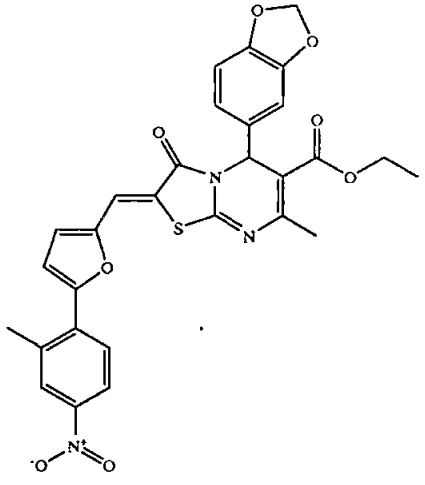
| ID | Structure | Name | Affinity |
|---|---|---|---|
| N |  | 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-5-(2-methoxy-naphthalen-1-yl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | ** |
| O |  | 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a] pyrimidine-6-carboxylic acid ethyl ester | *** |
| P |  | 5-Benzo[1,3]dioxol-5-yl-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a] pyrimidine-6-carboxylic acid ethyl ester | * |

TABLE 4-continued

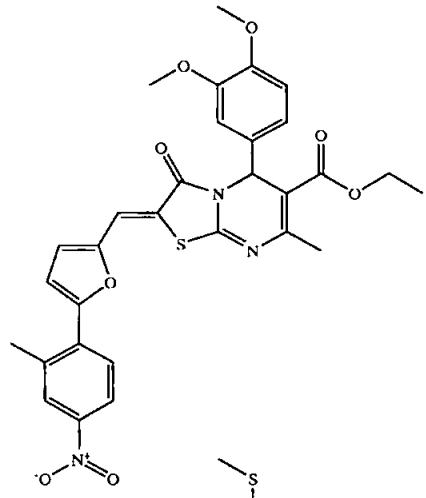
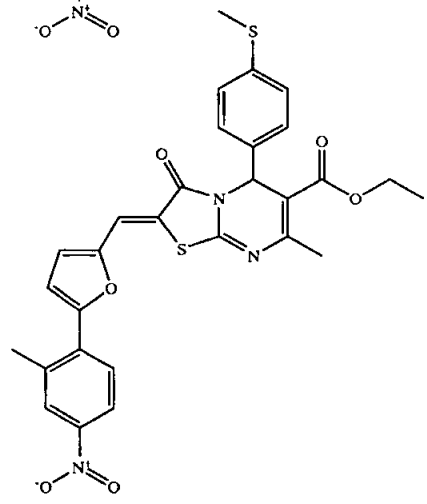
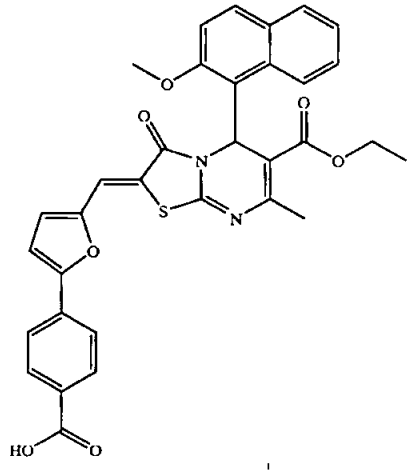
| ID | Structure | Name | Affinity |
|---|---|---|---|
| Q |  | 5-(3,4-Dimethoxy-phenyl)-7-methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | *** |
| R |  | 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-ylmethylene]-5-(4-methyl sulfanyl-phenyl)-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a] pyrimidine-6-carboxylic acid ethyl ester | *** |
| S |  | 2-[5-(4-Carboxy-phenyl)-furan-2-ylmethylene]-5-(2-methoxy-naphthalen-1-yl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | *** |

TABLE 4-continued

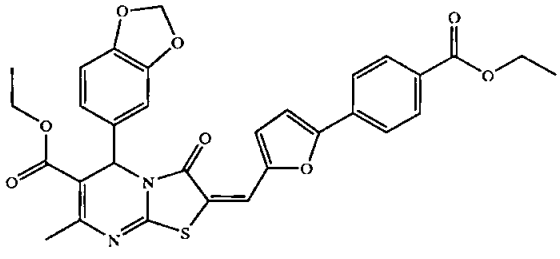
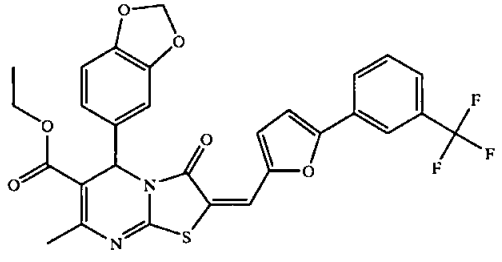
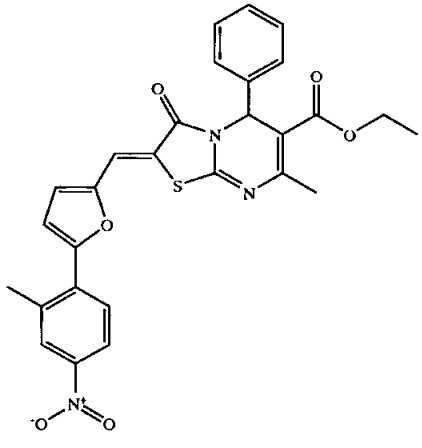
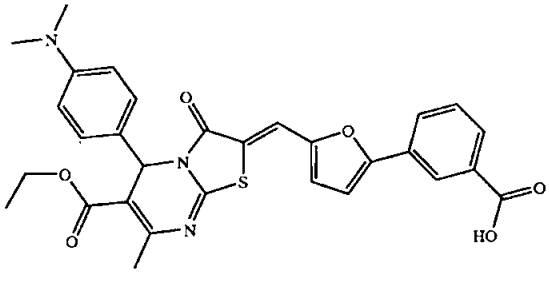
| ID | Structure | Name | Affinity |
|---|---|---|---|
| T |  | 5-Benzo[1,3]dioxol-5-yl-2-[5-(4-ethoxycarbonyl-phenyl)-furan-2-ylmethylene]-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | *** |
| U |  | 7-Methyl-3-oxo-5-phenyl-2-[5-(3-trifluoromethyl-phenyl)-furan-2-ylmethylene]-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | ** |
| V |  | 7-Methyl-2-[5-(2-methyl-4-nitro-phenyl)-furan-2-yl methylene]-3-oxo-5-phenyl-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | *** |
| W |  | 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-5-(4-dimethylamino-phenyl)-7-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | ** |

TABLE 4-continued

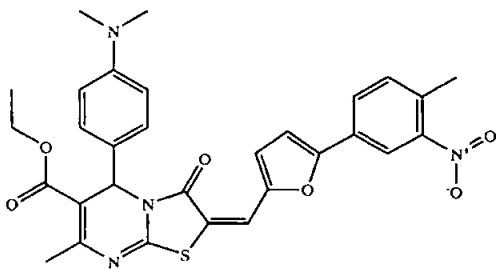
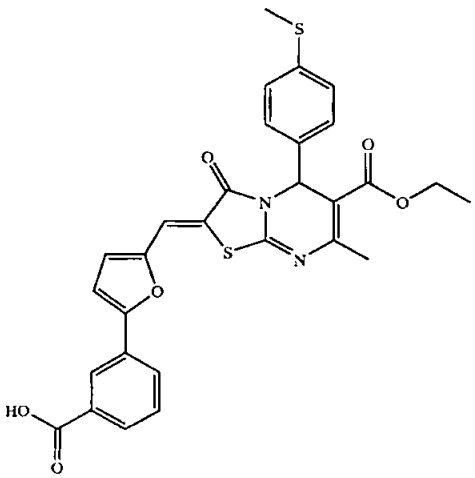
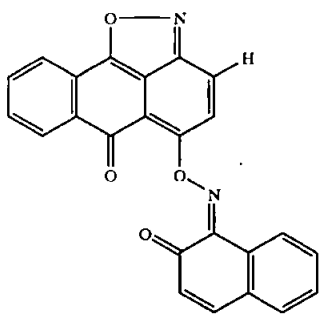
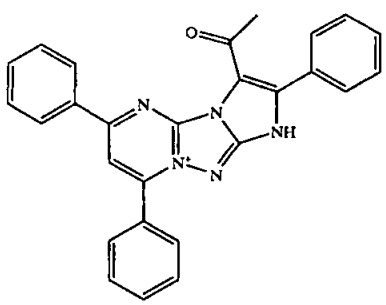
| ID | Structure | Name | Affinity |
|---|---|---|---|
| X |  | 5-(4-Dimethylamino-phenyl)-7-methyl-2-[5-(4-methyl-3-nitro-phenyl)-furan-2-yl methylene]-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | ** |
| Y |  | 2-[5-(3-Carboxy-phenyl)-furan-2-ylmethylene]-7-methyl-5-(4-methylsulfanyl-phenyl)-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]pyrimidine-6-carboxylic acid ethyl ester | *** |
| Z |  | [1,2]Naphthoquinone 1-[O-(6-oxo-6H-anthra[1,9-cd] isoxazol-5-yl)-oxime] | * |
| AA |  | 3-Acetyl-2,5,7-triphenyl-1H-1,3a,4,8-tetraaza-7a-azonia-cyclopenta[a]indene | *** |

TABLE 4-continued

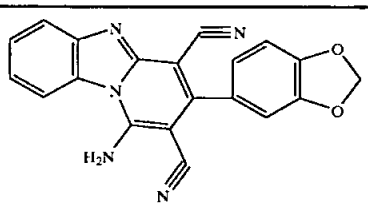
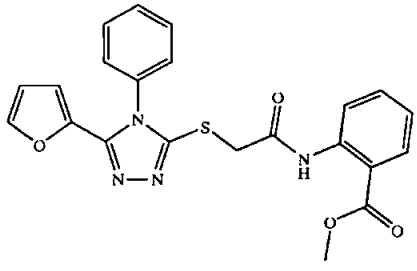
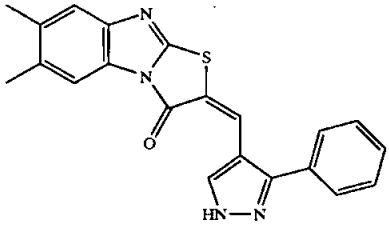
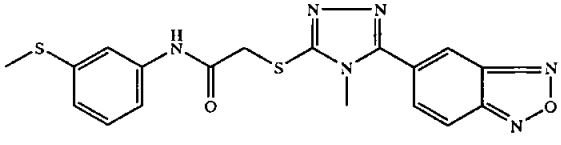
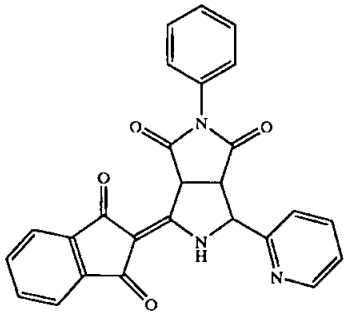
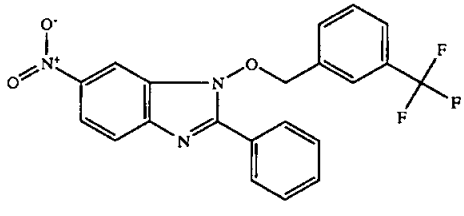
| ID | Structure | Name | Affinity |
|---|---|---|---|
| AB |  | 1-Amino-3-benzo[1,3]dioxol-5-yl-benzo[4,5]imidazo[1,2-a] pyridine-2,4-dicarbonitrile | ** |
| AC |  | 2-[2-(5-Furan-2-yl-4-phenyl-4H-[1,2,4]triazol-3-yl sulfanyl)-acetylamino]-benzoic acid methyl ester | * |
| AD |  | 6,7-Dimethyl-2-(3-phenyl-1H-pyrazol-4-ylmethylene)-benzo[4,5]imidazo[2,1-b] thiazol-3-one | * |
| AE |  | 2-(5-Benzo[1,2,5]oxadiazol-5-yl-4-methyl-4H-[1,2,4] triazol-3-ylsulfanyl)-N-(3-methylsulfanyl-phenyl)-acetamide | * |
| AF |  | 4-(1,3-Dioxo-indan-2-ylidene)-2-phenyl-6-pyridin-2-yl-tetrahydro-pyrrolo[3,4-c] pyrrole-1,3-dione | ** |
| AG |  | 6-Nitro-2-phenyl-1-(3-trifluoromethyl-benzyloxy)-1H-benzoimidazole | ** |

TABLE 4-continued

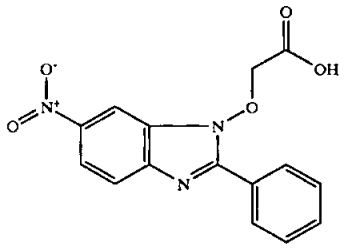
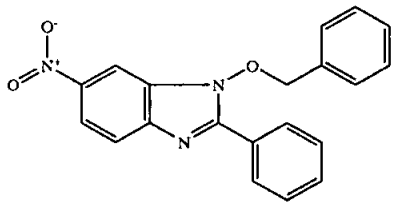
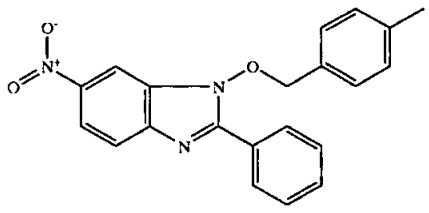
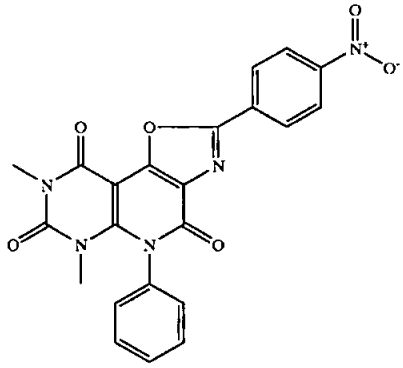
| ID | Structure | Name | Affinity |
|---|---|---|---|
| AH |  | (6-Nitro-2-phenyl-benzoimidazol-1-yloxy)-acetic acid | ** |
| AI |  | 1-Benzyloxy-6-nitro-2-phenyl-1H-benzoimidazole | ** |
| AJ |  | 1-(4-Methyl-benzyloxy)-6-nitro-2-phenyl-1H-benzoimidazole | * |
| AK |  | 6,8-Dimethyl-2-(4-nitro-phenyl)-5-phenyl-5H,6H-1-oxa-3,5,6,8-tetraza-cyclopenta[a]naphthalene-4,7,9-trione | ** |

TABLE 4-continued

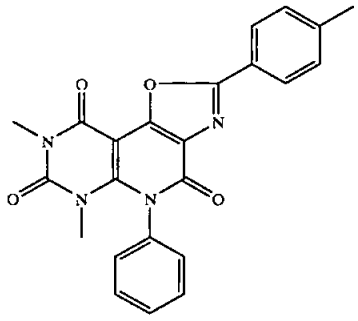
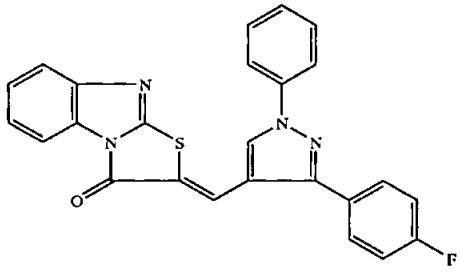
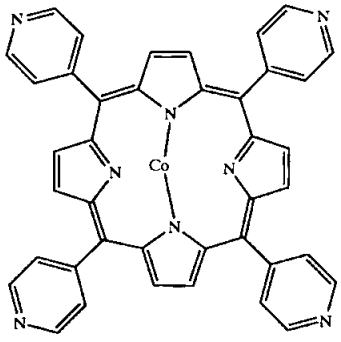
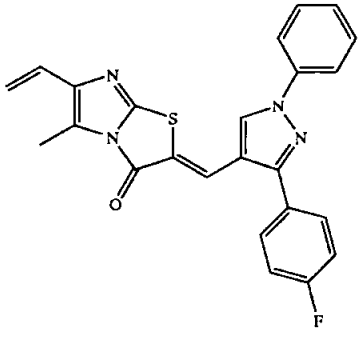
| ID | Structure | Name | Affinity |
|---|---|---|---|
| AL |  | 6,8-Dimethyl-5-phenyl-2-p-tolyl-5H,6H-1-oxa-3,5,6,8-tetraaza-cyclopenta [a]naphthalene-4,7,9-trione | * |
| AM |  | 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-yl methylene]-benzo [4,5] imidazo[2,1-b]thiazol-3-one | ** |
| AN |  | Cobalt 5,10,15,20-Tetra-pyridin-4-yl-porphyrine | *** |
| AO |  | 2-[3-(4-Fluoro-phenyl)-1-phenyl-1H-pyrazol-4-ylmethylene]-5-methyl-6-vinyl-imidazo[2,1-b]thiazol-3-one | ** |

TABLE 4-continued

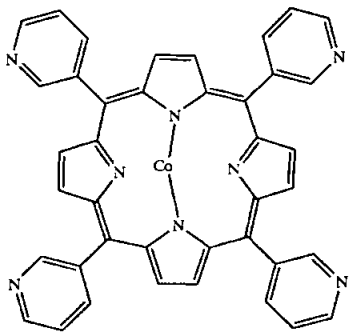
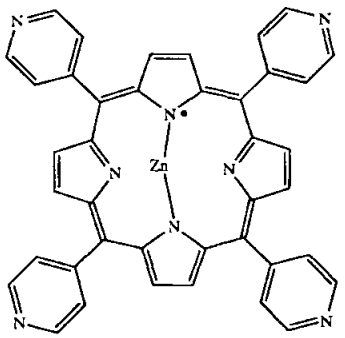
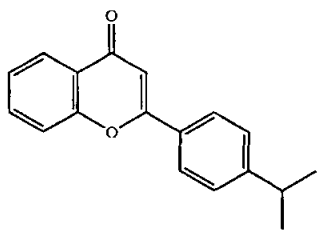
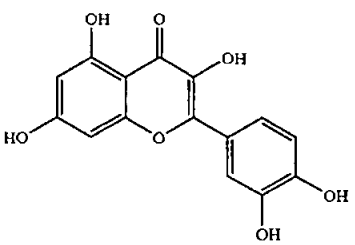
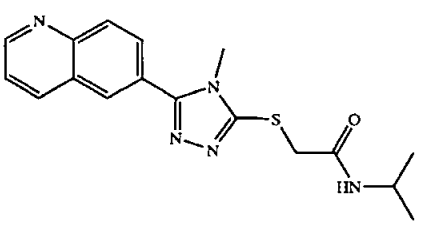
| ID | Structure | Name | Affinity |
|---|---|---|---|
| AP |  | Cobalt 5,10,15,20-Tetra-pyridin-3-yl-porphyrine | *** |
| AQ |  | Zinc 5,10,15,20-Tetra-pyridin-4-yl-porphyrine | *** |
| AR |  | 2-(4-isopropylphenyl)-4H-chromen-4-one | *** |
| AS |  | 2-(3,4-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one (luteolin) | *** |
| AT |  | N-isopropyl-2-[(4-methyl-5-quinolin-6-yl-4H-1,2,4-triazol-3-yl)thio]acetamide | *** |

TABLE 4-continued

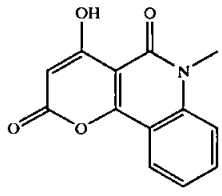
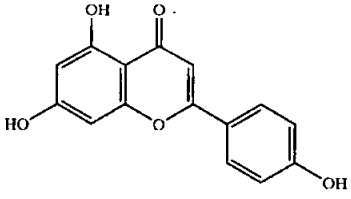
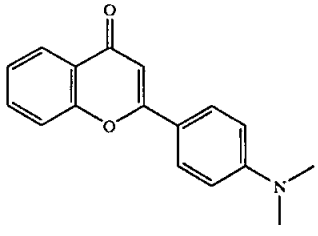
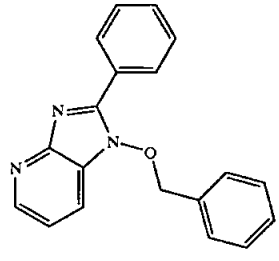
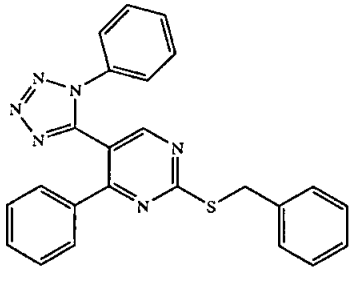
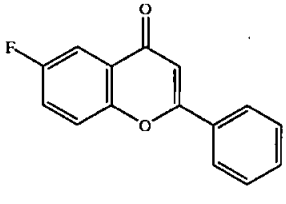
| ID | Structure | Name | Affinity |
|---|---|---|---|
| AU |  | 4-hydroxy-6-methyl-5,6-dihydro-2H-pyrano[3,2-c]quinoline-2,5-dione | *** |
| AV |  | 5,7-Dihydroxy-2-(4-hydroxy-phenyl)-chromen-4-one | *** |
| AW |  | 2-[4-(dimethylamino)phenyl]-4H-chromen-4-one | ** |
| AX |  | 1-(benzyloxy)-2-phenyl-1H-imidazo[4,5-b]pyridine | ** |
| AY |  | 2-(benzylthio)-4-phenyl-5-(1-phenyl-1H-1,2,3,4-tetraazol-5-yl)pyrimidine | ** |
| AZ |  | 6-fluoro-2-phenyl-4H-chromen-4-one | ** |

TABLE 4-continued

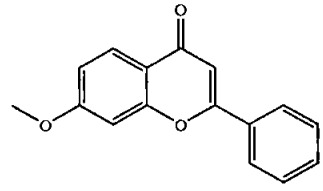
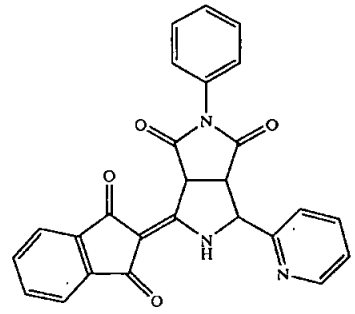
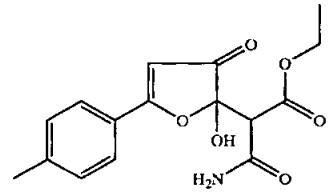
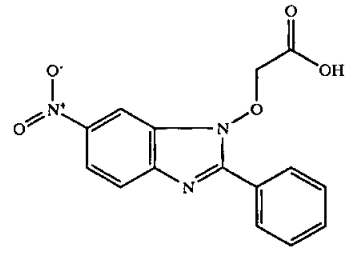
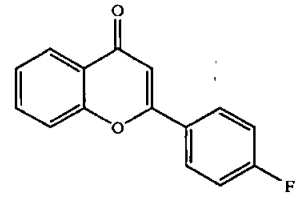
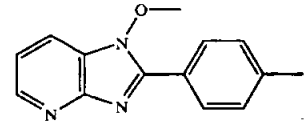
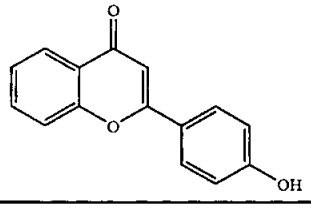
| ID | Structure | Name | Affinity |
|---|---|---|---|
| BA |  | 7-methoxy-2-phenyl-4H-chromen-4-one | * |
| BB |  | 4-(1,3-dioxo-1,3-dihydro-2H-inden-2-yliden)-2-phenyl-6-(2-pyridinyl) tetrahydro pyrrolo [3,4-c]pyrrole-1,3(2H,3aH)-dione | * |
| BC |  | 2-(2-Hydroxy-3-oxo-5-p-tolyl-2,3-dihydro-furan-2-yl)-malonamic acid ethyl ester | * |
| BD |  | 2-[(6-nitro-2-phenyl-1H-1,3-benzimidazol-1-yl)oxy]acetic acid | * |
| BE |  | 2-(4-fluorophenyl)-4H-chromen-4-one | * |
| BF |  | 1-methoxy-2-(4-methyl phenyl)-1H-imidazo [4,5-b]pyridine | * |

TABLE 4-continued

| ID | Structure | Name | Affinity |
|---|---|---|---|
| BG |  | 2-(4-hydroxyphenyl)-4H-chromen-4-one | * |

EXAMPLE 4

[0619] In this example, the expression of a selective marker (e.g., ccdB) is put under the direct control of a promoter repressed by MarA (e.g. fecA, purA, guaB). Under conditions of constitutive MarA synthesis (e.g., using a constitutive mar (marc) mutant), the expression of ccdB is silent. Following inactivation of MarA, the synthesis of ccdB results in cell death.

EXAMPLE 5

[0620] In this example, the expression of a selective marker (e.g., URA3) is put under the direct control of apromoter repressed by MarA (e.g., fecA, purA, guaB). Under conditions of constitutive MarA synthesis (e.g., using a constitutive mar (marc) mutant), the expression of URA3 is silent. Following inactivation of MarA, and in the presence of 5-FOA the synthesis of URA3 results in cell death.

EXAMPLE 6

[0621] In this example, a purine or guanine heterotroph is constructed by inactivation of either of the chromosomal guaB orpurA genes in *E. coli*. The guaB or purA gene is then placed into a suitable vector under the control of its natural promoter and transformed into the heterotrophic host.

EXAMPLE 7

*E. coli* Biofilm Assay

[0622] The biofilm assay screens test compounds for their ability to inhibit bacteria from forming a biofilm.

[0623] Materials:

[0624] The M9 media ("M9") contained M9, casamino acids, and glucose. The test compound was dissolved in 10 mg/mL DMSO stock solution.

[0625] Method.

[0626] Preparation of Inoculum

[0627] Inoculum was started the day of the experiment by adding a colony or glycerol stock stab to 2 mL M9. The tube was placed in the 37° C. shaker incubator for approximately 4-6 hours. This inoculum was referred to as the "Starter inoculum." The inoculum was then removed from the shaker incubator and diluted to $1\times10^6$ cells/mL in M9.

[0628] Preparation of Controls

[0629] Typically, there were eight of each control, including a positive and negative control. For both the positive and negative controls, 2.5 μL of DMSO was added to 200 μL of M9. 25 μL of the diluted DMSO was added to 50 PL of M9 in the assay plate.

[0630] Preparation of Test Compounds

[0631] The test compounds were screened at 20 tg/mL. 2.5 μL of the test compound were taken from a plate containing 10 mg/mL stock and added to 200 μL of M9 and mixed. 25 μL of the diluted test compound was added to 50 μL of M9 in the assay plate. The resulting concentration of the test compound was 40 μg/mL

[0632] Preparation of Plate

[0633] 75 μL of the inoculum at $1\times10^6$ cells/mL was added to each well containing compound and the positive controls. 75 μL M9 was added to the negative controls. The final concentration of the test compound was 20 μg/mL and the final concentration of the inoculum was $2\times10^5$ cells/mL. The plates were then placed in a microplate reader (Wallac Victor$^2$V) and read $OD_{535}$ ("Initial growth reading"). The plates were then placed in an incubator overnight at 35° C.

[0634] In the morning, the plates were read in a microplate reader at $OD_{535}$ ("Final growth reading.")

[0635] Addition of Crystal Violet

[0636] The inoculum was then removed from the wells and the plates were washed several times with tap water. 150 μL of Crystal Violet (0.02% Crystal Violet dissolved in water) was then added to each well.

[0637] Addition of Ethanol

[0638] The crystal violet was then removed and the plates were washed several times with tap water. 150 μL of ethanol were then added to each well, after mixing. The plates were then placed in a microplate reader and read the $OD_{535}$. This was referred to as the "Crystal Violet" reading.

[0639] Data Analysis

[0640] To determine whether a test compound inhibited growth, the Initial growth reading was subtracted from the Final growth reading ("Subtracted Growth").The same was done for the positive controls and averaged. The % inhibition of growth was determined by the following formula:

100−(100*Subtracted growth of sample/Average growth of Pos Controls)

[0641] To determine whether a test compound inhibited Biofilm formation, the % Inhibition of Biofilm Formation was determined using the following formula:

100–(100*Crystal Violet read of sample/Average crystal violet read of Pos Controls)

[0642] The results from the Crystal Violet assay are summarized in Table 5. In Table 5, ND indicates that a given compound did not inhibit biofilm formation in the CV assay. * indicates that the test compound inhibited some biofilm formation and ** indicates that the compound inhibited the formation of a biofilm well.

EXAMPLE 8

LANCE Screening Assay for Inhibitors of MarA, SoxS, or Rob DNA-Binding

[0643] This example describes a method for the identification of test compounds that inhibit the interactions of purified transcription factor such as MarA, SoxS and/or Rob with a target DNA sequence in an in vitro system. Such molecules will hopefully be able to inhibit this interaction in vivo, leading to inhibition of transcriptional regulation by these factors and ultimately in inhibition of the drug resistance and/or virulence phenotypes associated with MarA, SoxS and Rob.

[0644] Materials

[0645] The 6His-tagged MarA, SoxS and Rob purified according to respective protocol. The N-term-biotinylated double-stranded DNA has a sequence of CCG ATT TAG CAA AAC GTG GCA TCG GTC (SEQ ID NO. 5). The antibody used was the LANCE Eu-labeled anti-6x His Antibody (Eu-αHis) (Perkin Elmer cat # AD0110) which had at least 6 Europium molecules per antibody. Streptavidin conjugated to SureLight™-Allophycocyanin (SA-APC) was obtained from Perkin Elmer (cat # CR130-100). The Assay buffer contained 20 mM Hepes pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, and 30 mM KCl, and 0.2% Tween-20.

[0646] Method

[0647] The plates or vials of the compounds to be tested were thawed. These stocks were at a concentration of 10 mg/ml in DMSO. The solutions were allowed to thaw completely, and the plates were briefly shaken on the Titermix to redissolve any precipitated compound. Thawed aliquots of MarA, SoxS and Rob protein from the stock stored at −80° C. and 1M stock of dithiothreitol stored at −20° C. were then placed on ice.

[0648] Dilutions at 1:100 of the compounds were made into a fresh 96-well polystyrene plate. The dilutions were prepared with 100% DMSO to give a final concentration of 100 μg/ml solutions. The dilutions were vortexed on a Titermix.

[0649] Fresh DTT was added to 25-50 mL of assay buffer to produce a 1 mM final concentration. Next, 90 μl of assay buffer was added to each of the 10 μl protein aliquots, and the solution was mixed by pipetting. These proteins were diluted to give the required amount of each of the diluted proteins, resulting in 20 μl of diluted protein per well. In preparing the solutions, 20% excess was made to allow enough for control wells. Typically, depending on the protein preps and the initial binding curves that were performed, 1000-2000 fmoles of each protein was required per well. The diluted protein solutions were the placed on ice.

[0650] Three tests plates per plate of compound (for MarA, SoxS and Rob) were prepared. Using a multichannel pipet, 5 μl of the compound was added to each well. 5 μl of DMSO was added to the blank and control wells, and 5 μl of the control inhibitor was added to the respective wells.

[0651] Using the multichannel pipet, 20 μl of protein was added to all wells except those designated "blank". To these blank wells, 20 μl of assay buffer was added. The plates were covered with a plate sealer and incubated at room temperature, shaking on the Titermix, for 30 minutes.

[0652] Next, the DNA solution was prepared, with enough for at least 20% more wells than were tested. 15 μl (0.4 fmoles) was added per well. Then the DNA was diluted in assay buffer, and vortexed briefly to mix. The plate sealer was removed, and 15 μl of DNA solution was added to all of the wells. the plates were then resealed, and returned to the Titermix for a further 30 minutes.

[0653] After 25 minutes, the antibody solution was prepared. 0.4 fmoles of SA-APC and 0.125 fmoles of Eu-αHis were added per well in a total volume of 10 μl. Amounts were prepared sufficient for at least 20% excess. The plate sealer was the removed and 10 μl of antibody solution was added to every well. The plates were subsequently resealed, placed on the Titermix, and covered with aluminum foil. The plates were mixed for 1 hour. The plates were then read on the Wallac Victor V, using the LANCE 615/665 protocol.

[0654] Data Processing

[0655] For each plate, the mean control (i.e. signal from protein and DNA without inhibition), mean blank (background signal without protein) and mean inhibitor (P001407) $LANCE_{665}$ counts were determined. The percentage inhibition by each molecule (each test well) was then determined according to the following equation:

$$\%\ Inhibition = 100 - (((test - mean\ blank)/(mean\ control - mean\ blank)) * 100)$$

[0656] Compounds that gave 40% or greater inhibition were identified as hits and screened again for IC50.

[0657] $IC_{50}$ Screening

[0658] The protocol used was identical to that outlined above, except that only 10 compounds were assayed per plate. The testing concentrations started at 10 kg/ml and were diluted two-fold from 10 to 0.078 μg/ml.

[0659] $IC_{50}$ Data Processing

[0660] Percent inhibition was calculated as shown above. Percent inhibition was then plotted vs. log (conc. Inhibitor) using Graph pad Prism software. The $IC_{50}$ concentration was determined as the concentration that gives 50% inhibition.

[0661] The data from this example is also summarized in Table 5. *** indicates that a particular test compound inhibited the particular bacteria very well, ** indicates that the particular test compound inhibited the particular bacteria well, and * indicates that the particular test compound inhibited the particular bacteria to some extent.

EXAMPLE 9

Luciferase Assay

[0662] The luciferase assay is used to determine if any of the compounds tested reduce the luminescent signal. This indicates that the test compounds affect regulation of micF, which in turn is regulated by Mar.

[0663] Materials

[0664] The bacteria used were *E.coli* AG112 KmicF-Luc. The negative control Bacteria were *E.coli* AG112. The test compounds were prepared in a 10 mg/mL DMSO stock solution.

[0665] Methods

[0666] Preparation of Inoculum

[0667] Inoculum (or "Starter Inoculum") was started the night before the day of the experiment by adding either a colony or stab of a glycerol stock to 2 mL of LB Broth. The Starter Inoculum was then placed in a 37° C. shaker incubator and allowed to grow overnight.

[0668] The following day, the Starter Inoculum was removed from the shaker and added to fresh LB Broth. For each plate to be assayed, 6 mL of LB broth was prepared, with 5-10μL of Starter Inoculum being added per mL of added LB to form the "Test Inoculum". Typically, four plates of test compounds were assayed. In this typical example, 6 mL of LB Broth was used for each plate, or 24 mL of LB, followed by the addition of 5 μL/mL of Starter Inoculum, or 120 μL of Starter Inoculum to form the Test Inoculum.

[0669] Following preparation of the Test Inoculum, the Test Inoculum was placed in a 37 degree Celsius shaker and incubated for about 4 hours. The Test Inoculum was monitored for bacterial growth by taking OD readings at 535 nm on a spectrophotometer. The Test inoculum should be removed when the OD reaches between 0.6 and 1.5.

[0670] Preparation of Controls

[0671] Positive and negative controls were created by adding 2 uL DMSO to 198 uL LB Broth. At least 4 of each control were generated. Typically, there were 8 of each. 50 uL of diluted DMSO was added to 50 uL LB Broth in the assay plates.

[0672] Preparation of Compounds

[0673] The compounds were screened at 25 ug/mL. Two identical plates of each compound were set up: 1 clear plate for growth (or "Clear Plate"), 1 white plate for luminescence (or "White Plate"). Next, 2 μL of each compound was taken from the daughter plate (containing 10 mg/mL stock) and added to 198 μL of LB Broth. The sample was then mixed. Next, 25 μL of the diluted test compound was added to 25 μL of LB Broth in all of the assay plates. The concentration of the compound at this stage was 50 μg/mL.

[0674] Preparation of Plate

[0675] 50 μL of the Test Inoculum was added to each well of the plates, except for the negative controls. Half of the negative controls received 50 μL of AG112, while the other half of the negative controls received 50 μL LB Broth. The final concentration of the test compound was 25 μg/mL.

[0676] The Clear Plates were placed in the plate reader and read at $OD_{535}$. This was the "Initial" growth read. The plates were then incubated plates for 5 hours at 37 degrees Celsius. After 5 hours, the plates were removed from the incubator. The Clear Plates were placed in the plate reader and read at $OD_{535}$. This was the "Final" growth read.

[0677] 100 μL of Promega Steady-Glo reagent was added to each well (including all controls) in the White Plates. The plates, covered with aluminum foil, were then shaken on a plate shaker set at 10000 rpm for 10 min. The plates were then placed in plate reader and read on luminescence for 1sec per well. This was the LUMINESCENT read.

[0678] Data Analysis

[0679] To determine whether the test compound inhibited growth, the Initial growth read was subtracted from the Final growth read. This was the Subtracted Growth. The same calculation was performed for the positive controls. The results for the positive controls were averaged. The % Inhibition of Growth was determined using the following formula:

100-(100*Subtracted growth of sample/Average growth of Pos Controls)

[0680] To determine whether compound inhibits Luciferase, use the following equation:

100-(100*Luminescence for Compound/Average Luminescence of Pos Controls)

[0681] ND indicates that a particular test compound did not appear to decrease the lumninesce of in this particular assay. * indicates that the luminescence was decreased somewhat and ** indicates that the luminescence was decreased a substantial amount. The results from this assay are also shown in Table 5.

[0682] Equivalents

[0683] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

TABLE 5

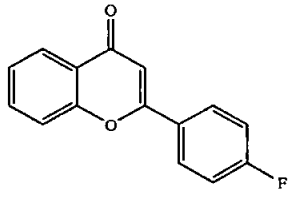
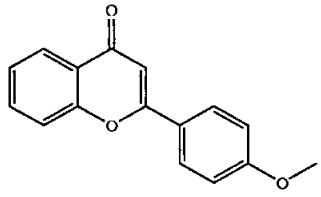
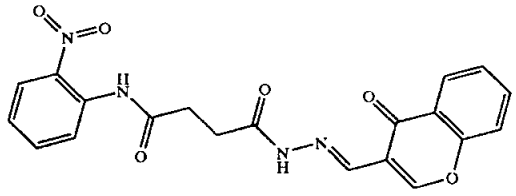
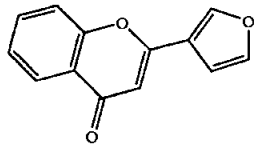
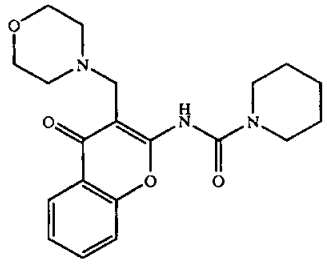
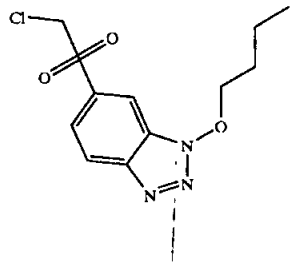
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|---|---|---|---|---|---|---|---|
| BH |  | ** | * | ** | ** | ** | ** |
| BI |  | ** | NT | NT | ** | ** | ND |
| BJ |  | *** | *** | * | ** | ND | ** |
| BK |  | * | * | ** | ** | ND | ND |
| BL |  | * | ** | * | ** | ** | ND |
| BN |  | * | * | * | ** | ** | ND |

TABLE 5-continued

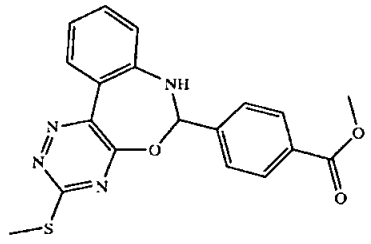
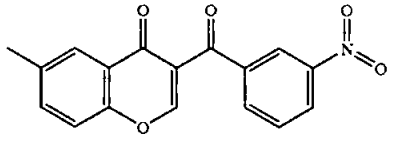
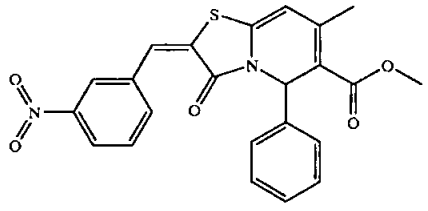
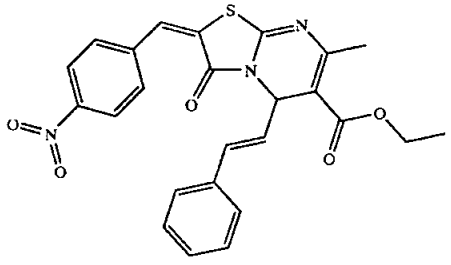
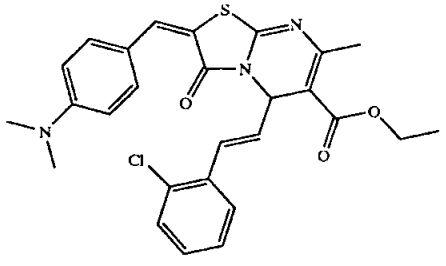
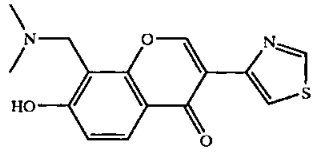
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| BO |  | * | ** | * | ** | ** | ND |
| BP |  | * | NT | NT | ** | * | ** |
| BQ |  | * | * | *** | ** | ND | ** |
| BR |  | *** | *** | *** | ** | ND | ND |
| BS |  | * | * | * | ** | ** | ** |
| BT |  | * | ** | * | ** | * | * |

TABLE 5-continued

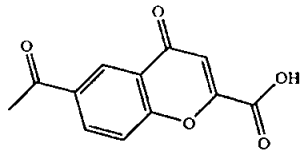
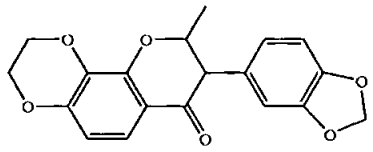
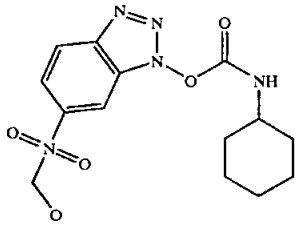
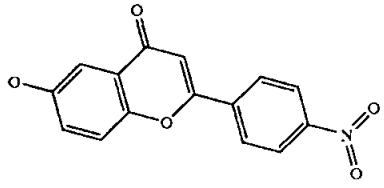
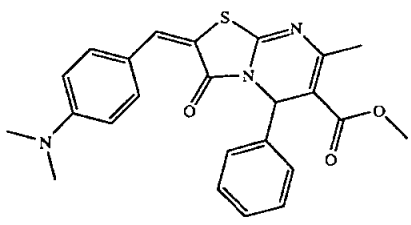
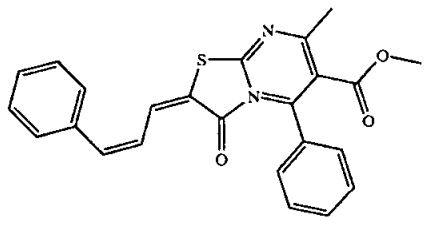
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
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| BU |  | * | NT | NT | ** | ND | ND |
| BV |  | * | NT | NT | ** | ** | ** |
| BW |  | NT | *** | ** | ** | ND | ND |
| BX |  | * | NT | NT | ** | ND | ND |
| BY |  | * | * | *** | ** | ND | ** |
| BZ |  | *** | * | ** | ** | ND | ND |

TABLE 5-continued

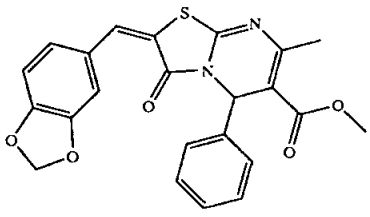
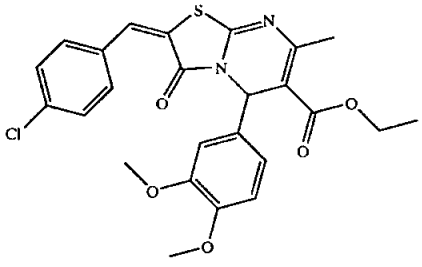
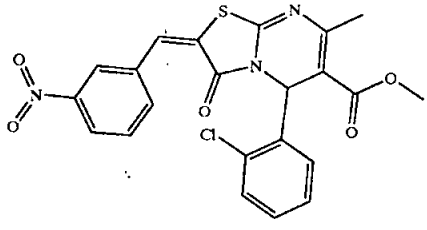
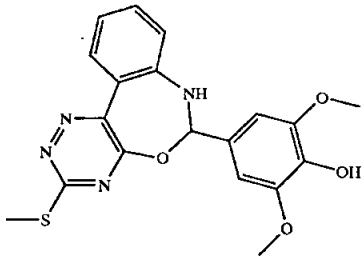
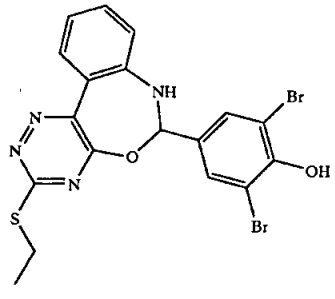
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| CB |  | * | - | * | ** | ND | ** |
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| CD |  | NT | *** | *** | ** | ND | ** |
| CE |  | ** | ** | * | ** | * | ND |

TABLE 5-continued

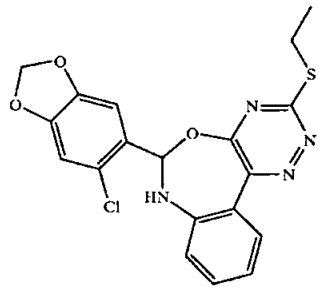
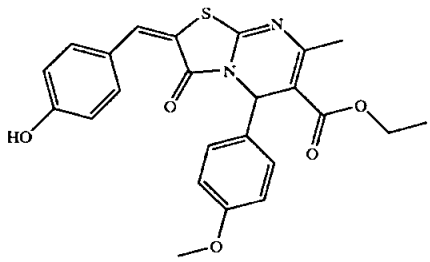
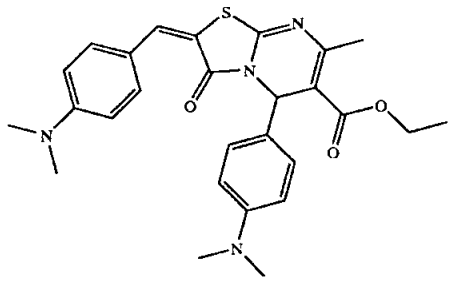
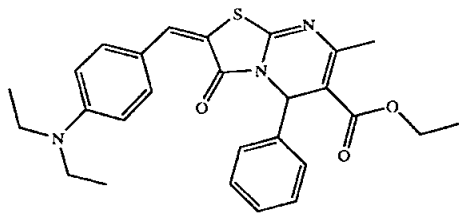
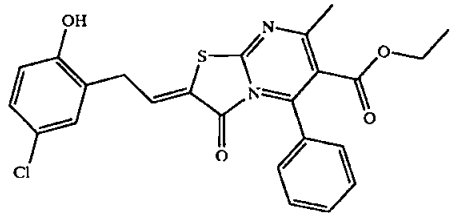
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| CF |  | * | NT | NT | ** | ND | ** |
| CG |  | * | NT | NT | ** | ND | ** |
| CH |  | * | NT | NT | ** | ND | ND |
| CI |  | * | NT | NT | ** | ND | ** |
| CJ |  | ** | * | * | ** | ND | ND |

TABLE 5-continued

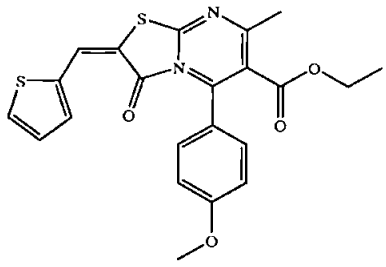
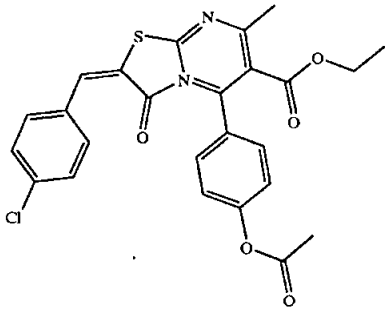
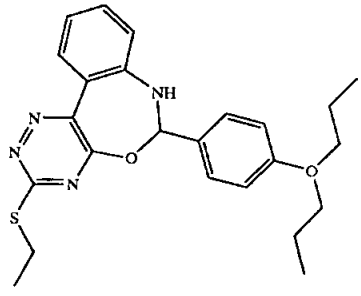
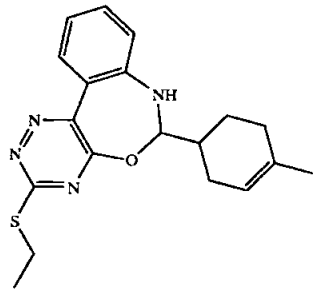
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| CK |  | ** | *** | ** | ** | ND | ND |
| CL |  | ** | * | * | ** | ND | ND |
| CM |  | * | * | * | ** | ** | ** |
| CN |  | *** | *** | * | ** | ** | ND |

TABLE 5-continued

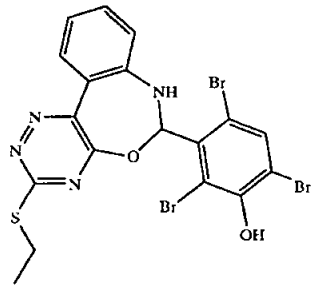
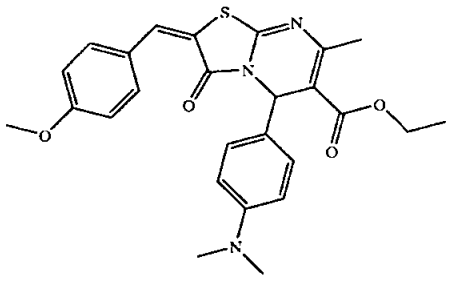
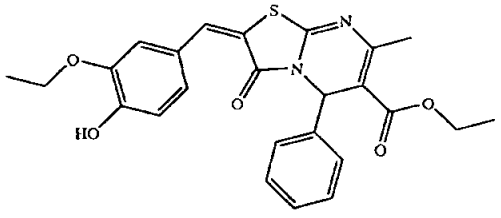
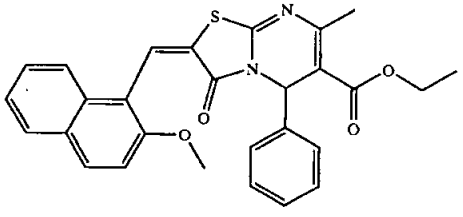
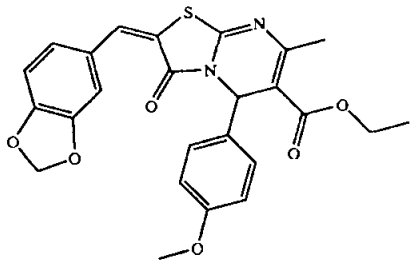
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| CO |  | * | * | ** | ** | ** | * |
| CP |  | * | - | * | ** | ** | * |
| CQ |  | * | * | * | ** | ** | * |
| CR |  | * | ** | *** | ** | ** | ND |
| CS |  | *** | *** | * | ** | ** | ND |

TABLE 5-continued

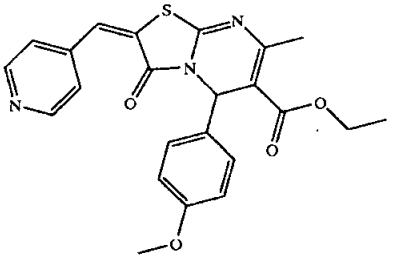
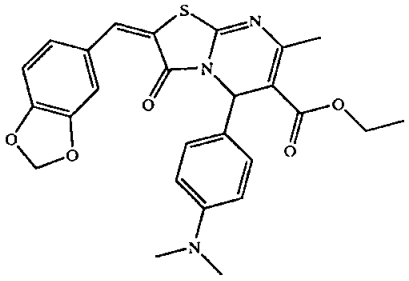
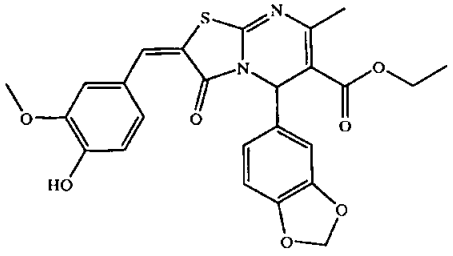
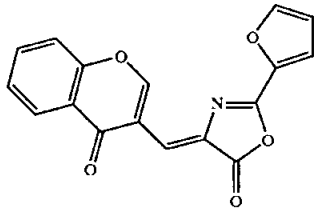
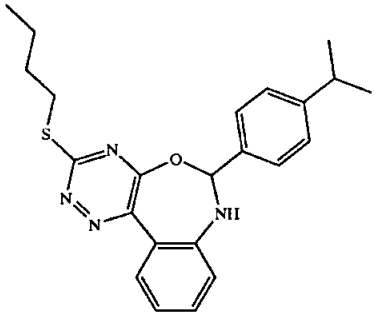
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| CT |  | NT | *** | *** | ** | ND | * |
| CU |  | NT | ** | * | ** | ND | ND |
| CV |  | * | NT | NT | ** | ND | ND |
| CW |  | * | * | *** | ** | ND | ** |
| CX |  | * | *** | *** | ** | ND | ** |

TABLE 5-continued

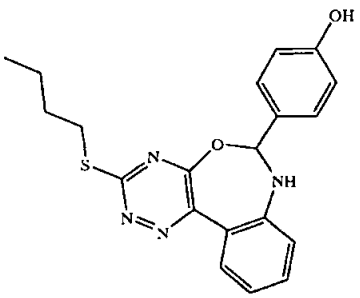
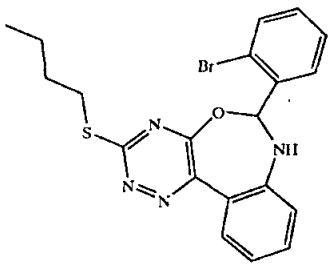
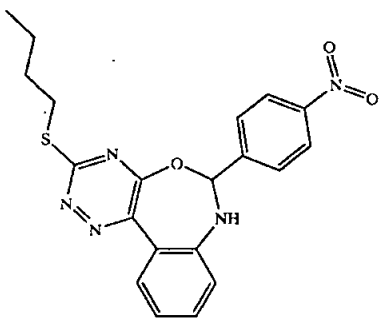
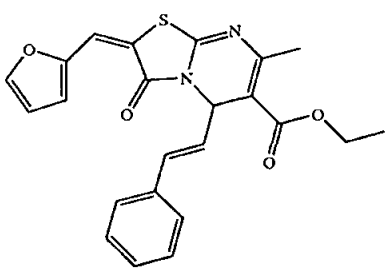
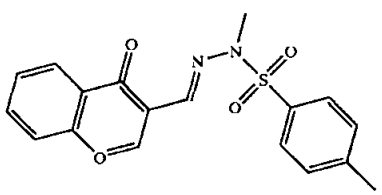
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
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| CZ |  | * | * | ** | ** | ND | ND |
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| DB |  | ** | * | * | ** | ND | ND |
| DC |  | * | * | * | ** | ND | * |

TABLE 5-continued

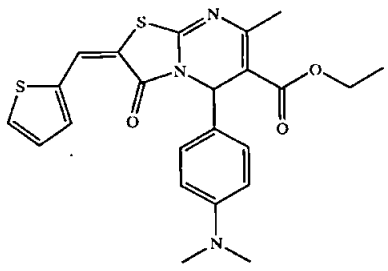
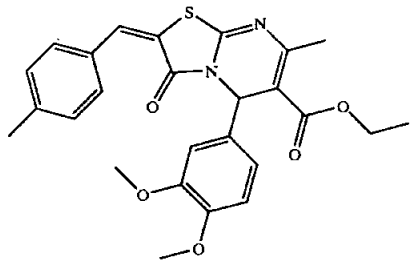
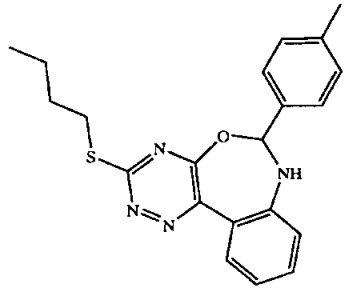
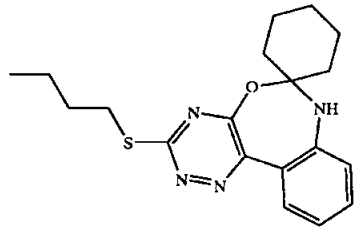
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| DD |  | * | * | * | ** | ND | * |
| DE |  | * | * | ** | ** | ** | ND |
| DF |  | * | * | * | ** | ND | ** |
| DG |  | * | * | * | ** | ** | ** |

TABLE 5-continued

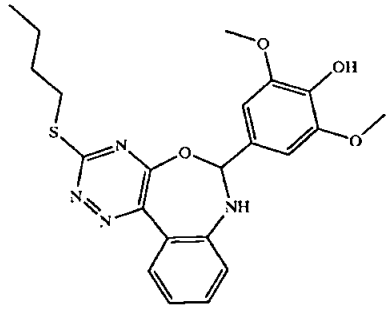
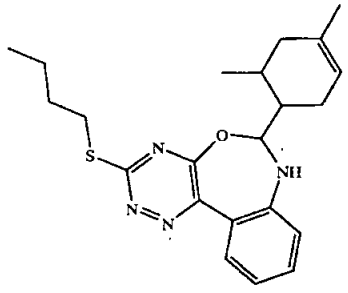
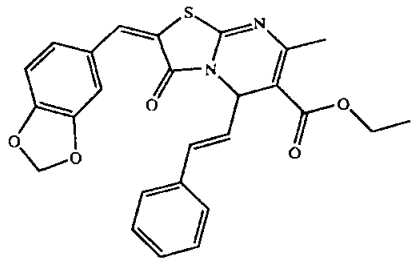
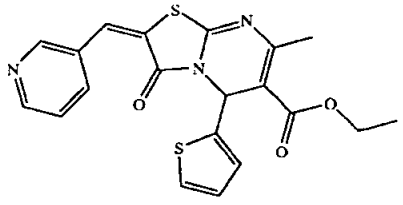
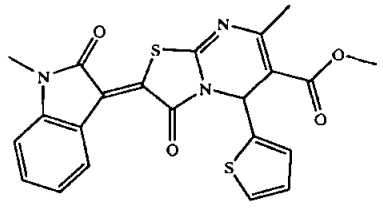
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| DH |  | * | * | ** | ** | ** | ND |
| DI |  | * | * | * | ** | ** | ** |
| DJ |  | ** | NT | NT | ** | ND | ND |
| DK |  | * | NT | NT | ** | ND | ND |
| DL |  | *** | * | *** | ** | ND | ** |

TABLE 5-continued

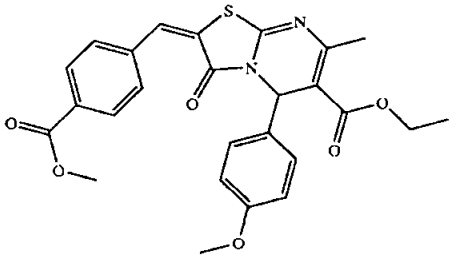
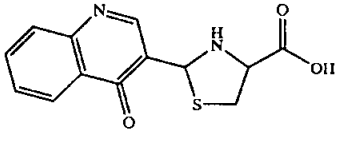
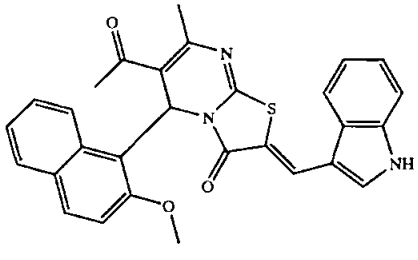
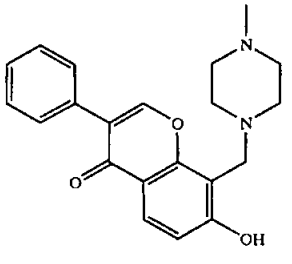
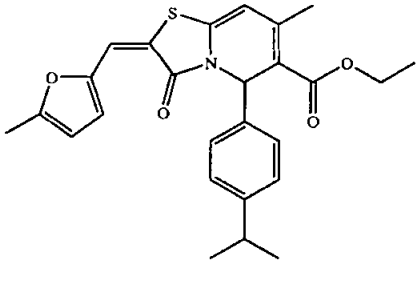
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| DM |  | *** | * | * | ** | ND | ND |
| DN |  | * | * | * | ** | ND | ND |
| DO |  | * | * | * | ** | ND | * |
| DP |  | *** | * | * | ** | ND | ND |
| DQ |  | * | * | * | ** | ND | ND |

TABLE 5-continued

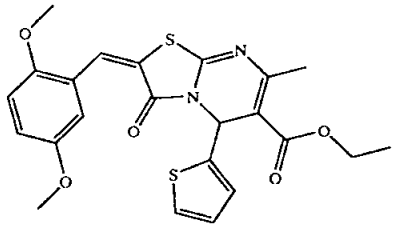
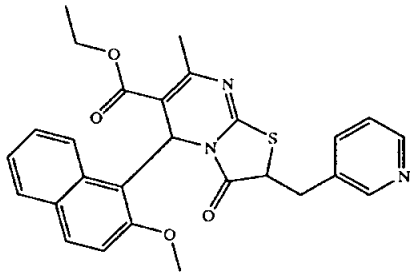
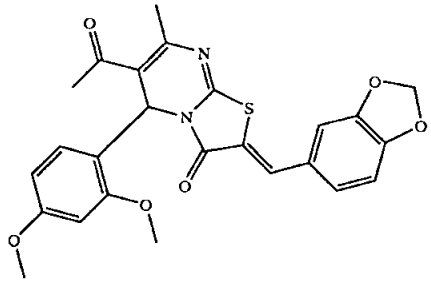
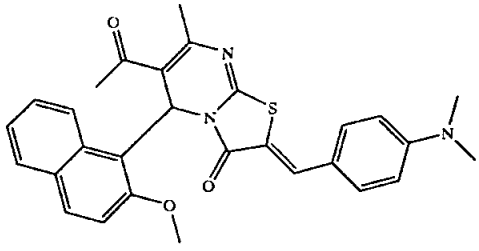
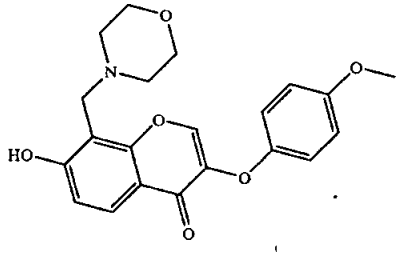
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| DR |  | *** | * | * | ** | ND | ND |
| DS |  | * | * | * | ** | ND | * |
| DT |  | * | * | * | ** | ND | * |
| DU |  | *** | *** | * | ** | ND | ** |
| DV |  | *** | *** | ** | ** | ND | ** |

TABLE 5-continued

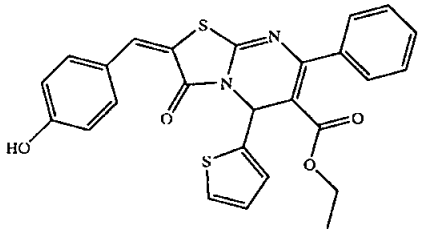
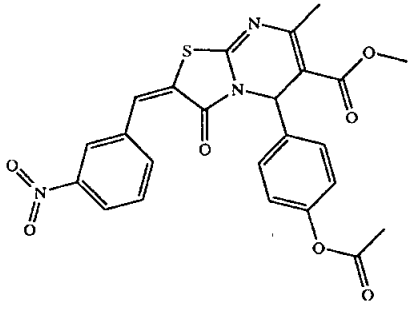
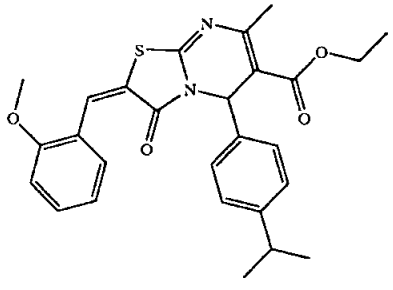
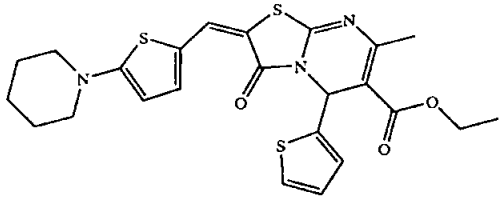
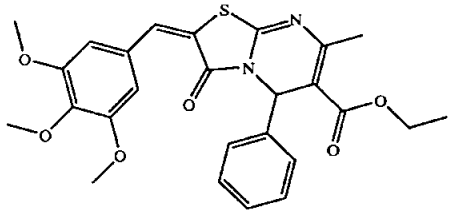
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| DW |  | ** | * | ** | ** | ND | ** |
| DX |  | *** | *** | ** | ** | ND | ** |
| DY |  | * | * | ** | ** | ND | ** |
| DZ |  | * | * | ** | ** | ** | ** |
| EA |  | * | NT | NT | ** | * | * |

TABLE 5-continued

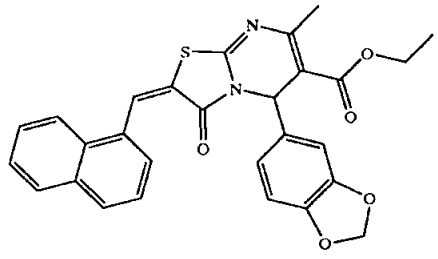
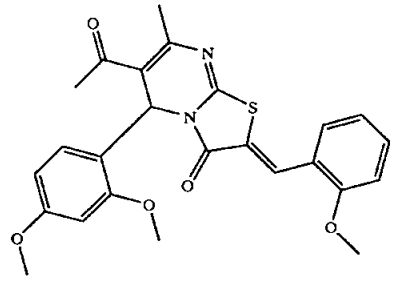
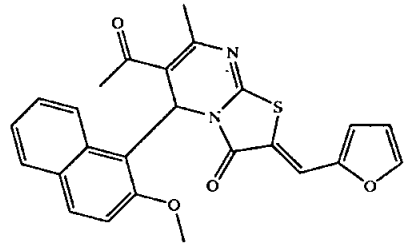
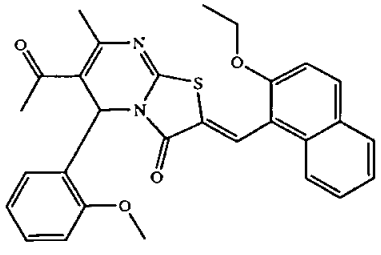
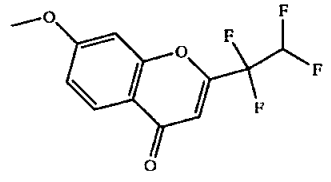
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| EB |  | * | NT | NT | ** | * | ** |
| EC |  | * | NT | NT | ** | ** | ND |
| ED |  | *** | *** | * | ** | ** | ** |
| EF |  | ** | *** | *** | ** | * | ** |
| EG |  | * | ** | * | ** | ** | ** |

TABLE 5-continued

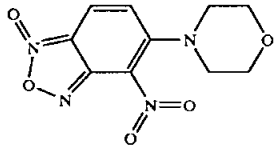
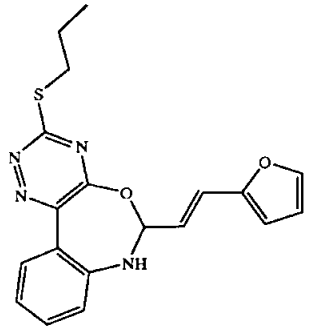
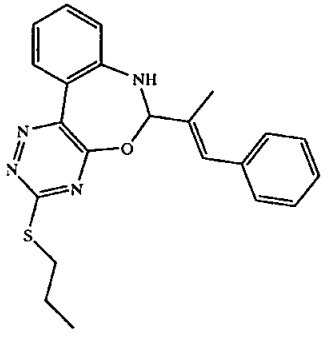
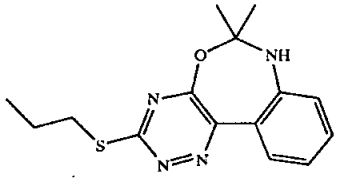
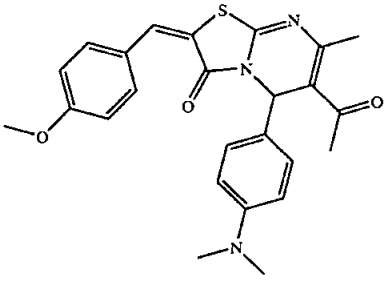
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| EH |  | *** | *** | ** | ** | * | ** |
| EI |  | * | NT | NT | ** | ND | ND |
| EJ |  | * | NT | NT | ** | ND | ND |
| EK |  | *** | NT | NT | ** | ND | * |
| EL |  | * | NT | NT | ** | ND | ND |

TABLE 5-continued

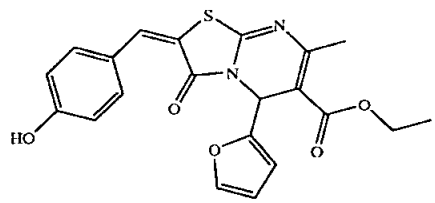
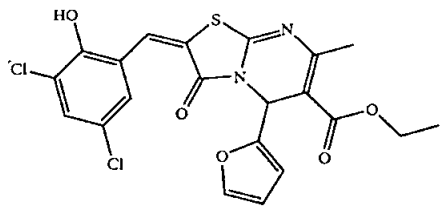
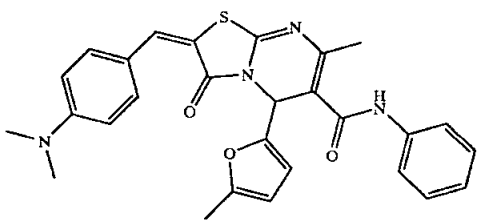
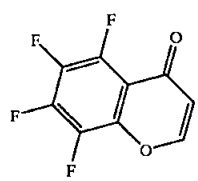
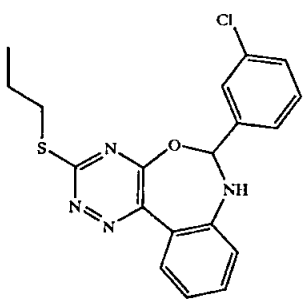
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| EM |  | ** | NT | NT | ** | ND | ND |
| EN |  | * | NT | NT | ** | ND | * |
| EO |  | * | * | * | ** | ND | ** |
| EQ |  | * | * | * | ** | ND | ND |
| ER |  | * | * | * | ** | ND | ** |

TABLE 5-continued

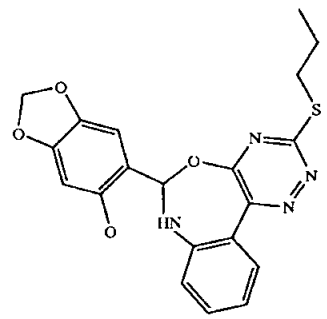
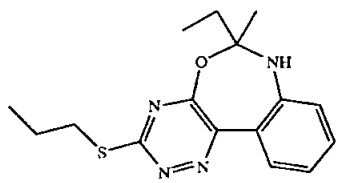
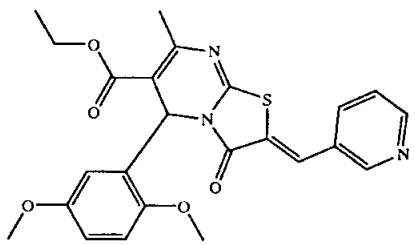
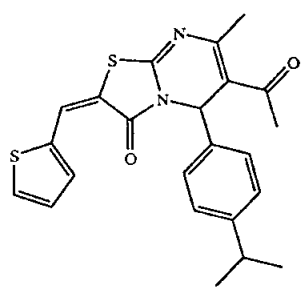
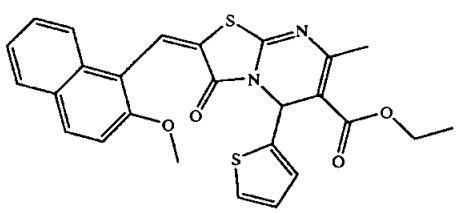
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ES |  | * | * | *** | ** | ND | ND |
| ET |  | ** | * | * | ** | ND | ** |
| EU |  | * | * | * | ** | ND | ND |
| EV |  | *** | * | * | ** | ND | ND |
| EW |  | * | * | ** | ** | ND | ** |

TABLE 5-continued

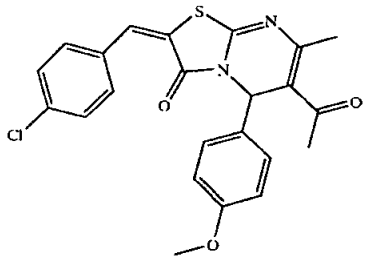
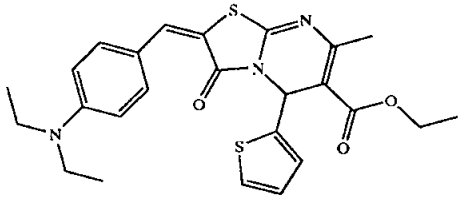
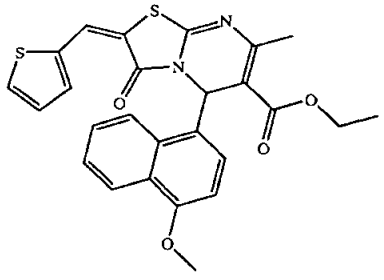
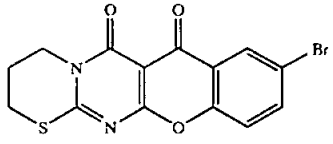
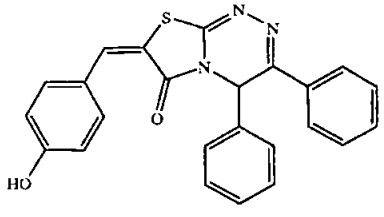
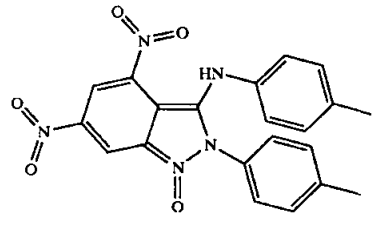
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| EX |  | * | * | ** | ** | ND | ND |
| EY |  | * | * | * | ** | ND | * |
| EZ |  | * | * | * | ** | ND | * |
| FA |  | * | * | * | ** | ND | * |
| FB |  | * | * | * | ** | ND | * |
| FC |  | * | * | * | ** | * | * |

TABLE 5-continued

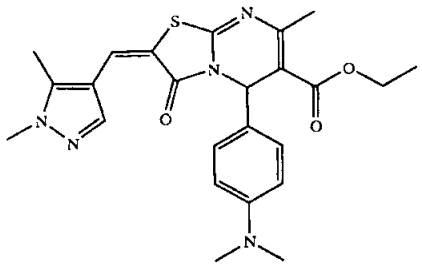
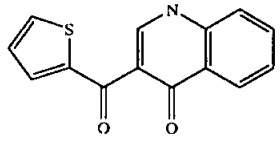
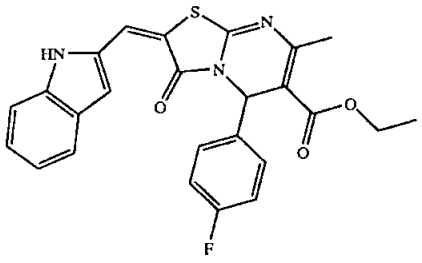
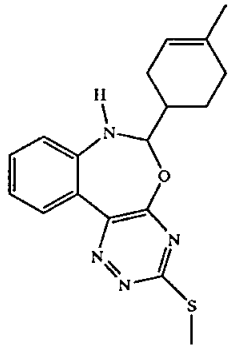
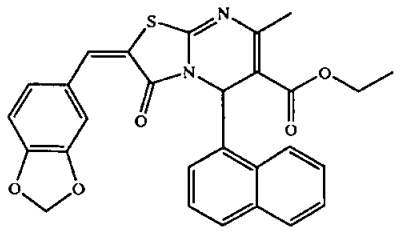
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| FD |  | * | * | * | ** | * | ** |
| FE |  | * | ** | * | ** | ** | ND |
| FG |  | ** | *** | ** | ** | * | ND |
| FH |  | * | * | * | ** | ND | ** |
| FI |  | * | * | * | ** | ND | ND |

TABLE 5-continued

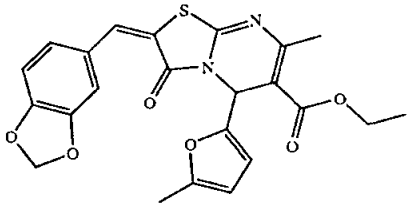
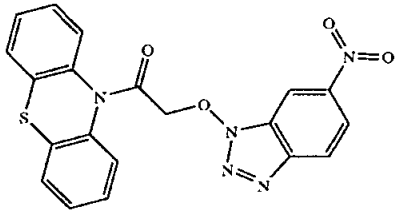
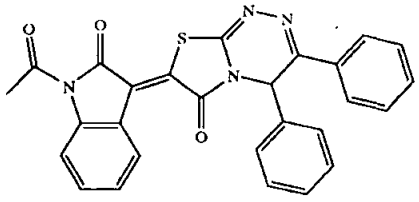
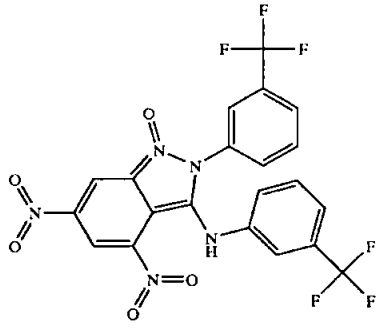
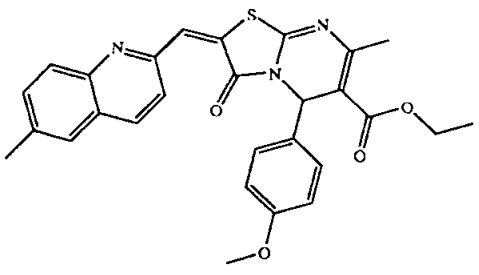
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| FJ |  | ** | ** | * | ** | ND | ** |
| FK |  | *** | * | *** | ** | ND | ** |
| FL |  | * | * | ** | ** | ND | ** |
| FM |  | * | * | * | ** | ND | ** |
| FN |  | *** | ** | NT | ** | ND | * |

TABLE 5-continued

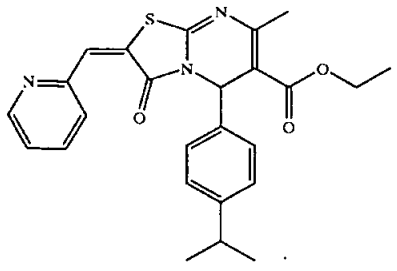
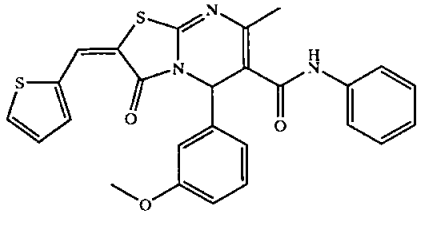
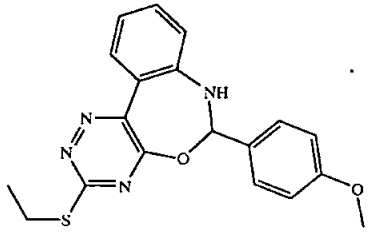
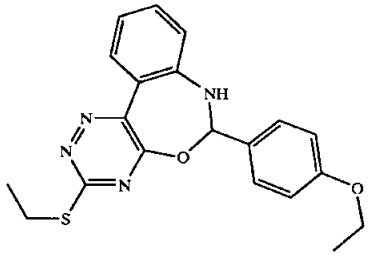
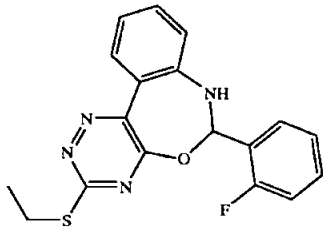
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| FO |  | *** | ** | * | ** | * | * |
| FP |  | *** | *** | * | ** | ** | ** |
| FQ |  | * | ** | ** | ** | ** | * |
| FR |  | * | NT | NT | ** | * | ** |
| FS |  | * | ** | * | ** | ** | ND |

TABLE 5-continued

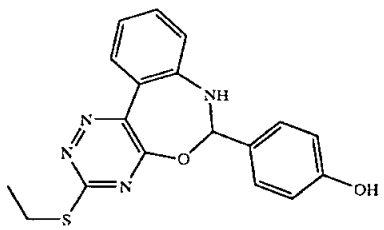
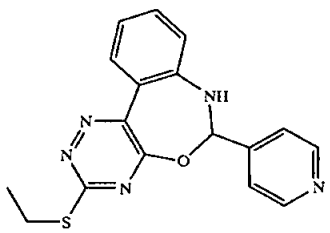
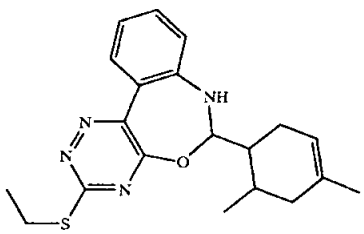
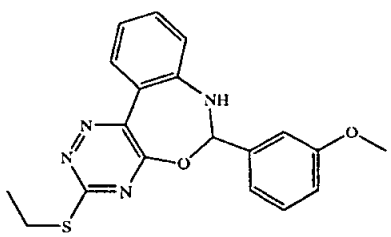
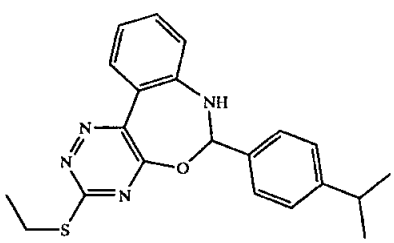
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| FT |  | NT | * | * | ** | ND | ** |
| FU |  | ** | * | *** | ** | ND | ** |
| FV |  | * | * | * | ** | ND | ** |
| FW |  | * | * | * | ** | ND | ND |
| FX |  | * | * | * | ** | ND | ** |

TABLE 5-continued

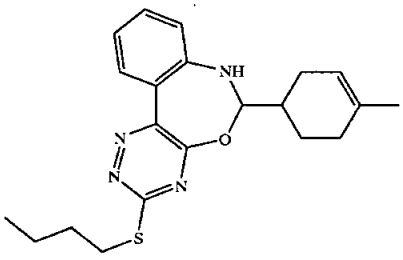
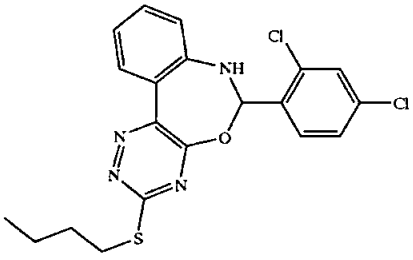
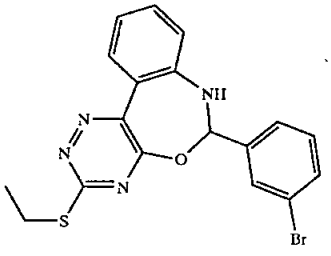
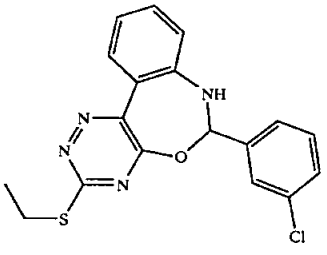
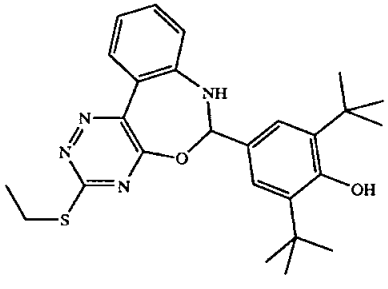
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| FY |  | * | * | * | ** | ND | ** |
| FZ |  | * | * | * | ** | ND | ** |
| GA |  | * | * | ** | ** | ND | ND |
| GB |  | * | * | * | ** | ND | * |
| GC |  | * | * | * | ** | ND | ** |

TABLE 5-continued

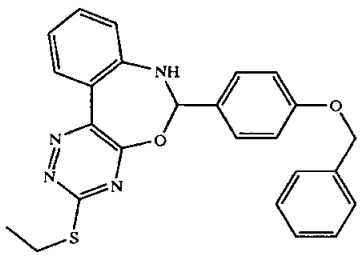
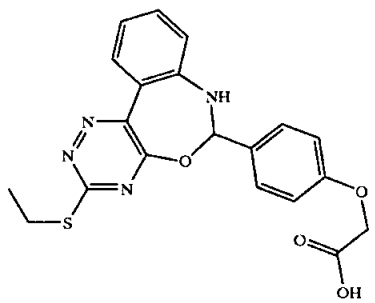
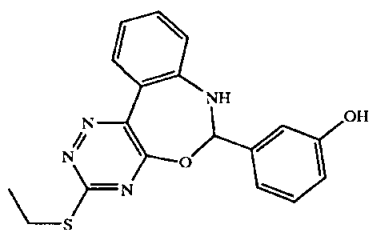
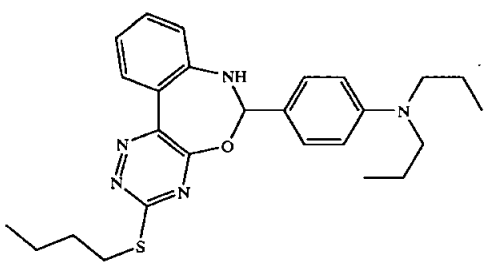
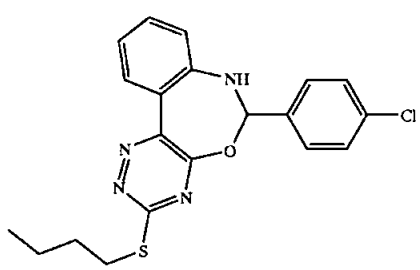
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| GD |  | * | * | * | ** | ND | ND |
| GE |  | * | * | * | ** | ND | ND |
| GF |  | ** | * | * | ** | ND | ND |
| GG |  | * | * | * | ** | ND | * |
| GH |  | * | * | * | ** | ND | ND |

TABLE 5-continued

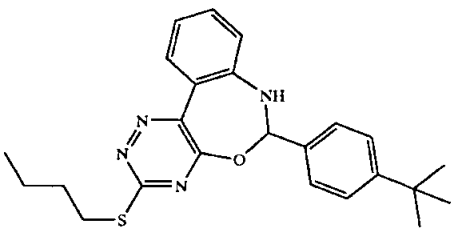
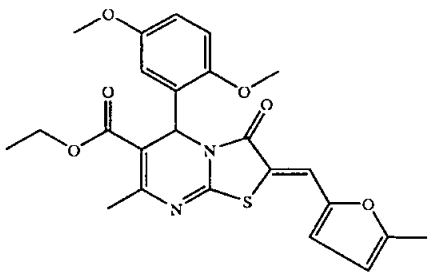
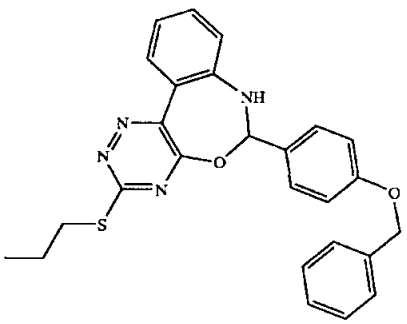
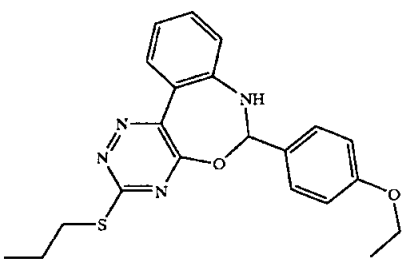
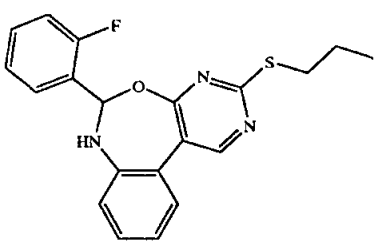
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| GI |  | ** | ** | *** | ** | ND | ** |
| GJ |  | * | * | ** | ** | ND | ND |
| GK |  | * | * | * | ** | ND | * |
| GL |  | * | * | * | ** | ND | ND |
| GM |  | * | * | *** | ** | ND | ND |

TABLE 5-continued

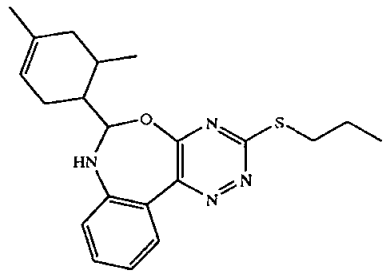
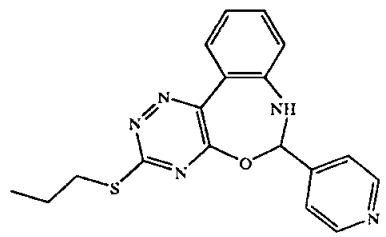
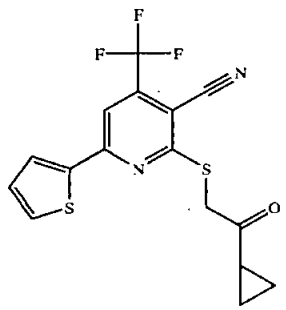
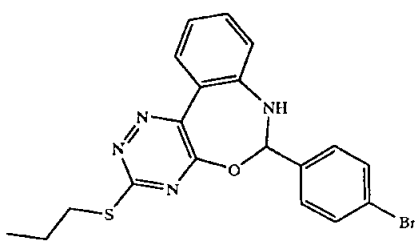
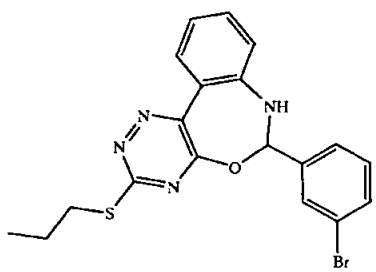
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| GN |  | * | * | * | ** | ND | ND |
| GO |  | * | * | * | ** | ND | * |
| GP |  | * | ** | ** | ** | ** | ** |
| GQ |  | ** | *** | ** | ** | * | ** |
| GR |  | * | *** | *** | ** | * | ND |

TABLE 5-continued

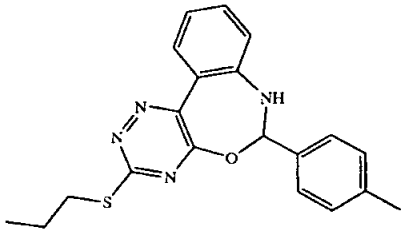
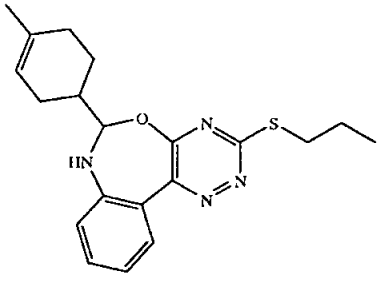
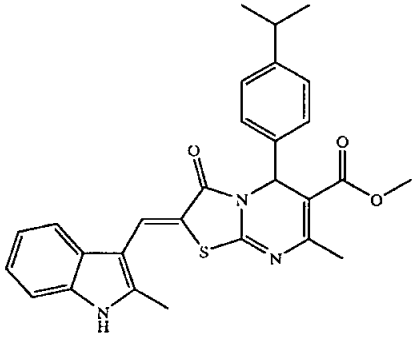
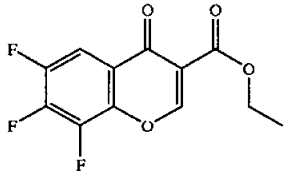
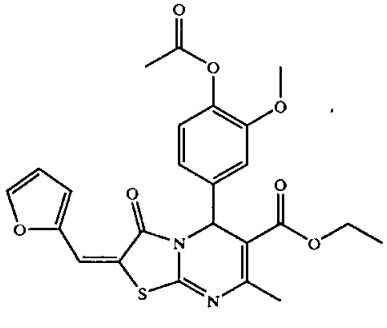
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| GS |  | *** | *** | *** | ** | ** | ND |
| GT |  | * | ** | ** | ** | * | ND |
| GU |  | ** | *** | * | ** | ** | ND |
| GV |  | * | * | * | ** | ND | ND |
| GW |  | * | * | ** | ** | ND | ** |

TABLE 5-continued

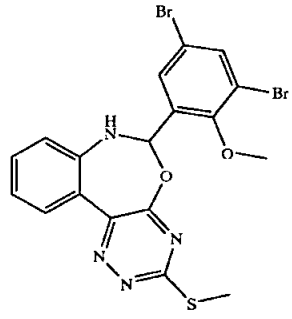
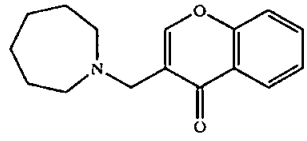
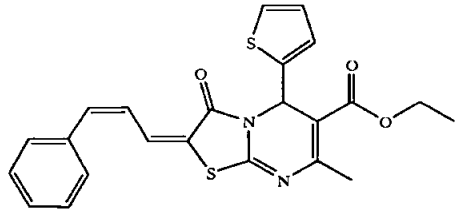
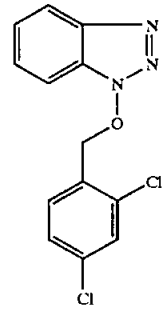
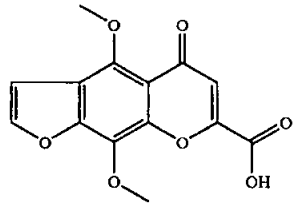
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| GX |  | * | * | *** | ** | ND | ** |
| GY |  | * | * | * | ** | ND | ** |
| GZ |  | ** | * | ** | ** | ND | ** |
| HA |  | * | * | * | ** | ND | ** |
| HB |  | * | * | * | ** | ND | * |

TABLE 5-continued

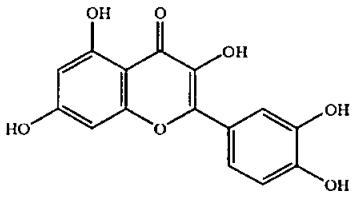
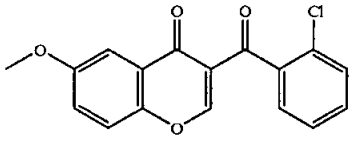
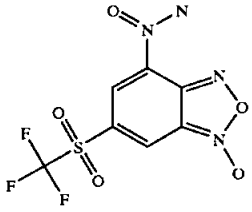
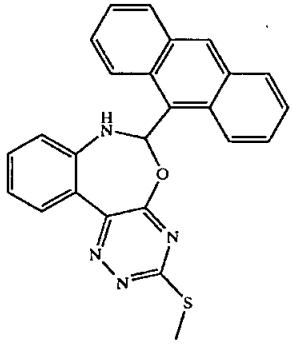
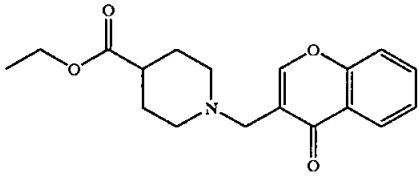
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| HC |  | *** | * | NT | ** | ND | ** |
| HD |  | ** | * | *** | ** | ND | * |
| HE |  | * | * | * | ** | ** | ** |
| HF |  | ** | * | * | ** | ** | ND |
| HG |  | * | * | * | ** | ** | ** |

TABLE 5-continued

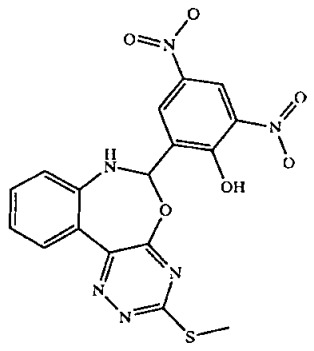
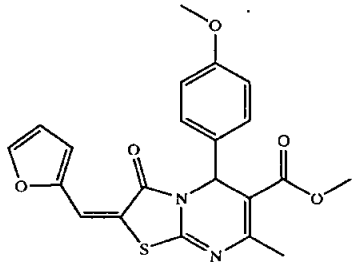
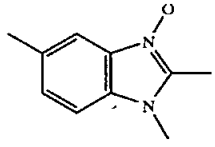
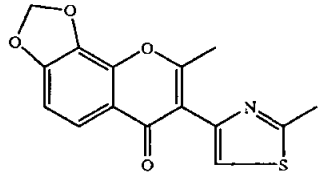
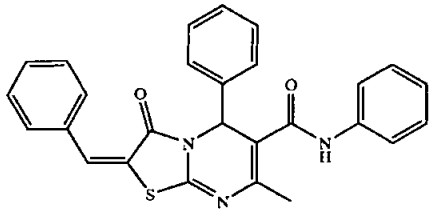
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| HH |  | * | * | * | ** | ** | ** |
| II |  | * | * | * | ** | ** | ** |
| HJ |  | * | ** | * | ** | ** | ** |
| HK |  | * | ** | ** | ** | ** | * |
| HL |  | *** | *** | * | ** | ** | ** |

TABLE 5-continued

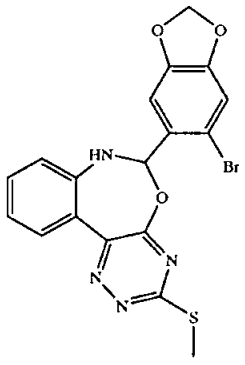
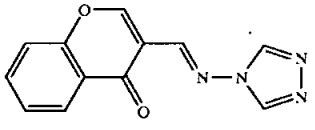
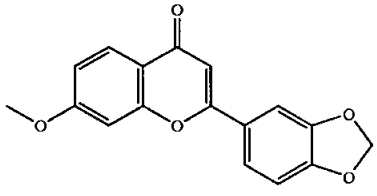
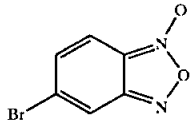
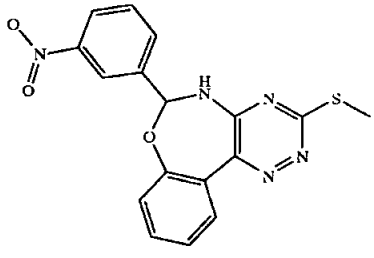
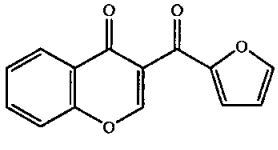
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| HM |  | NT | ** | * | ** | ND | ** |
| HN |  | NT | ** | ** | ** | ND | ND |
| HO |  | NT | * | ** | ** | ND | ND |
| HP |  | NT | * | * | ** | ND | * |
| HQ |  | * | ** | * | ** | ** | ** |
| HR |  | * | * | * | ** | ** | ** |

TABLE 5-continued

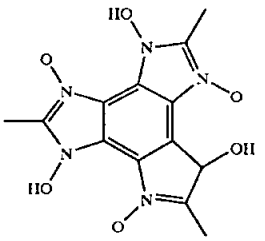
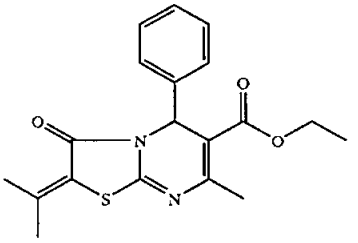
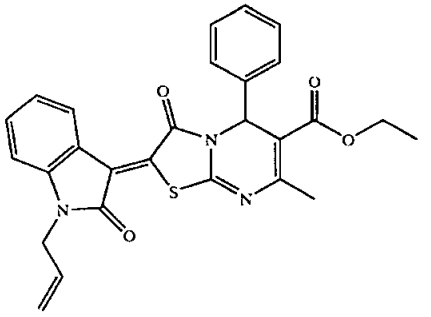
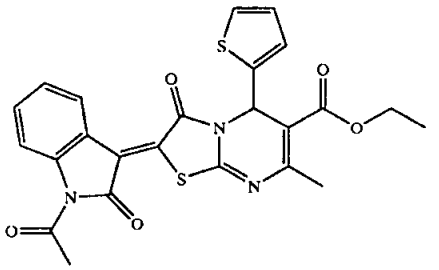
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| HS |  | ** | NT | NT | ** | ND | * |
| HT |  | * | NT | NT | ** | ND | ** |
| HU |  | *** | *** | * | ** | ND | ** |
| HV |  | * | * | * | ** | ND | ** |

TABLE 5-continued

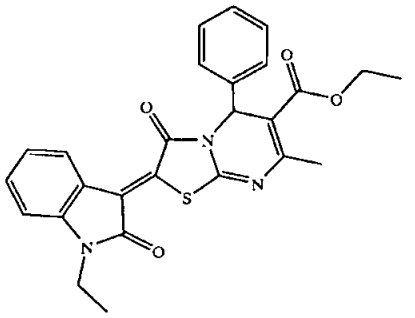
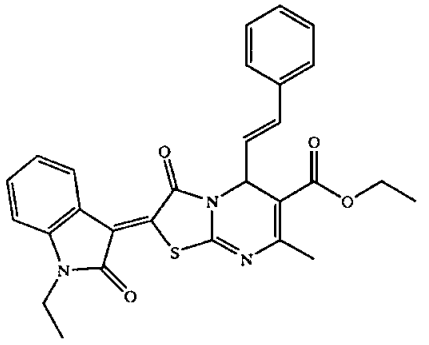
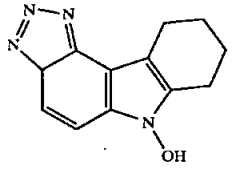
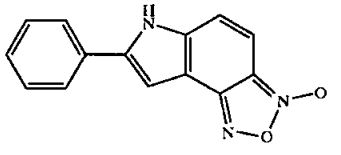
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| HW |  | * | *** | * | ** | ND | ** |
| HX |  | * | * | * | ** | ND | ** |
| HY |  | * | * | * | ** | ND | * |
| HZ |  | * | * | * | ** | ND | ** |

TABLE 5-continued

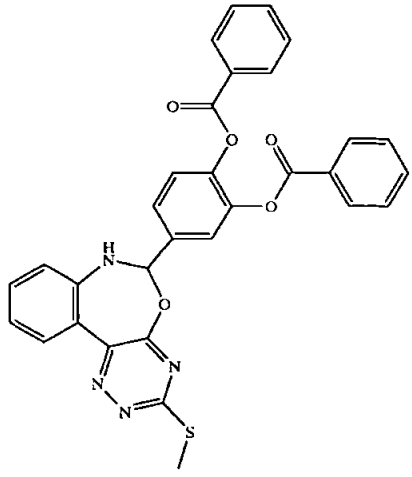
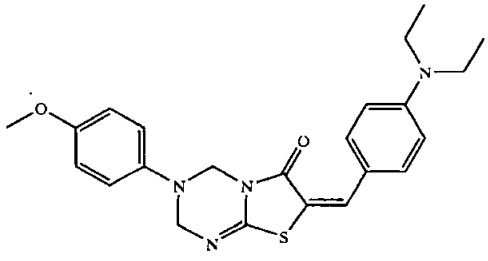
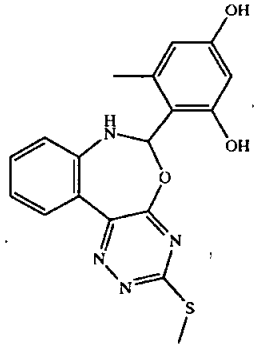
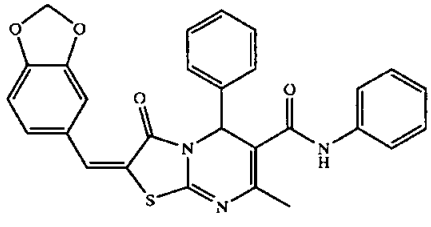
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| IA |  | * | * | * | ** | ND | * |
| IB |  | * | * | * | ** | ND | ND |
| IC |  | * | * | * | ** | ND | ND |
| ID |  | * | * | * | ** | ND | * |

TABLE 5-continued

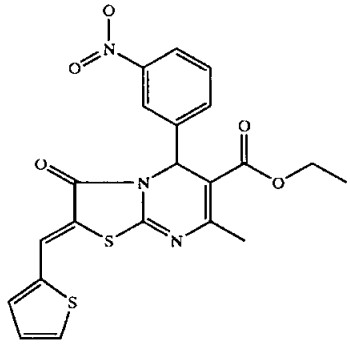
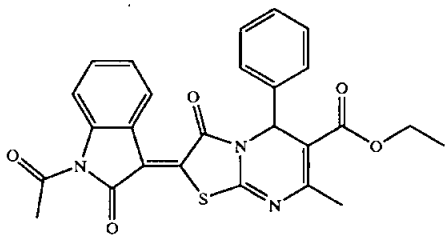
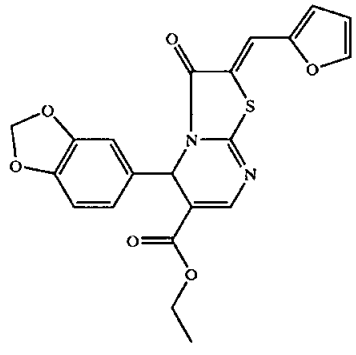
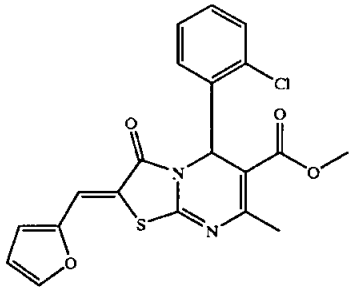
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| IE |  | *** | *** | * | ** | ND | ** |
| IF |  | * | * | * | ** | ND | ND |
| IG |  | * | * | * | ** | ND | ND |
| IH |  | * | * | * | ** | ND | * |

TABLE 5-continued

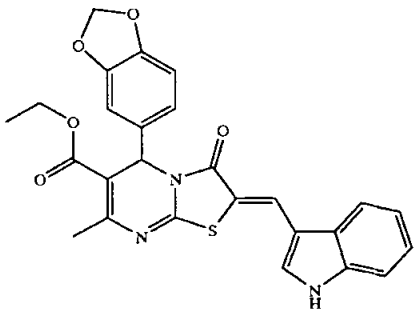
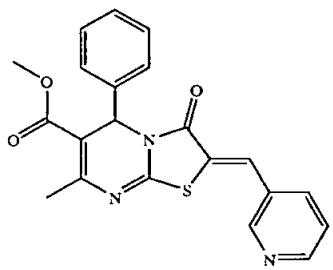
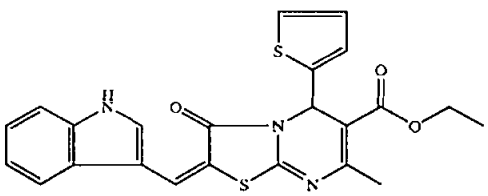
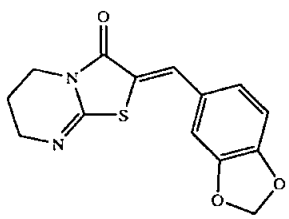
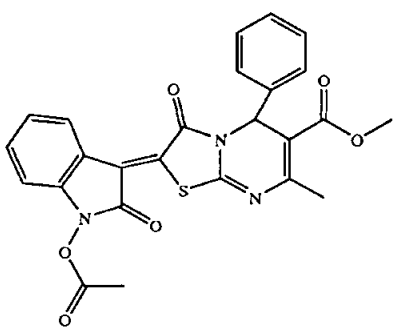
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| II |  | ** | * | * | ** | ND | ** |
| IJ |  | ** | * | * | ** | ND | * |
| IK |  | * | * | * | ** | ND | ND |
| IL |  | *** | * | * | ** | ND | ** |
| IM |  | * | * | * | ** | ND | ** |

TABLE 5-continued

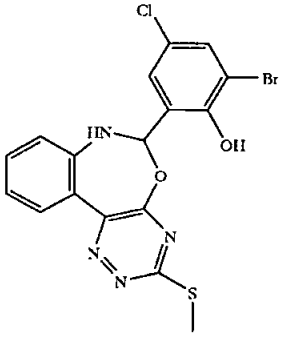
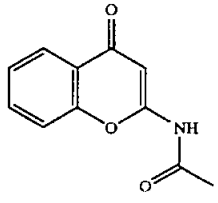
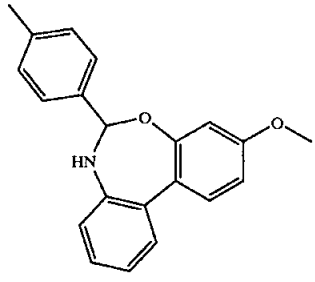
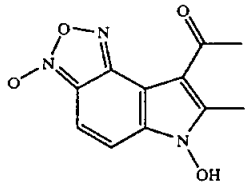
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| IN |  | * | * | * | ** | ND | ND |
| IO |  | * | * | * | ** | ND | ND |
| IP |  | * | * | * | ** | ND | ND |
| IQ |  | * | * | * | ** | ND | ND |

TABLE 5-continued

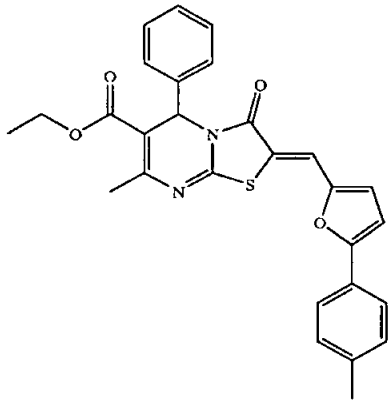
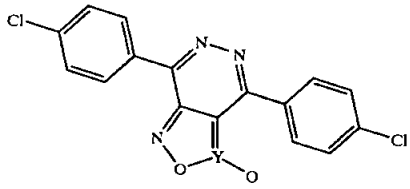
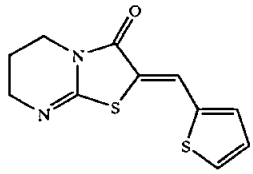
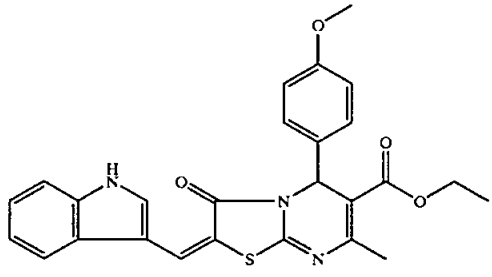
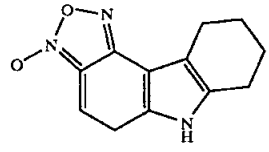
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| IR |  | * | * | ** | ** | ND | ** |
| IS |  | * | *** | ** | ** | ND | * |
| IT |  | * | * | * | ** | ND | * |
| IU |  | *** | * | ** | ** | ND | ND |
| IV |  | * | * | * | ** | ND | ND |

TABLE 5-continued

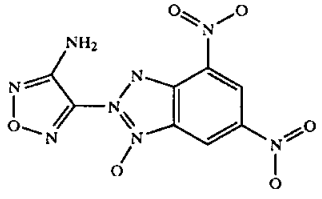
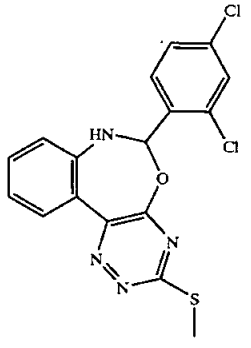
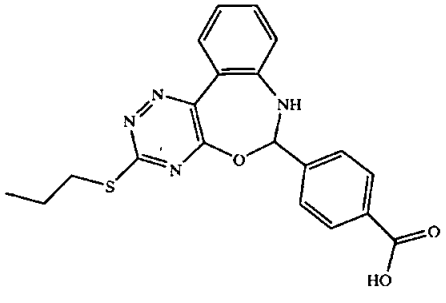
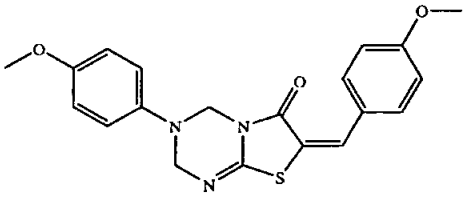
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| IW |  | * | * | * | ** | ND | ND |
| IX |  | ** | * | * | ** | ND | * |
| IY |  | * | * | * | ** | ND | ** |
| IZ |  | * | * | * | ** | ND | * |

TABLE 5-continued

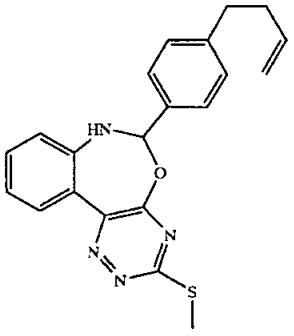
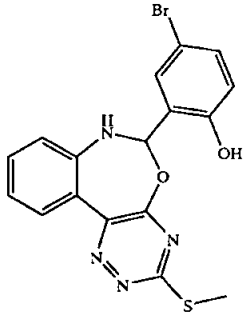
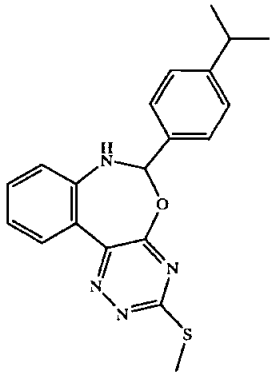
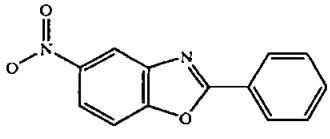
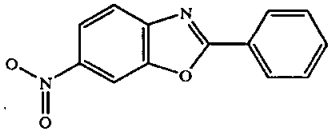
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| JA |  | * | * | * | ** | ND | * |
| JB |  | ** | * | * | ** | ND | ND |
| JD |  | * | * | * | ** | ND | * |
| JE |  | * | * | * | ** | ** | * |
| JF |  | ** | ** | *** | ** | * | * |

TABLE 5-continued

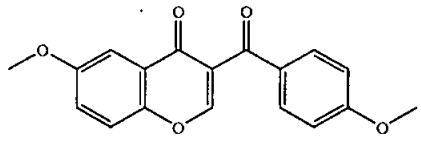
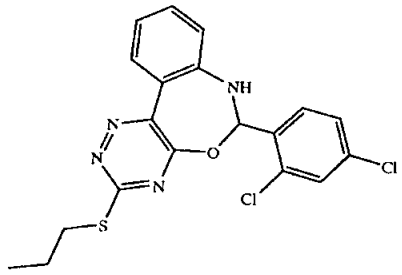
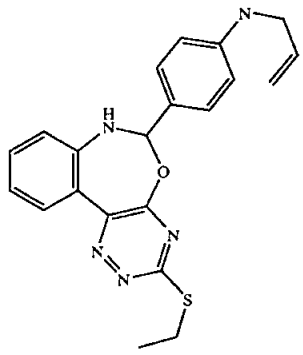
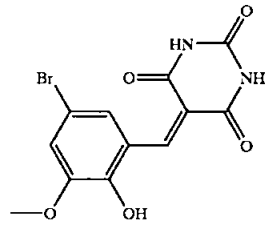
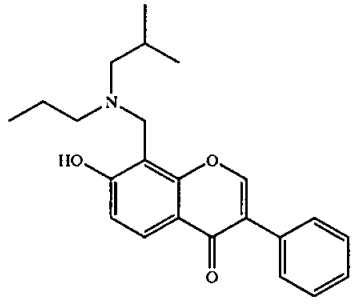
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| JG |  | * | - | ** | ** | ** | ND |
| JH |  | * | - | * | ** | ** | ** |
| JI |  | * | - | ** | ** | ** | ** |
| JJ |  | ** | - | ** | ** | ND | ** |
| JK |  | ** | - | * | ** | ND | ** |

TABLE 5-continued

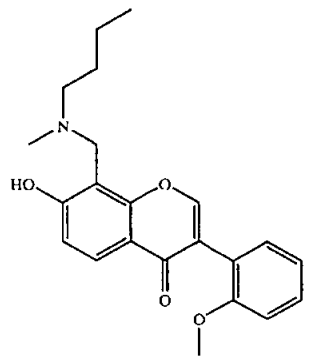
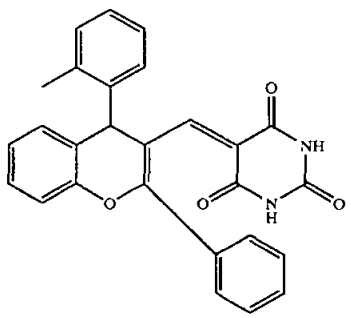
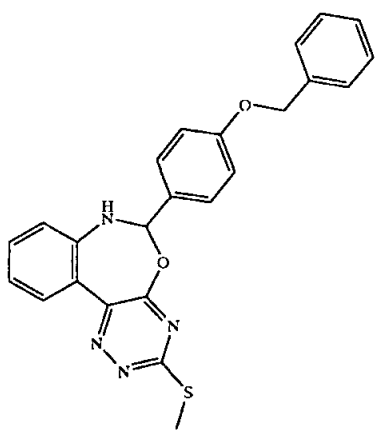
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| JL |  | ** | * | * | ** | ND | ND |
| JM |  | * | - | * | ** | ND | ** |
| JN |  | *** | * | NT | ** | ND | ** |

TABLE 5-continued

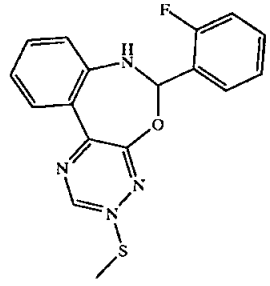
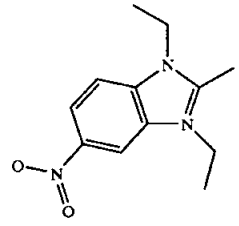
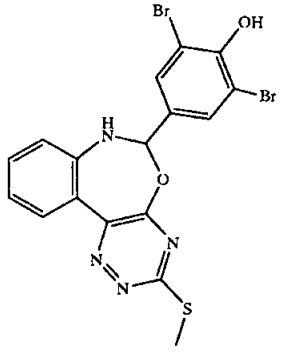
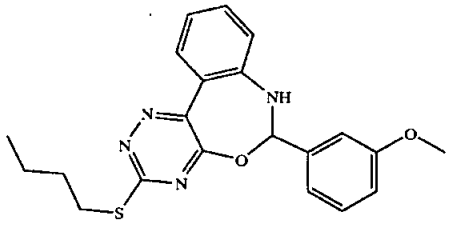
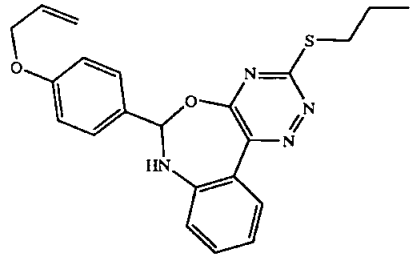
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| JO |  | *** | * | NT | ** | ND | ** |
| JP |  | * | * | * | ** | * | * |
| JQ |  | * | * | * | ** | ** | ** |
| JR |  | *** | *** | *** | ** | ** | ND |
| JZ |  | ** | * | * | ** | ** | ** |

TABLE 5-continued

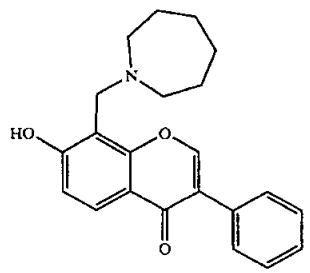
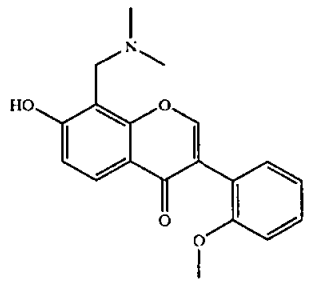
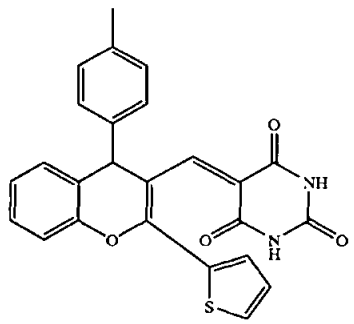
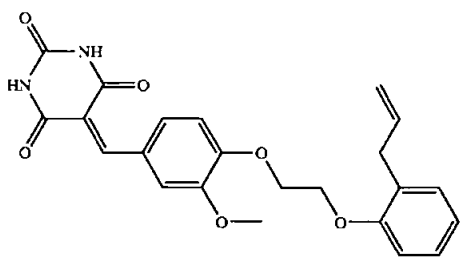
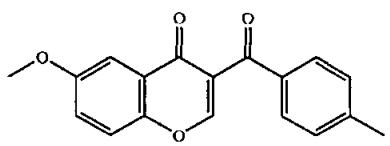
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KA |  | * | * | * | ** | ** | ** |
| KB |  | * | * | * | ** | ** | ** |
| KC |  | * | * | * | ** | ** | ** |
| KD |  | * | NT | NT | ** | * | ** |
| KE |  | * | NT | NT | ** | ** | * |

TABLE 5-continued

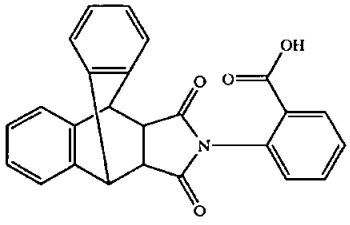
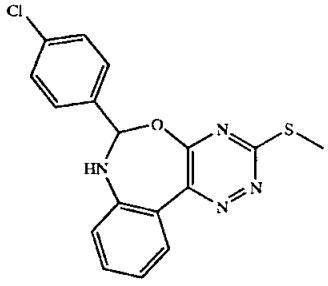
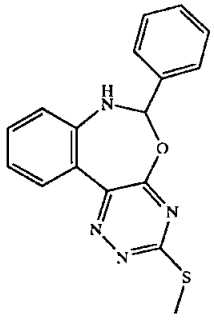
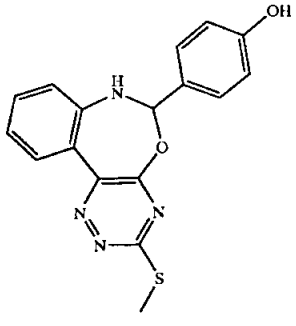
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KF |  | *** | *** | ** | ** | ** | ** |
| KG |  | NT | - | * | ** | ND | * |
| KH |  | NT | - | *** | ** | ND | ** |
| KI |  | NT | - | * | ** | ND | ND |

TABLE 5-continued

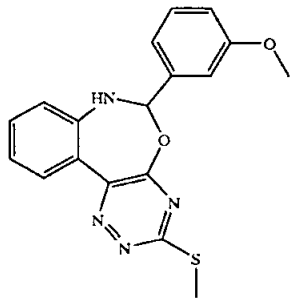
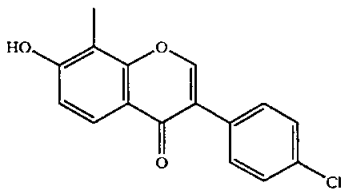
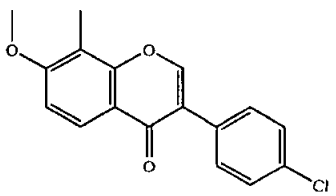
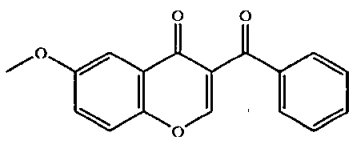
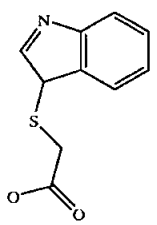
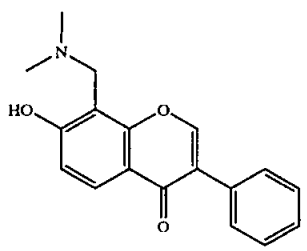
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KJ |  | NT | ** | *** | ** | ND | ND |
| KK |  | * | NT | NT | ** | ND | ** |
| KL |  | * | * | * | ** | ND | ND |
| KM |  | * | * | ** | ** | ND | ND |
| KN |  | *** | * | * | ** | ND | ** |
| KO |  | * | *** | *** | ** | ND | ** |

TABLE 5-continued

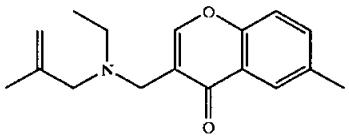
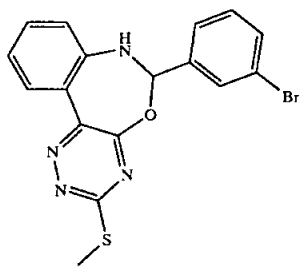
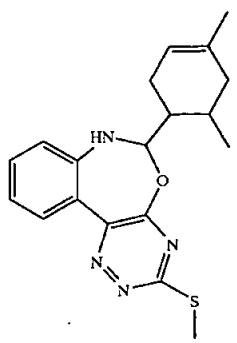
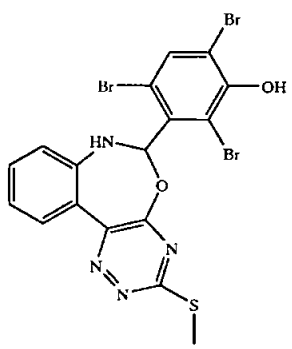
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KP |  | * | * | ** | ** | ND | ** |
| KQ |  | * | * | * | ** | ND | * |
| KR |  | *** | ** | * | ** | ND | ND |
| KS |  | * | * | * | ** | ND | * |

TABLE 5-continued

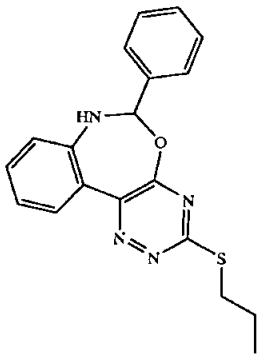
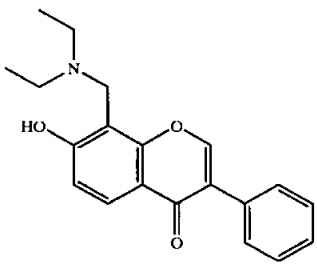
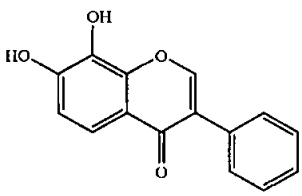
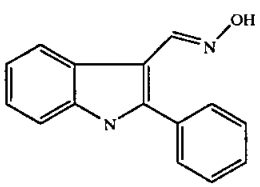
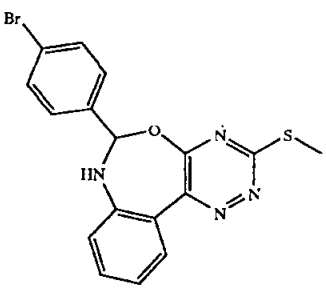
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KT |  | * | * | * | ** | ND | * |
| KU |  | * | * | ** | ** | ND | ** |
| KV |  | * | * | * | ** | ND | * |
| KW |  | * | * | * | ** | ND | ND |
| KX |  | * | ** | * | ** | ND | ND |

TABLE 5-continued

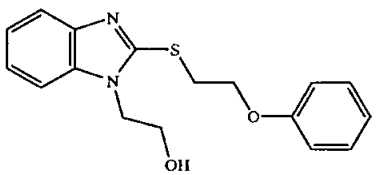
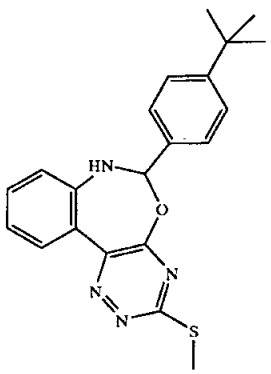
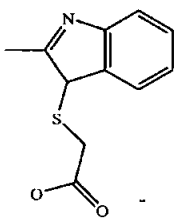
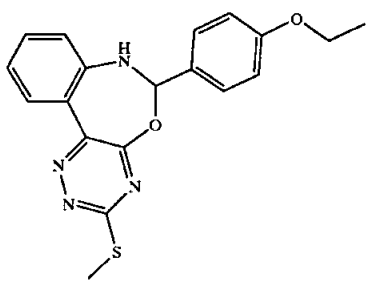
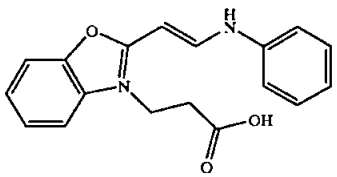
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| KY |  | • | •• | • | •• | ND | •• |
| KZ |  | •• | • | •• | •• | ND | ND |
| LA |  | • | • | • | •• | ND | ND |
| LB |  | • | • | • | •• | ND | ND |
| LC |  | • | • | • | •• | ND | ND |

TABLE 5-continued

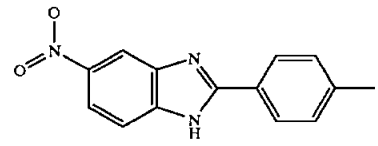
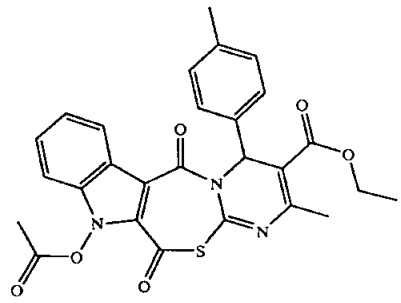
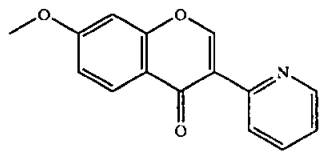
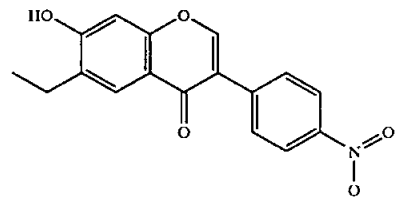
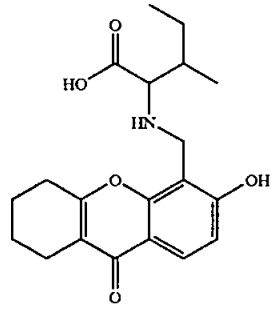
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| LD |  | * | * | * | ** | ND | * |
| LE |  | * | * | * | ** | ND | ND |
| LF |  | * | * | ** | ** | ND | ND |
| LG |  | * | * | * | ** | ND | * |
| LH |  | ** | * | * | ** | ND | * |

TABLE 5-continued

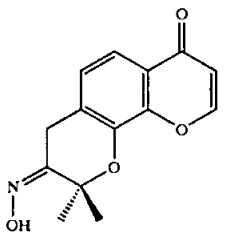
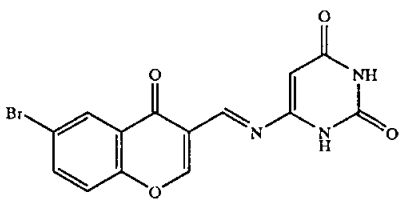
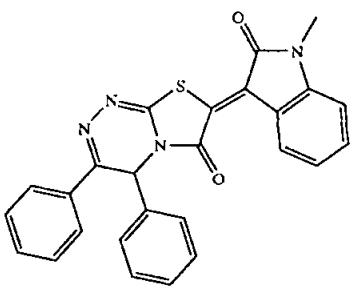
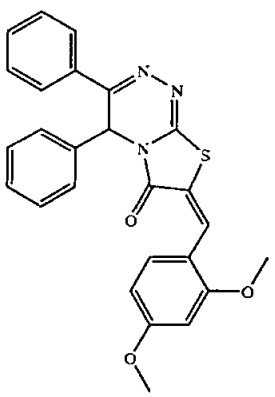
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| LI |  | * | * | * | ** | ND | * |
| LJ |  | * | ** | * | ** | * | ** |
| LK |  | * | * | * | ** | ** | * |
| LL |  | * | * | * | ** | ** | ** |

TABLE 5-continued

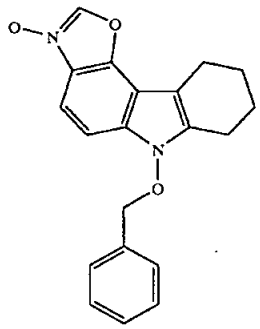
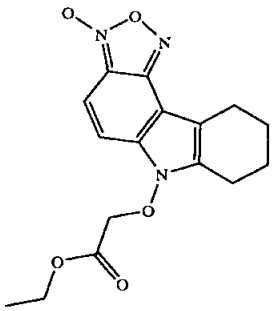
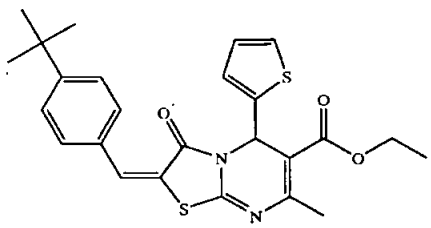
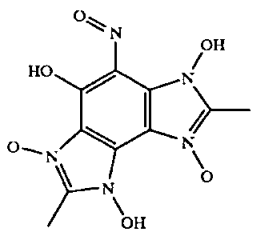
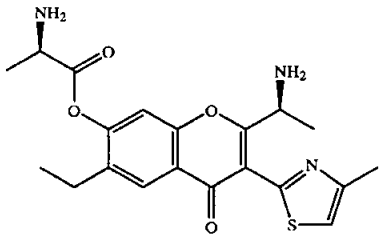
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| LM |  | ** | *** | * | ** | ** | ND |
| LN |  | ** | * | * | ** | ND | ** |
| LO |  | *** | *** | ** | ** | ND | ** |
| LP |  | *** | * | * | ** | ND | * |
| LQ |  | ** | * | *** | ** | ND | * |

TABLE 5-continued

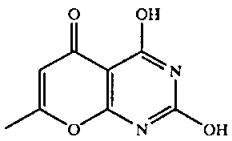
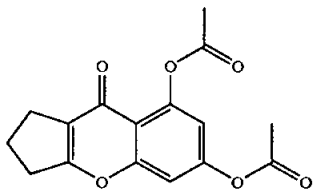
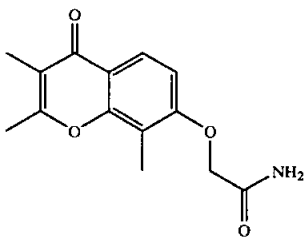
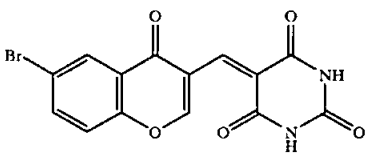
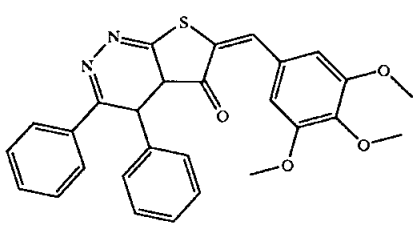
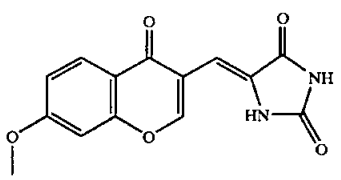
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| LR |  | * | * | * | ** | ND | ** |
| LS |  | ** | ** | * | ** | ND | * |
| LT |  | ** | ** | ** | ** | ND | ND |
| LU |  | *** | *** | NT | ** | ND | ND |
| LV |  | * | * | ** | ** | ** | * |
| LW |  | * | * | * | ** | ** | ** |

TABLE 5-continued

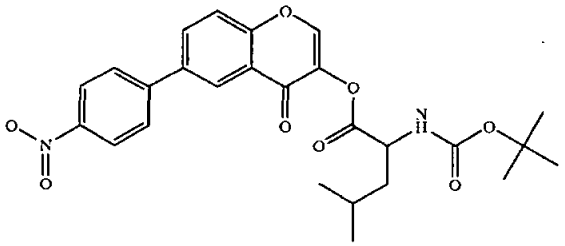
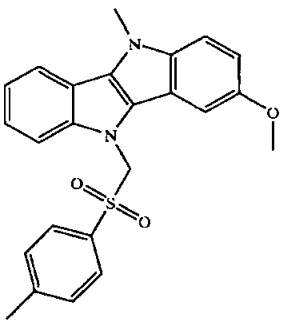
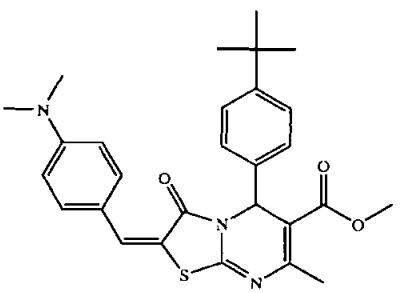
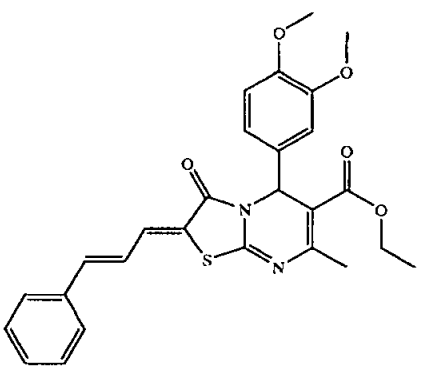
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| LX |  | *** | * | * | ** | ** | ** |
| LY |  | * | * | * | ** | ** | ** |
| LZ |  | * | * | * | ** | ** | ** |
| MA |  | * | NT | NT | ** | * | * |

TABLE 5-continued

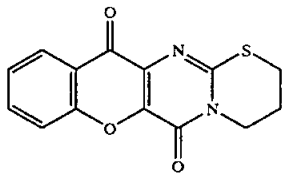
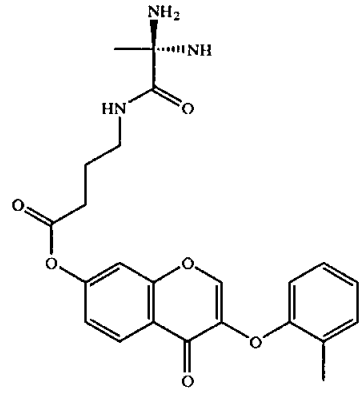
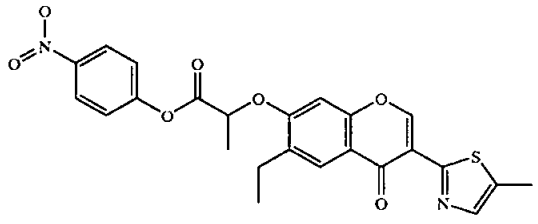
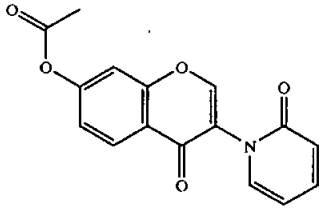
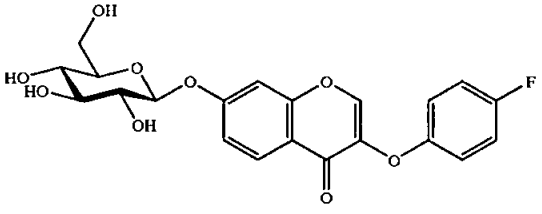
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| MB |  | * | NT | NT | ** | ** | ND |
| MC |  | * | ** | * | ** | * | * |
| MD |  | *** | *** | ** | ** | ** | * |
| ME |  | *** | *** | ** | ** | ** | * |
| MF |  | *** | *** | *** | ** | ** | ND |

TABLE 5-continued

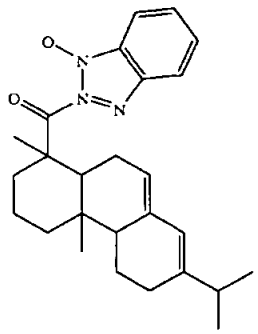
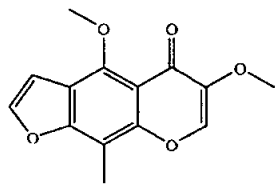
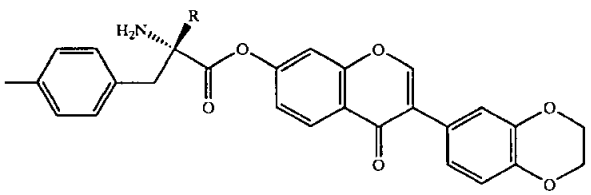
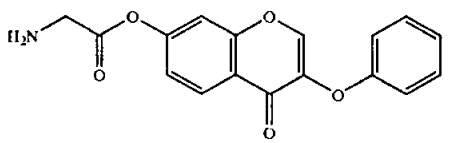
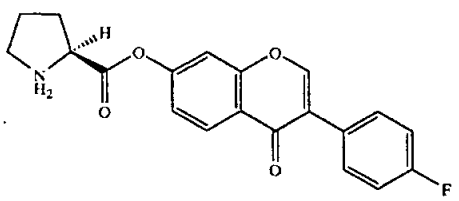
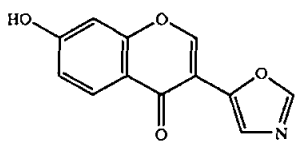
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| MG |  | * | ** | ** | ** | ** | * |
| MH |  | *** | *** | *** | ** | ** | * |
| MI |  | NT | * | * | ** | ND | ** |
| MJ |  | NT | * | * | ** | ND | * |
| MK |  | NT | ** | ** | ** | ND | ND |
| ML |  | NT | ** | *** | ** | ND | ** |

TABLE 5-continued

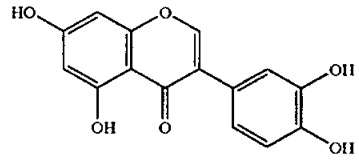
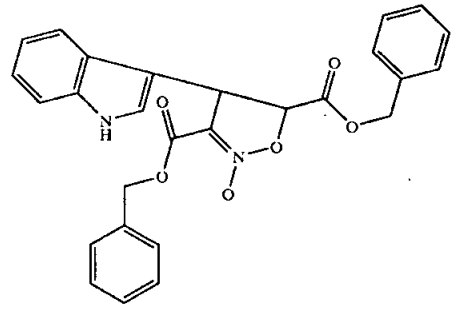
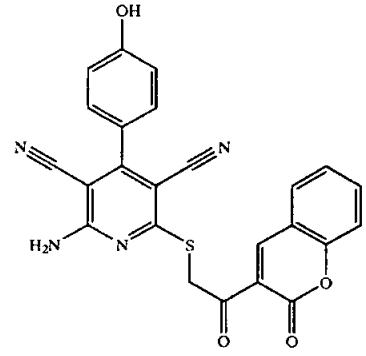
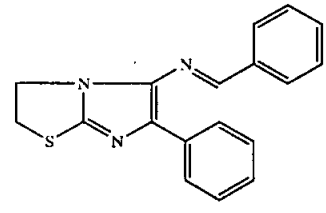
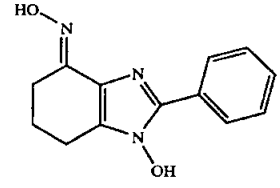
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| MM |  | NT | *** | *** | ** | ND | ND |
| MN |  | * | ** | * | ** | * | ND |
| MO |  | * | * | * | ** | ** | ** |
| MP |  | * | ** | * | ** | ** | ** |
| MQ |  | * | *** | * | ** | ** | ND |

TABLE 5-continued

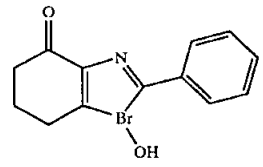
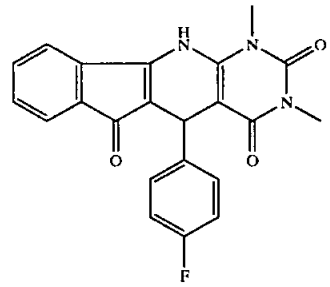
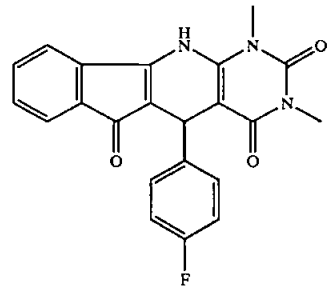
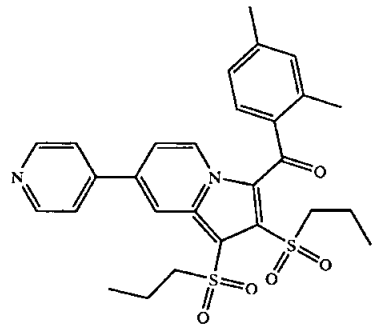
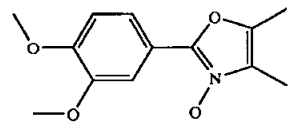
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| MR |  | ** | NT | NT | ** | ND | ** |
| MS |  | * | NT | NT | ** | ND | ND |
| MT |  | * | NT | NT | ** | ND | ND |
| MU |  | ** | * | * | ** | ND | * |
| MV |  | * | * | * | ** | ND | ** |

TABLE 5-continued

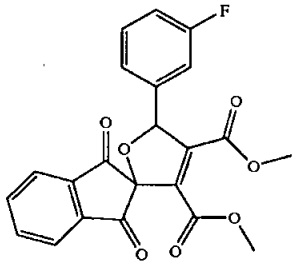
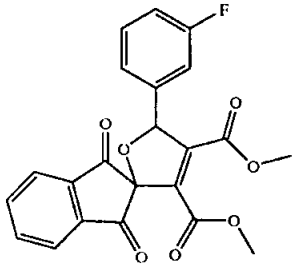
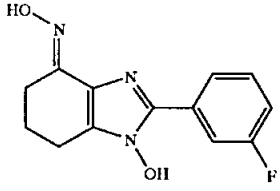
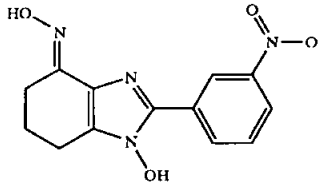
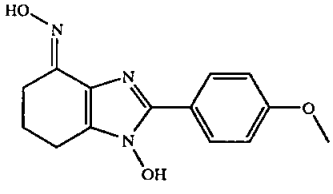
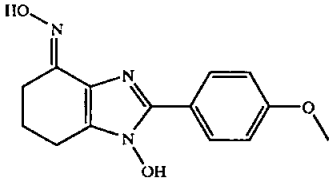
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| MX |  | *** | *** | ** | ** | ND | ND |
| MY |  | *** | * | * | ** | ND | ** |
| MZ |  | * | * | * | ** | ND | * |
| NA |  | *** | * | * | ** | ND | * |
| NB |  | ** | * | ** | ** | ND | * |
| NC |  | * | * | * | ** | ND | * |

TABLE 5-continued

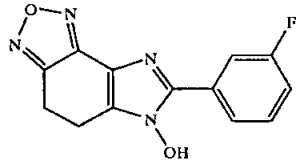
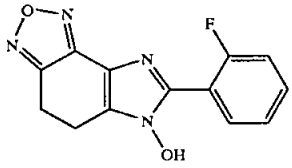
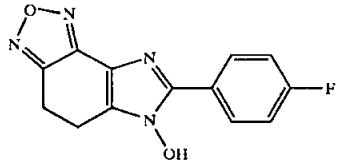
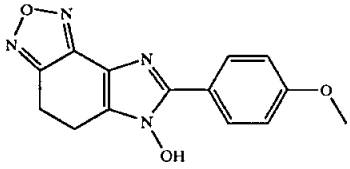
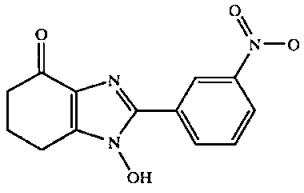
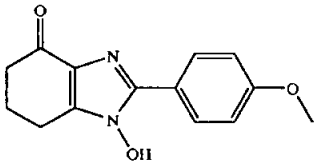
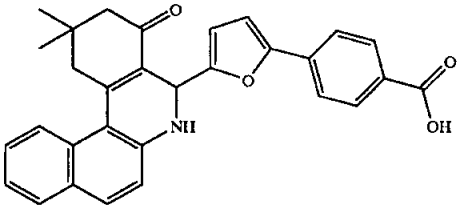
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ND |  | *** | * | * | ** | ND | ND |
| NE |  | * | * | * | ** | ND | ** |
| NF |  | *** | ** | * | ** | ND | ** |
| NG |  | * | * | * | ** | ND | ** |
| NH |  | * | * | * | ** | ND | ND |
| NI |  | * | * | * | ** | ND | ND |
| NJ |  | * | * | * | ** | ND | ND |

TABLE 5-continued

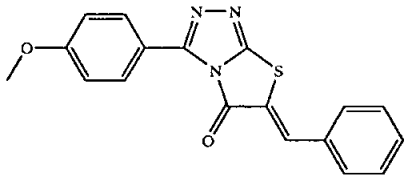
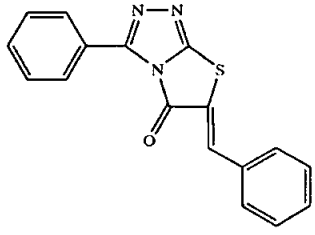
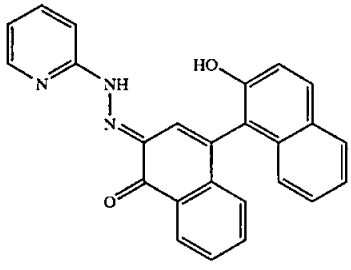
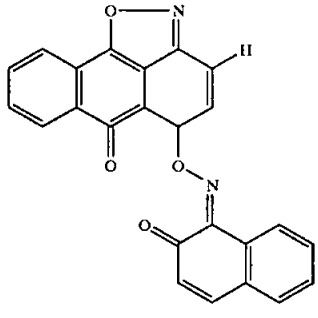
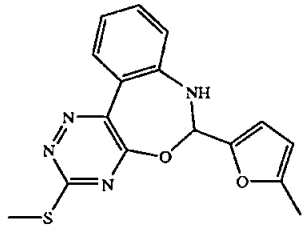
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| NK |  | * | * | * | ** | ND | ND |
| NL |  | * | * | *** | ** | ND | * |
| NM |  | * | ** | * | ** | ND | ** |
| NN |  | * | * | ** | ** | ND | * |
| NO |  | * | ** | ** | ** | ND | * |

TABLE 5-continued

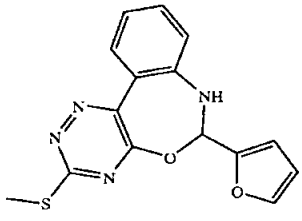
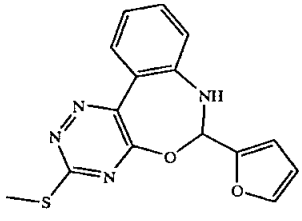
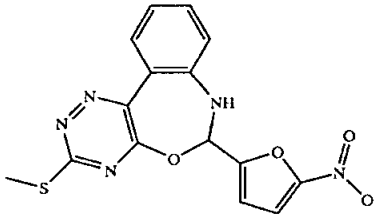
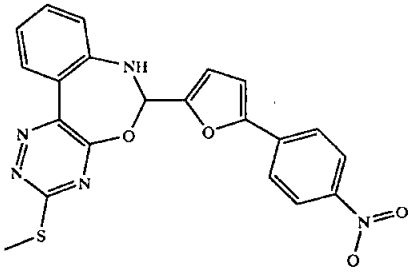
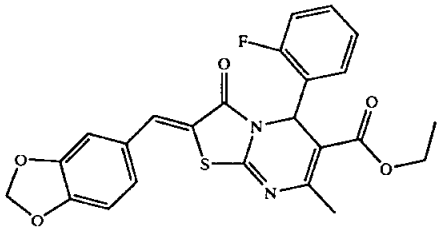
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| NP |  | * | ** | * | ** | ND | ND |
| NQ |  | * | * | * | ** | ND | ND |
| NR |  | * | * | * | ** | ND | ND |
| NS |  | * | * | * | ** | ND | ND |
| NT |  | * | * | * | ** | ND | ND |

TABLE 5-continued

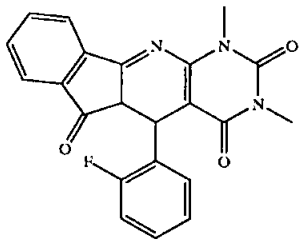
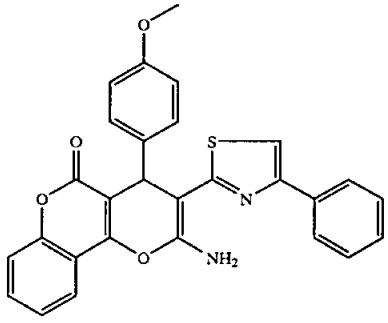
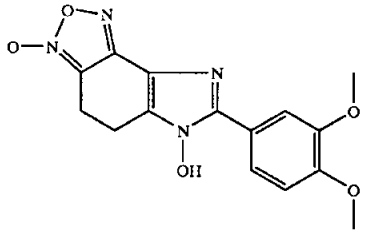
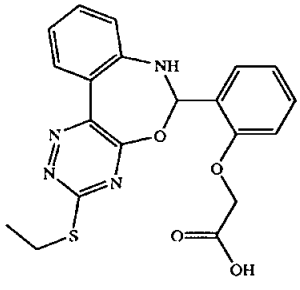
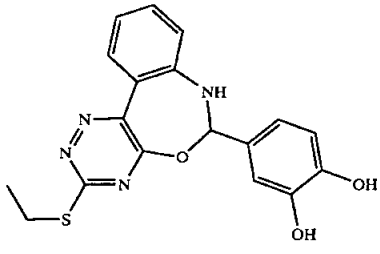
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| NU |  | * | * | * | ** | ND | ND |
| NV |  | * | * | * | ** | ND | ND |
| NW |  | * | * | * | ** | ND | * |
| NX |  | ** | * | * | ** | ND | ** |
| NY |  | * | * | * | ** | ND | * |

TABLE 5-continued

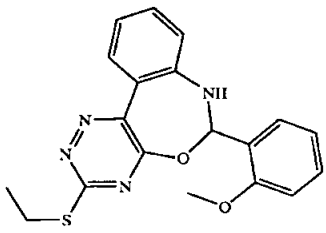
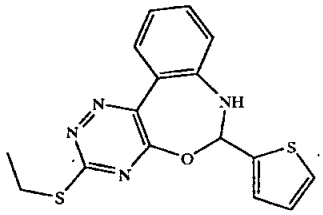
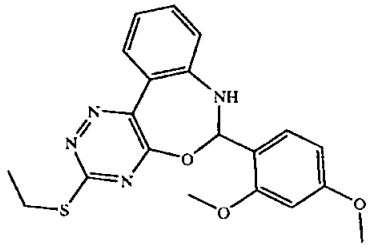
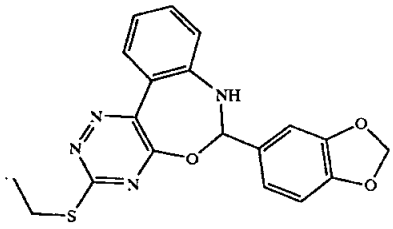
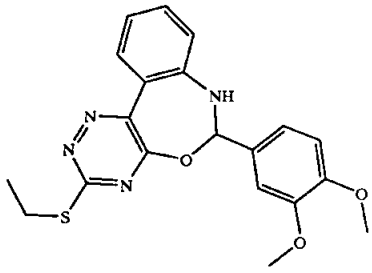
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| NZ |  | * | * | * | ** | ND | ND |
| OA |  | *** | *** | * | ** | ND | ND |
| OB |  | * | * | * | ** | ND | ** |
| OC |  | ** | * | * | ** | ND | ND |
| OD |  | *** | * | ** | ** | ND | * |

TABLE 5-continued

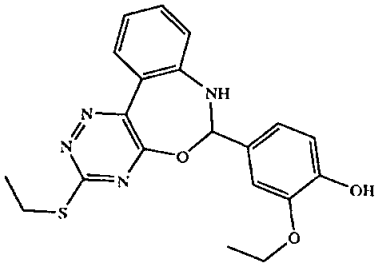
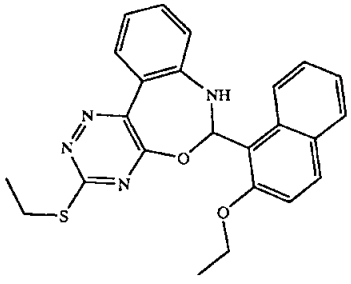
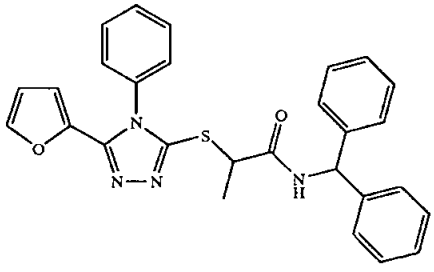
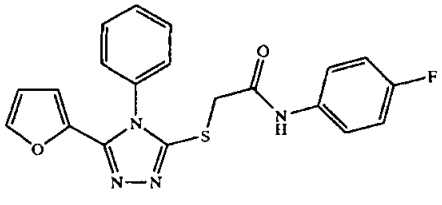
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| OE |  | * | * | * | ** | ND | ND |
| OF |  | * | * | *** | ** | * | ND |
| OG |  | * | * | * | ** | * | ** |
| OH |  | * | * | * | ** | * | ** |

TABLE 5-continued

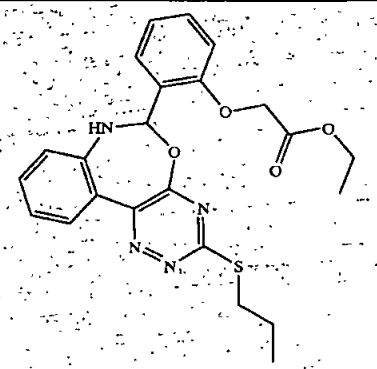
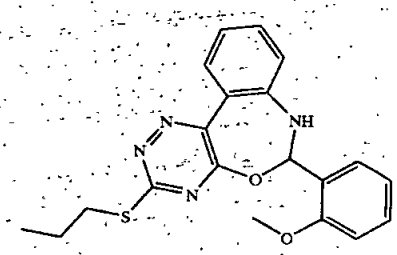
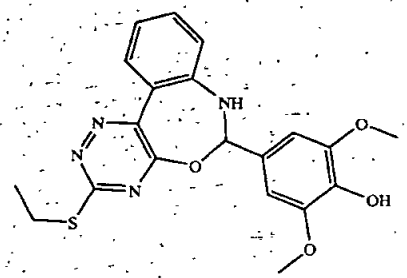
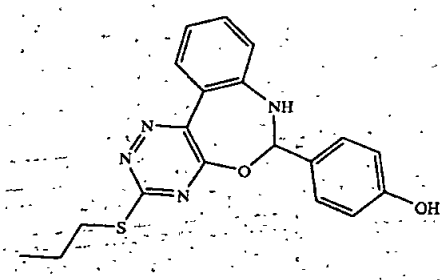
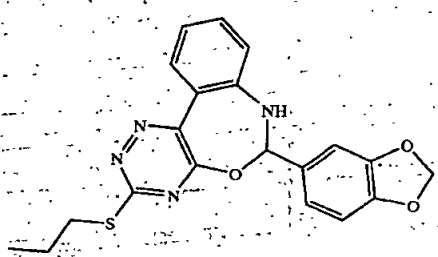
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| OI |  | * | ** | ** | ** | ** | ND |
| OJ |  | * | ** | ** | ** | * | ND |
| OK |  | *** | *** | ** | ** | ** | ND |
| OL |  | * | ** | *** | ** | ** | * |
| OM |  | * | ** | *** | ** | * | ND |

TABLE 5-continued

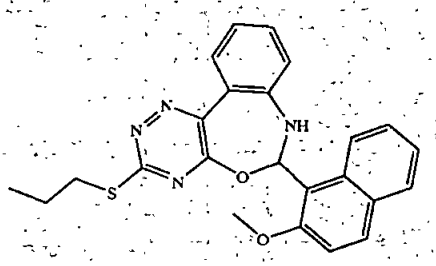
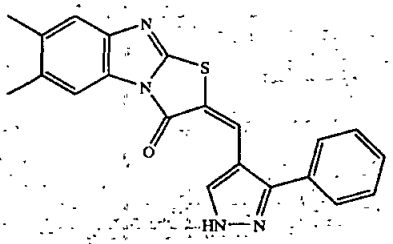
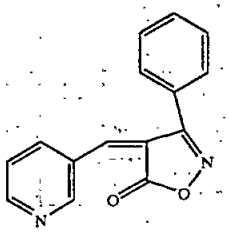
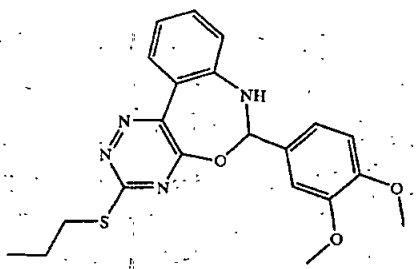
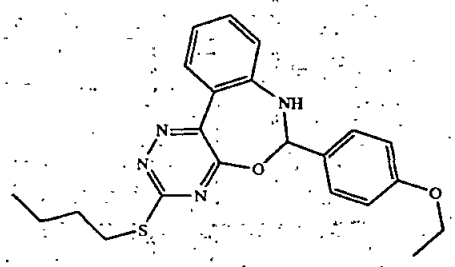
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ON |  | ** | * | *** | ** | ND | ** |
| OO |  | * | * | * | ** | ND | * |
| OP |  | * | * | * | ** | ND | ** |
| OQ |  | ** | * | * | ** | ND | * |
| OR |  | ** | * | * | ** | ND | ** |

TABLE 5-continued

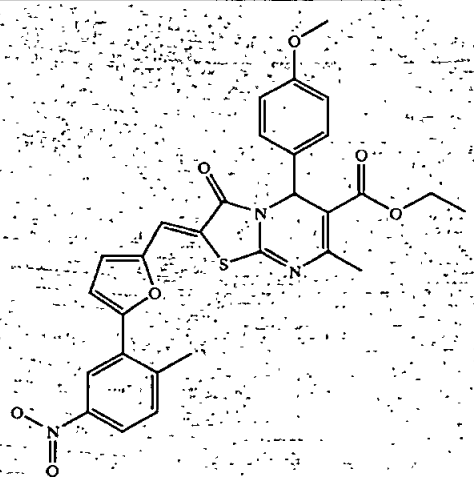
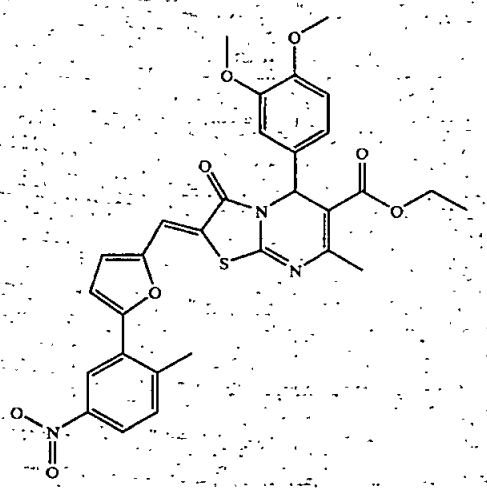
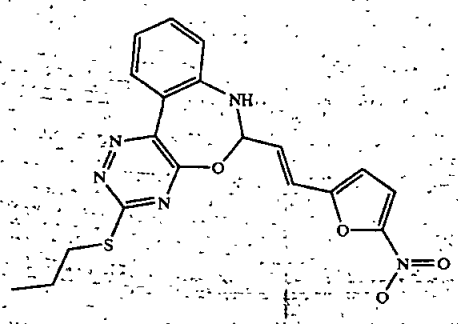
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| OS |  | ** | * | * | ** | ND | * |
| OT |  | ** | ** | ** | ** | ND | ** |
| OU |  | * | ** | ** | ** | ND | ND |

TABLE 5-continued

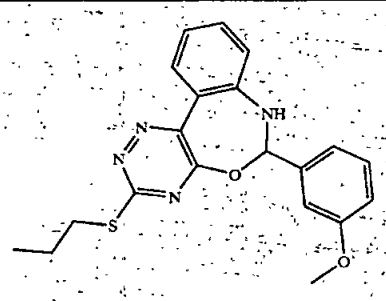
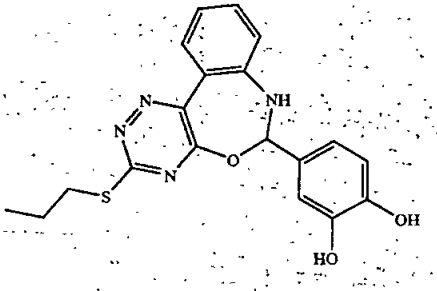
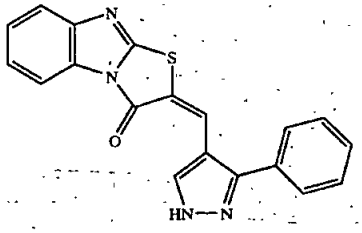
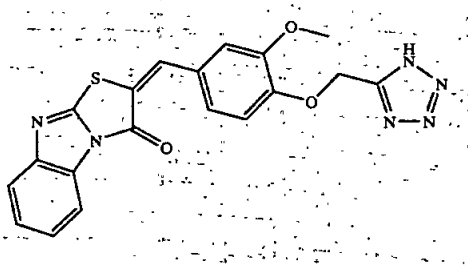
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| OV |  | *** | ** | NT | ** | ND | ** |
| OW |  | * | * | ** | ** | ** | ND |
| OX |  | * | * | * | ** | ** | * |
| OY |  | * | * | * | ** | ** | ND |

TABLE 5-continued

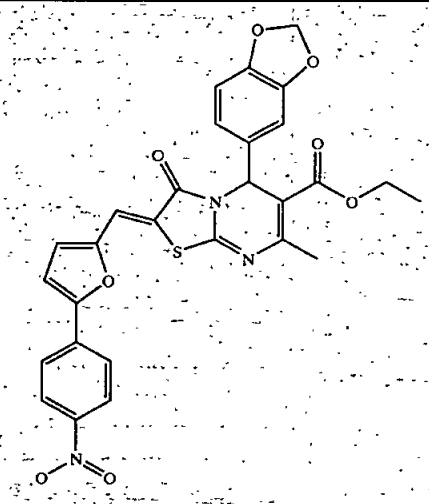
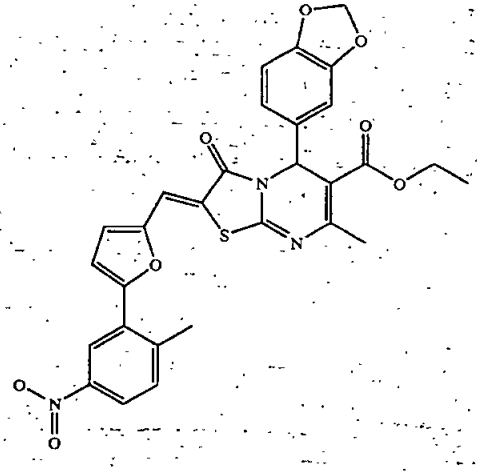
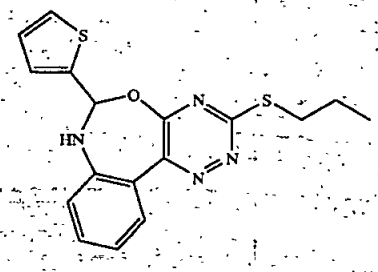
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| OZ |  | * | * | * | ** | * | ** |
| PA |  | *** | NT | NT | ** | ** | * |
| PB |  | *** | *** | ** | ** | ** | ND |

TABLE 5-continued

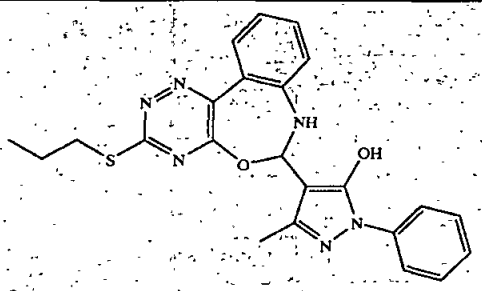
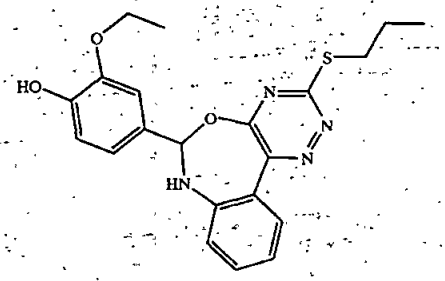
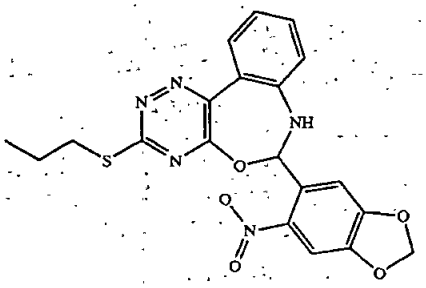
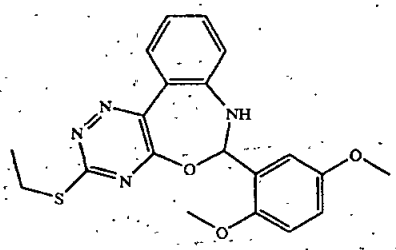
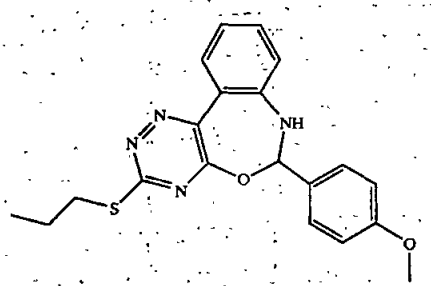
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PC |  | ** | *** | * | ** | * | ** |
| PD |  | NT | *** | * | ** | ND | ND |
| PD |  | NT | *** | * | ** | ND | ** |
| PE |  | NT | ** | * | ** | ND | ND |
| PF |  | NT | *** | ** | ** | ND | * |

TABLE 5-continued

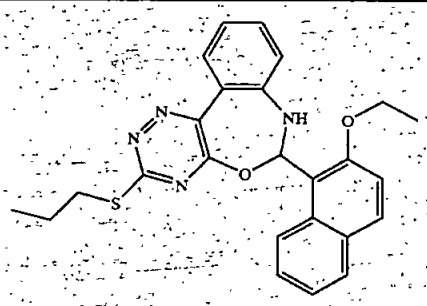
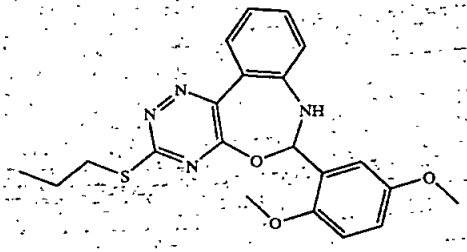
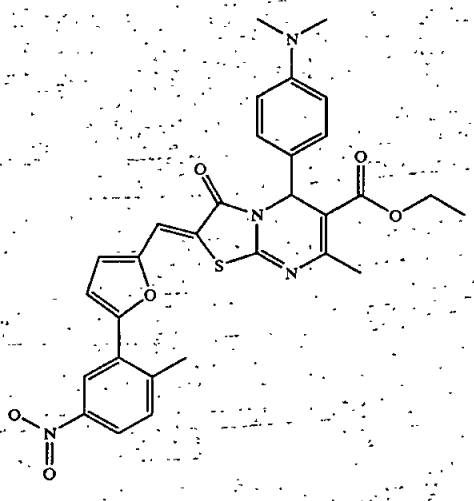
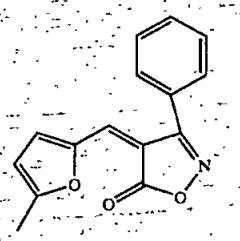
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PG |  | NT | * | * | ** | ND | ND |
| PH |  | NT | * | * | ** | ND | ND |
| PI |  | NT | ** | * | ** | ND | ND |
| PJ |  | NT | * | ** | ** | ND | ** |

TABLE 5-continued

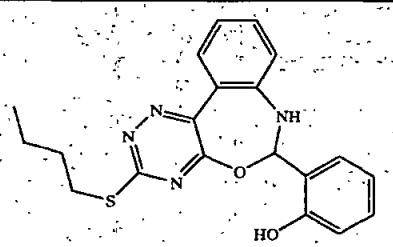
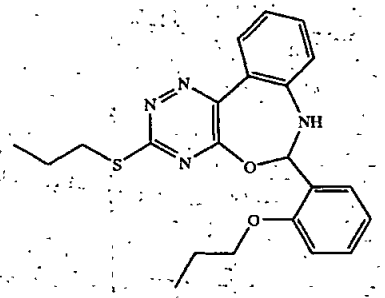
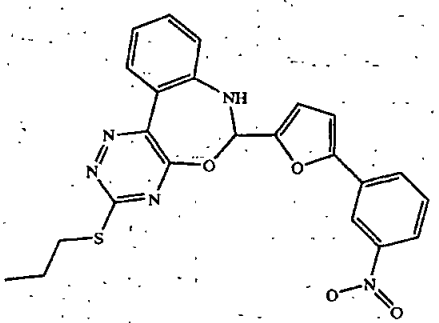
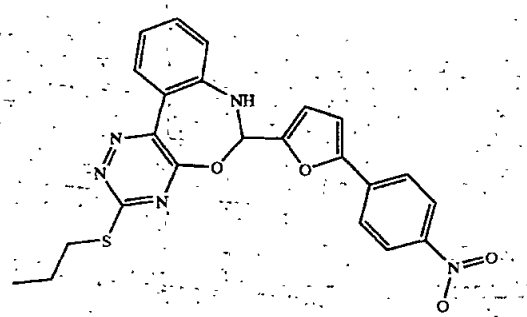
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PK |  | * | ** | * | ** | ** | ** |
| PL |  | * | ** | * | ** | ** | ** |
| PM |  | * | ** | * | ** | * | ** |
| PN |  | * | ** | ** | ** | ** | * |

TABLE 5-continued

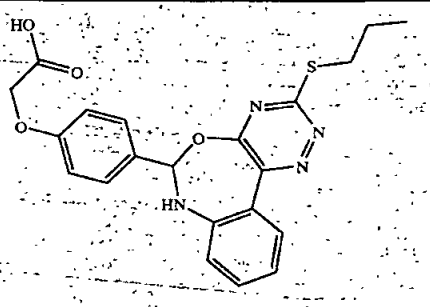
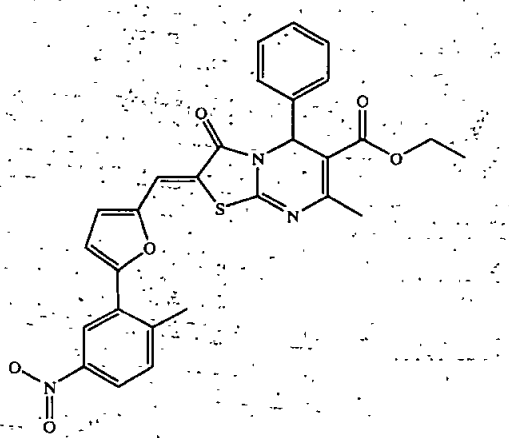
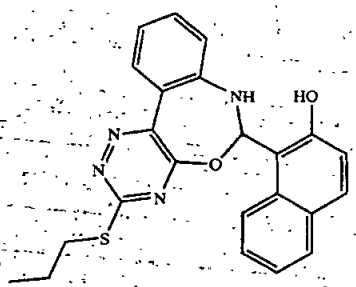
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PO |  | * | * | * | ** | * | ND |
| PP |  | * | NT | NT | ** | ND | ND |
| PQ |  | ** | NT | NT | ** | ND | ND |

TABLE 5-continued

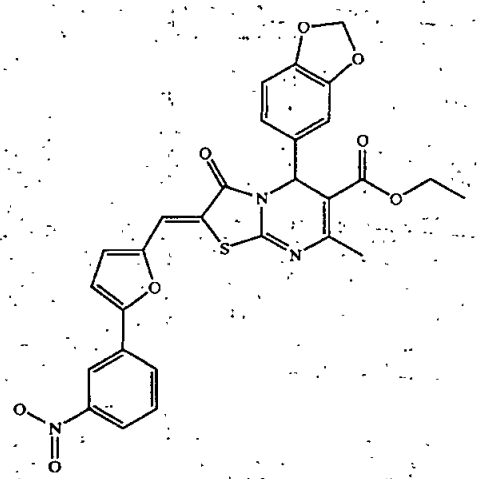
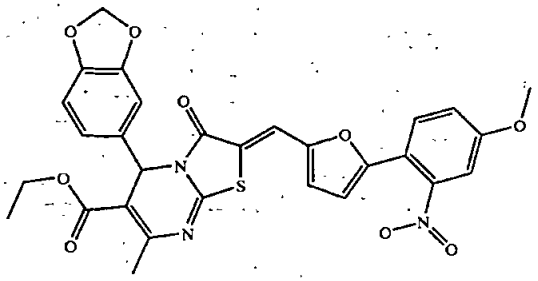
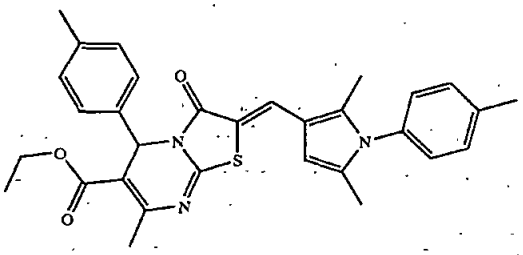
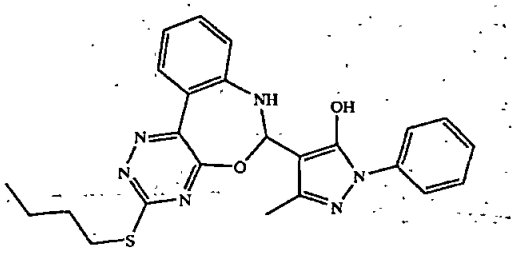
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PR |  | *** | NT | NT | ** | ND | ND |
| PS |  | * | NT | NT | ** | ND | ** |
| PT |  | * | * | * | ** | ND | ND |
| PU |  | * | * | * | ** | ND | ** |

TABLE 5-continued

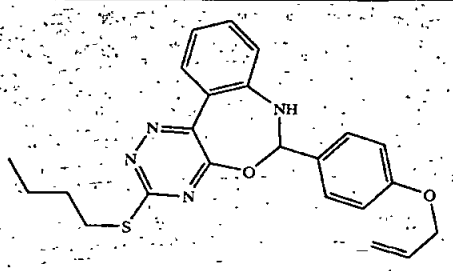
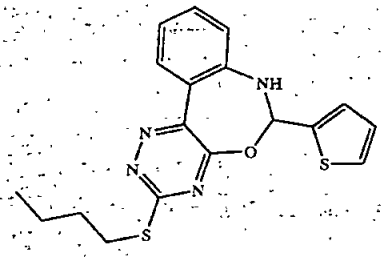
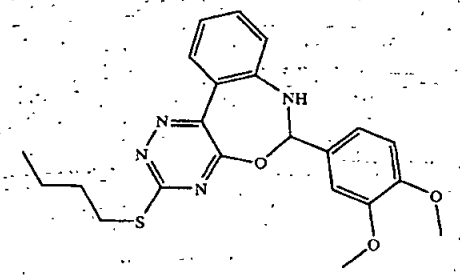
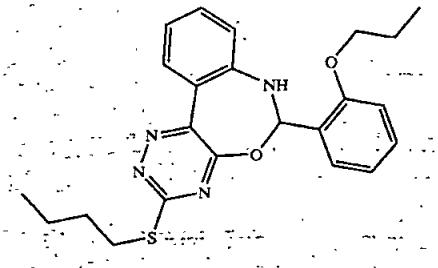
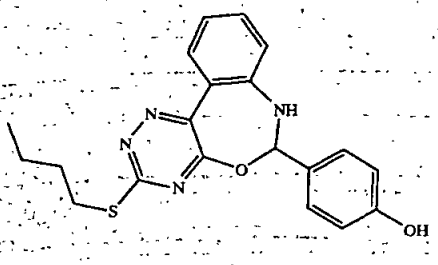
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| PV |  | * | ** | * | ** | ND | ** |
| PW |  | ** | * | * | ** | ND | * |
| PX |  | * | * | * | ** | ND | ** |
| PY |  | * | * | * | ** | ND | ** |
| PZ |  | * | * | * | ** | ND | ** |

TABLE 5-continued

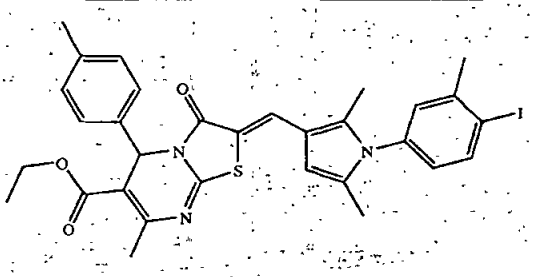
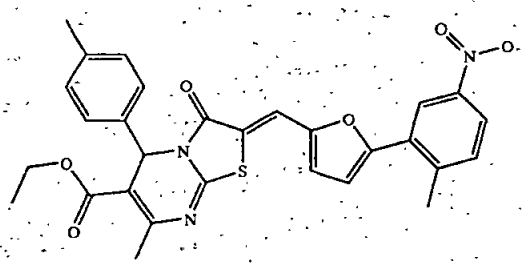
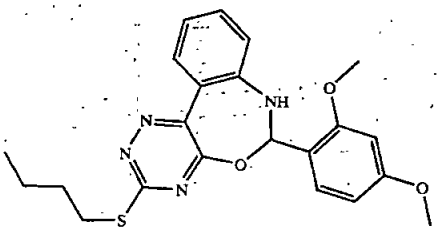
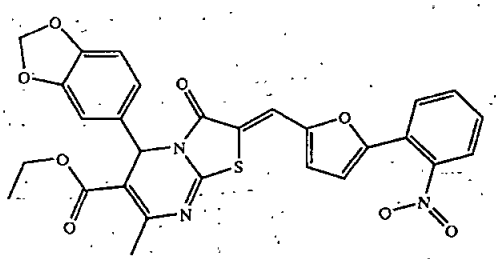
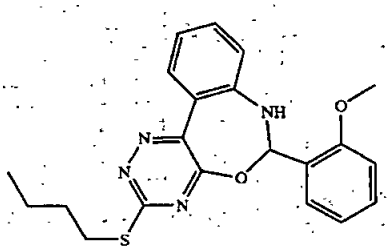
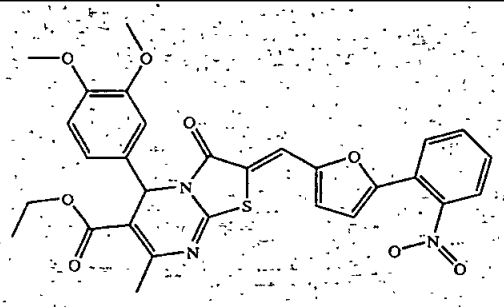
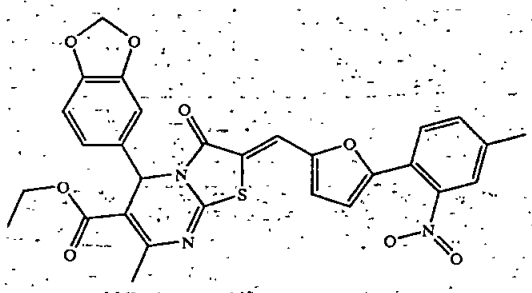
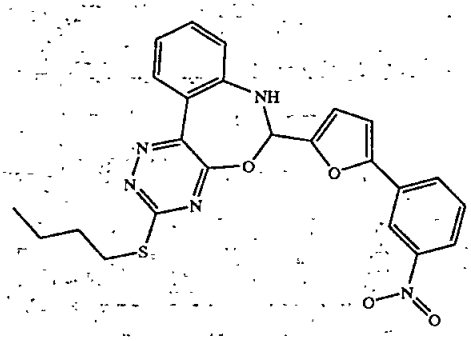
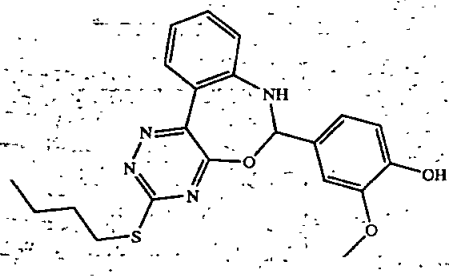
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QA |  | * | * | ** | ** | ND | ** |
| QB |  | *** | * | ** | ** | ND | * |
| QC |  | *** | * | * | ** | ND | ND |
| QD |  | * | * | * | ** | ND | ND |
| QE |  | * | * | * | ** | ND | * |

TABLE 5-continued

| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QF |  | *** | * | * | ** | ND | ND |
| QG |  | * | * | * | ** | ND | ** |
| QH |  | * | * | * | ** | ND | * |
| QI |  | *** | *** | *** | ** | ND | * |

[0684]

TABLE 5

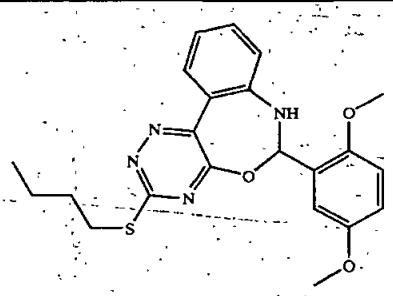
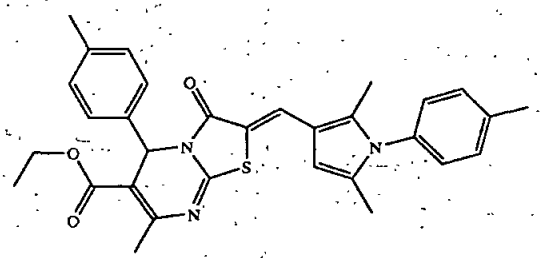
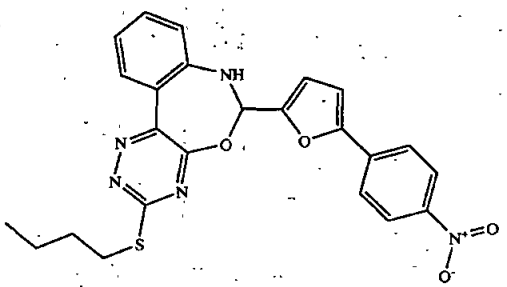
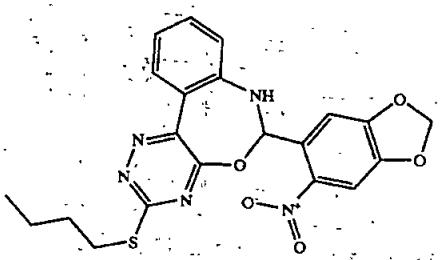
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QJ |  | * | * | ** | ** | ND | * |
| QK |  | * | * | * | ** | ND | ** |
| QL |  | ** | * | * | ** | ND | ND |
| QM |  | * | * | * | ** | ND | ND |

TABLE 5-continued

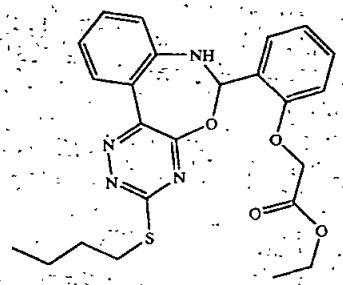
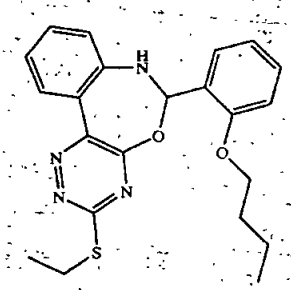
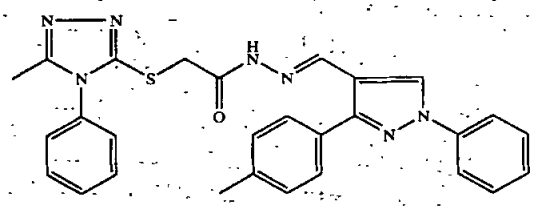
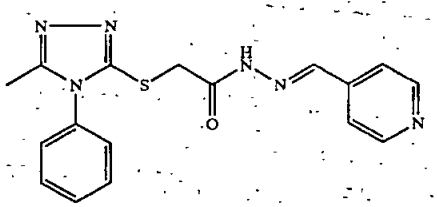
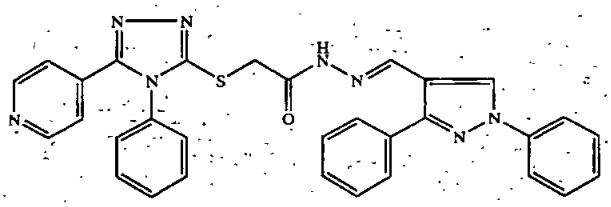
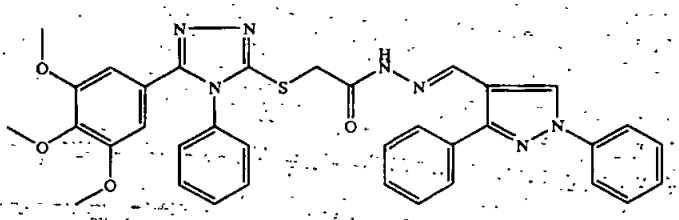
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QN |  | * | * | * | ** | ND | ND |
| QO |  | * | * | * | *** | ND | * |
| QP |  | * | ** | * | ** | ND | * |
| QQ |  | * | * | ** | *** | ND | ND |
| QR |  | ** | * | *** | ** | ND | ND |
| QS |  | * | ** | *** | ** | ND | ND |

TABLE 5-continued

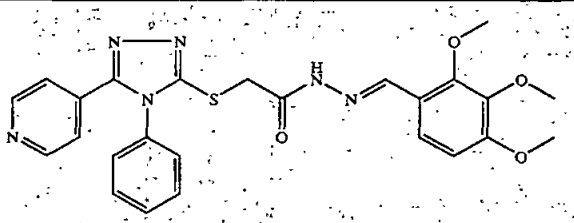
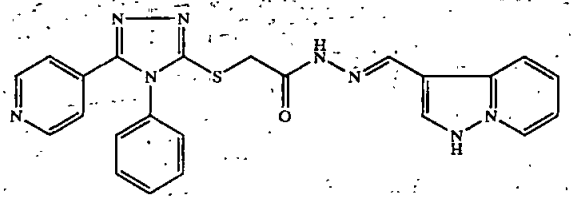
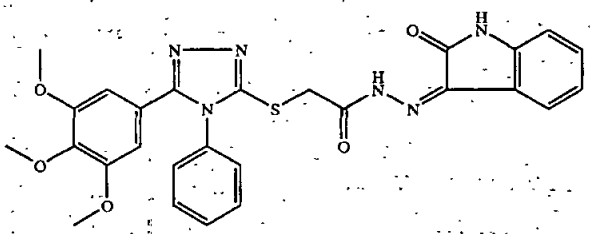
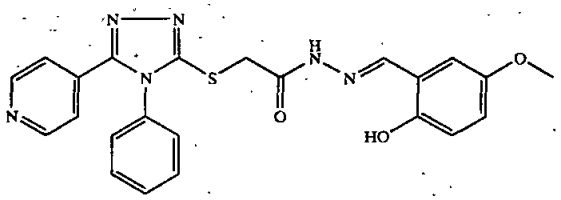
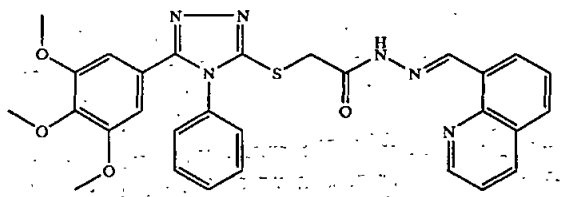
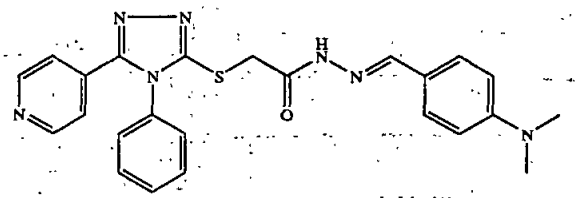
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QT |  | * | * | *** | ** | ND | ND |
| QU |  | ** | ** | ** | ** | ND | * |
| QV |  | * | ** | ** | ** | ND | * |
| QW |  | * | * | ** | ** | ND | ND |
| QX |  | ** | * | ** | ** | ND | ND |
| QY |  | * | * | ** | ** | ND | ND |

TABLE 5-continued

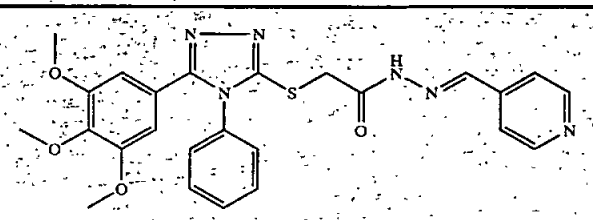
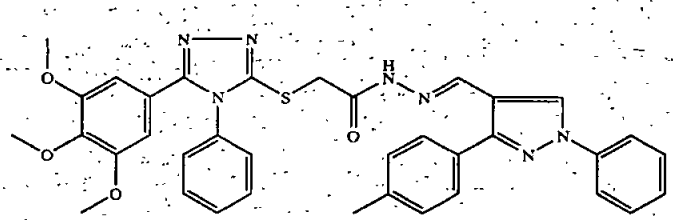
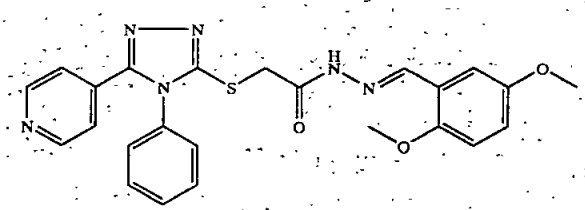
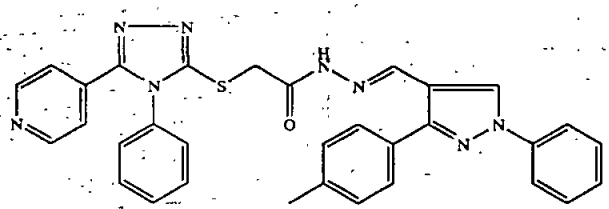
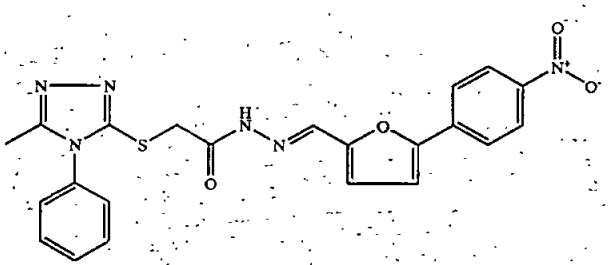
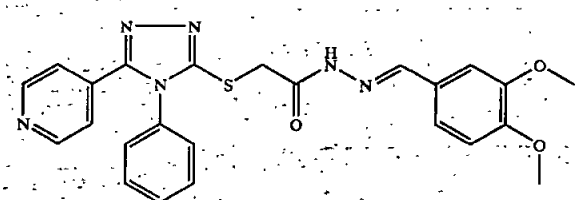
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| QZ |  | ** | * | * | ** | ND | ND |
| RA |  | ** | * | ** | ** | ND | ** |
| RB |  | * | * | * | ** | ND | * |
| RC |  | * | ** | * | ** | ND | ND |
| RD |  | ** | *** | *** | ** | * | ND |
| RE |  | * | ** | *** | ** | * | ND |

TABLE 5-continued

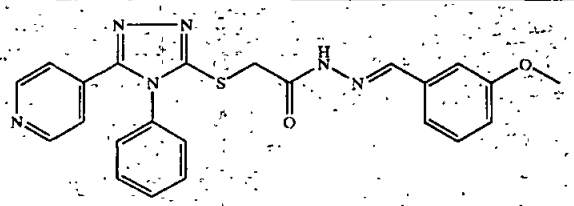
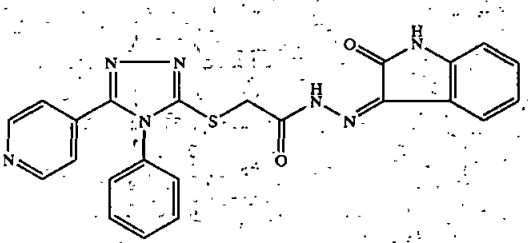
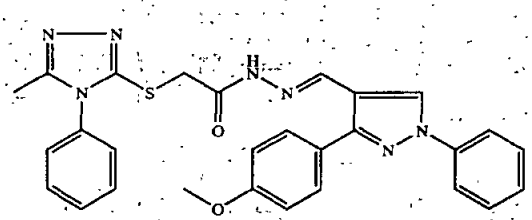
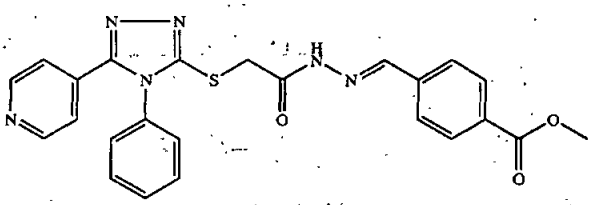
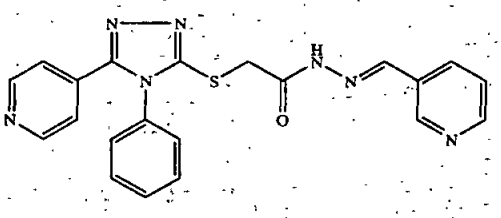
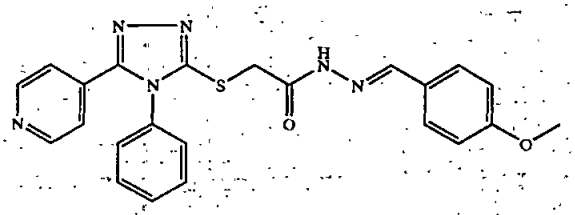
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| RF |  | ** | *** | ** | ** | * | ** |
| RG |  | * | ** | ** | ** | * | ** |
| RH |  | * | * | ** | ** | * | ** |
| RI |  | * | * | * | ** | * | ND |
| RJ |  | ** | ** | * | ** | * | ** |
| RK |  | * | *** | ** | ** | * | ** |

TABLE 5-continued

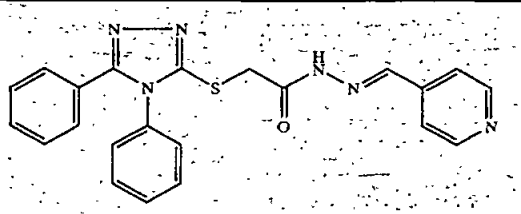
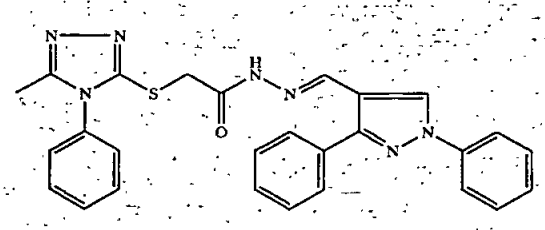
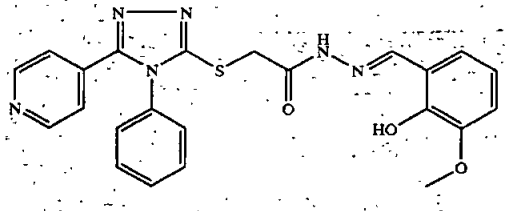
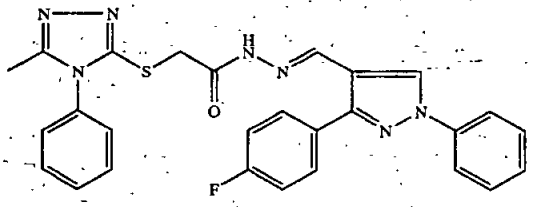
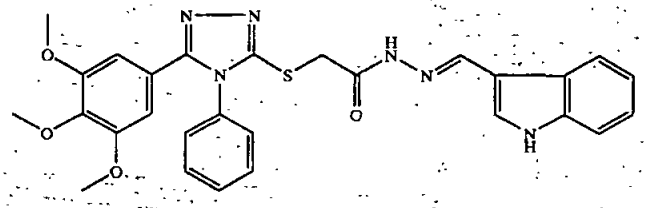
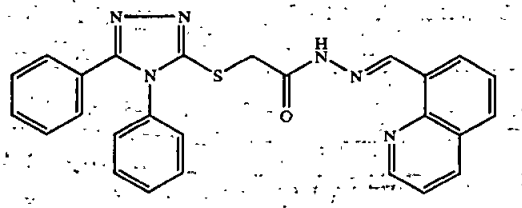
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc... |
|---|---|---|---|---|---|---|---|
| RL |  | ** | *** | *** | ** | ** | ND |
| RM |  | * | * | * | ** | * | ND |
| RN |  | * | * | ** | ** | * | * |
| RO |  | * | * | ** | ** | * | * |
| RP |  | ** | ** | *** | ** | * | ** |
| RQ |  | *** | *** | * | ** | * | ** |

TABLE 5-continued

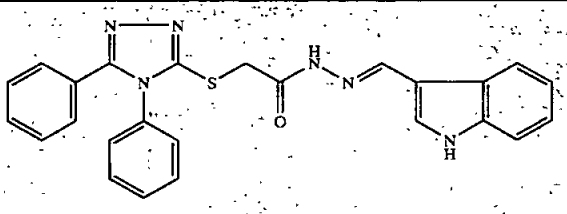
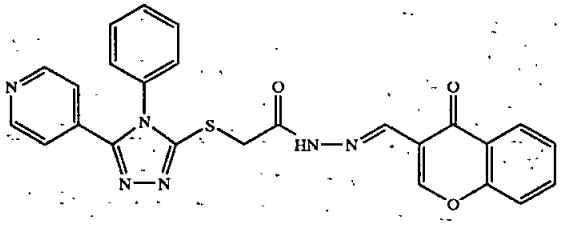
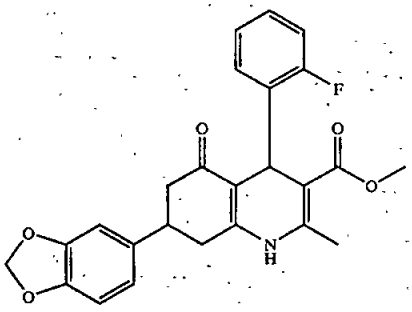
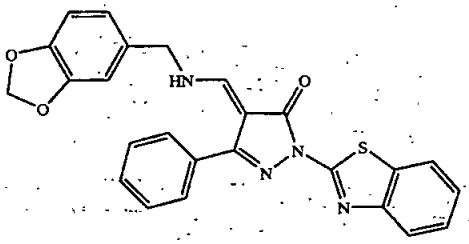
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| RS |  | *** | * | *** | ** | ND | ** |
| RT |  | *** | *** | *** | ** | ND | * |
| RU |  | *** | ** | ** | ** | ND | * |
| RV |  | ** | * | * | ** | ND | ** |

TABLE 5-continued

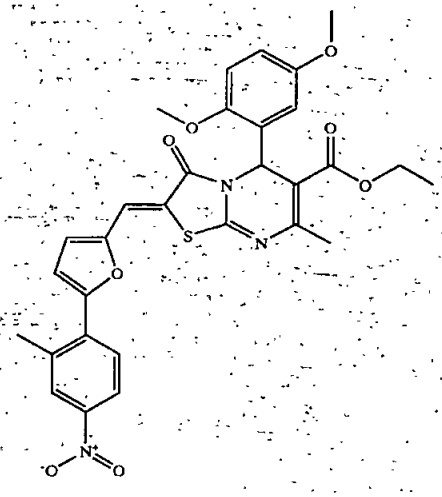
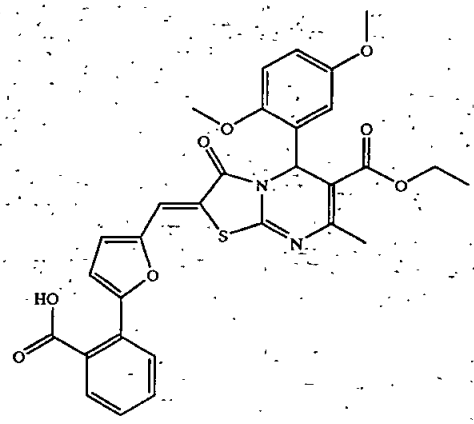
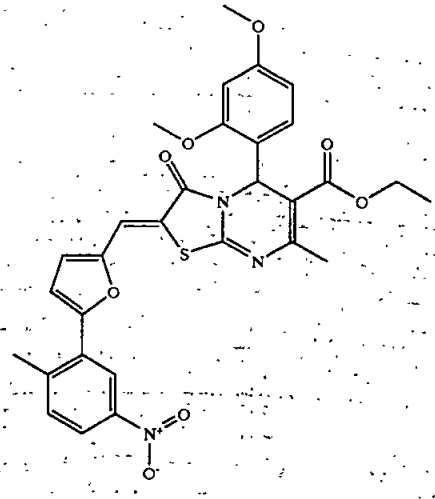
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| RW |  | ** | ** | * | ** | ND | ** |
| RX |  | ** | *** | * | ** | ND | ND |
| RY |  | ** | * | * | ** | ND | ** |

TABLE 5-continued

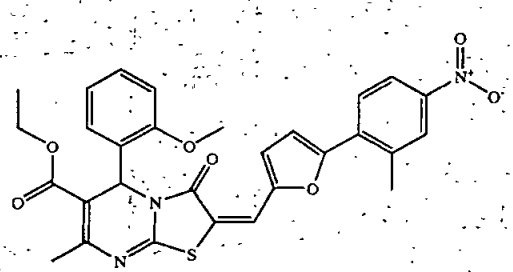
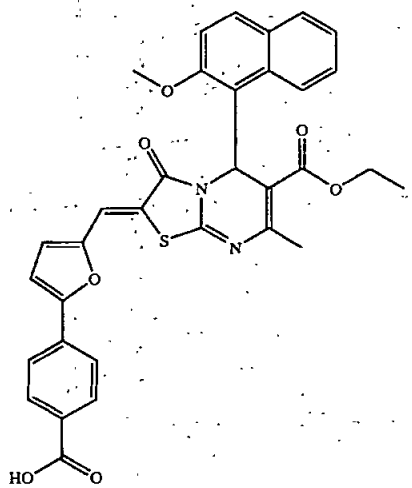
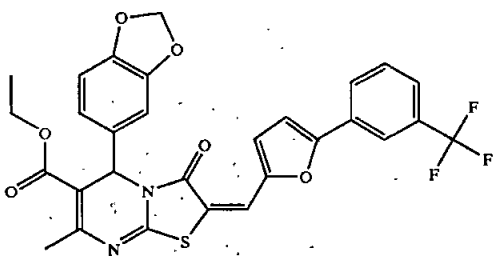
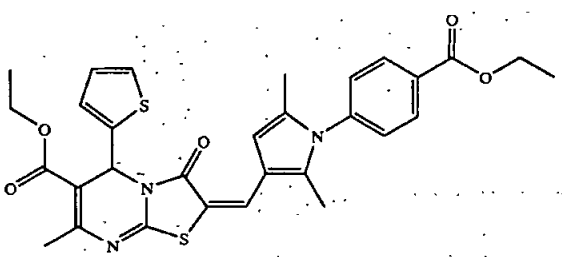
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| RZ |  | * | * | * | ** | ND | ** |
| SA |  | * | * | * | ** | ND | * |
| SB |  | ** | * | * | ** | ND | ** |
| SC |  | *** | * | * | ** | ND | ** |

TABLE 5-continued

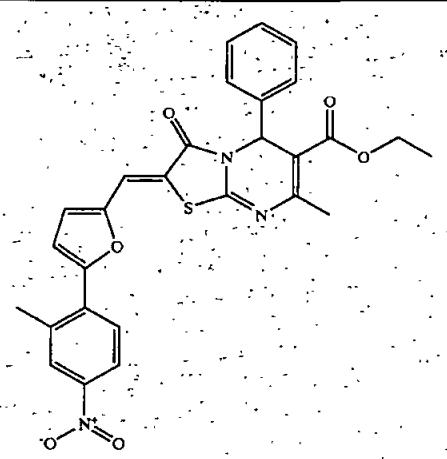
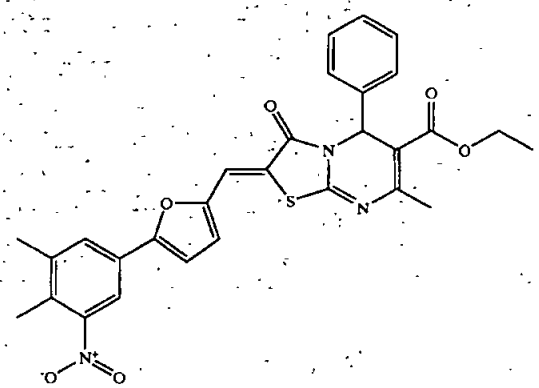
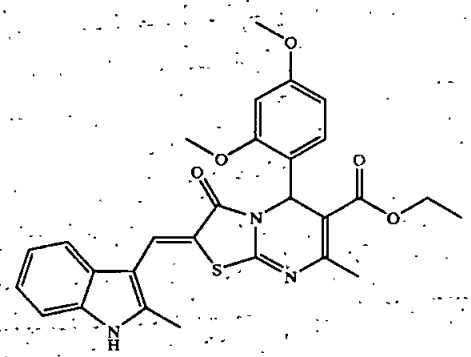
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SD |  | * | *** | * | ** | ND | * |
| SE |  | ** | * | * | ** | ND | ND |
| SF |  | * | * | ** | ** | ND | ND |

TABLE 5-continued

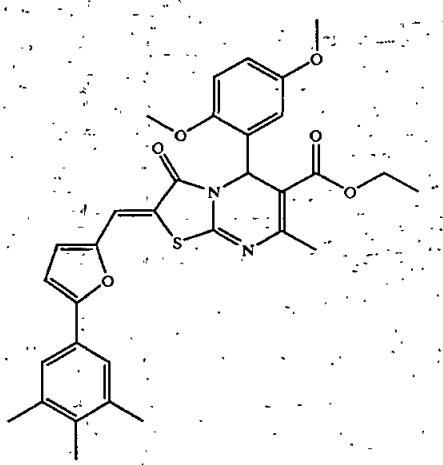
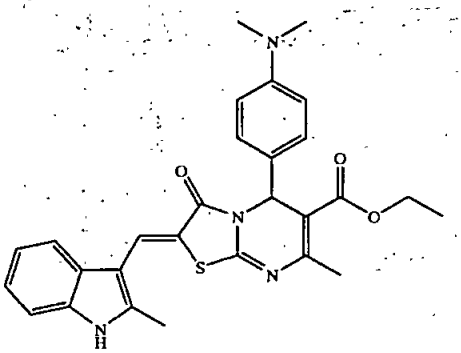
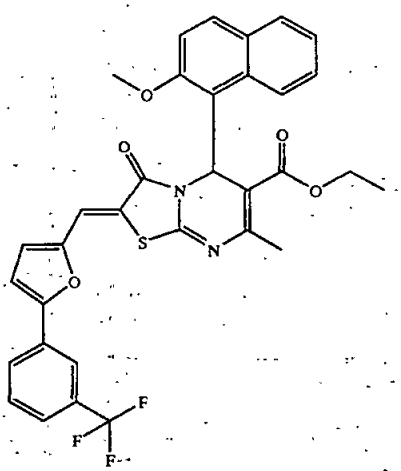
| ID. | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SG |  | ** | * | NT | ** | ND | * |
| SH |  | ** | ** | NT | ** | ND | ND |
| SI |  | ** | *** | NT | ** | ND | ND |

TABLE 5-continued

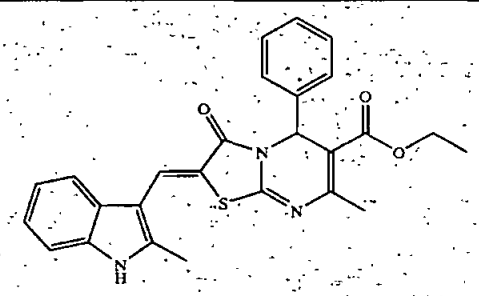
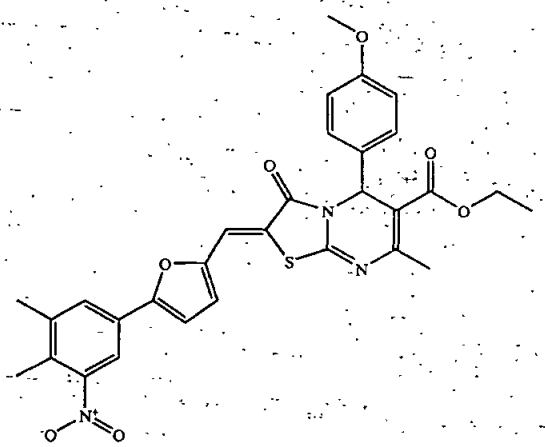
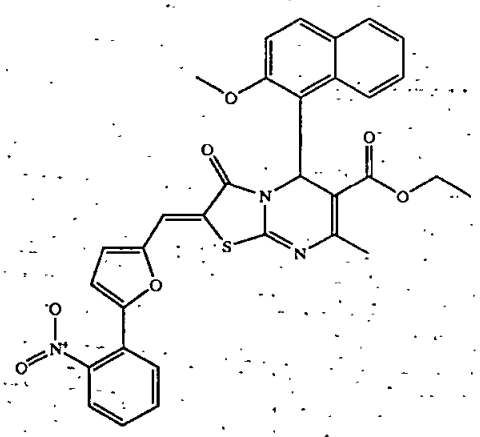
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SJ |  | * | *** | * | ** | ** | ND |
| SK |  | * | * | * | ** | ** | ND |
| SL |  | * | * | * | ** | ** | ND |

TABLE 5-continued

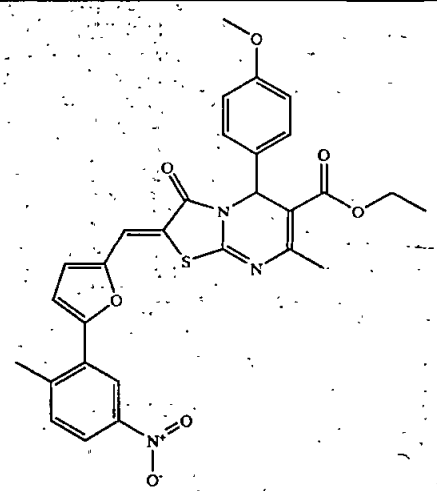
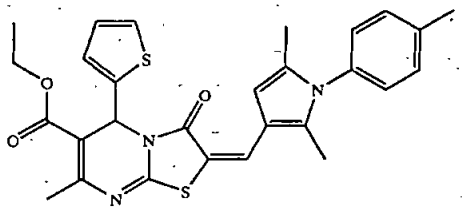
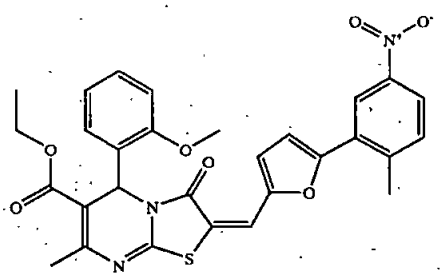
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SM |  | * | NT | NT | ** | * | ** |
| SN |  | * | NT | NT | ** | * | * |
| SO |  | * | NT | NT | ** | ** | * |

TABLE 5-continued

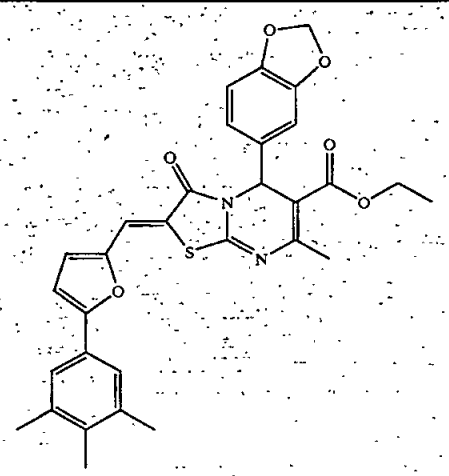
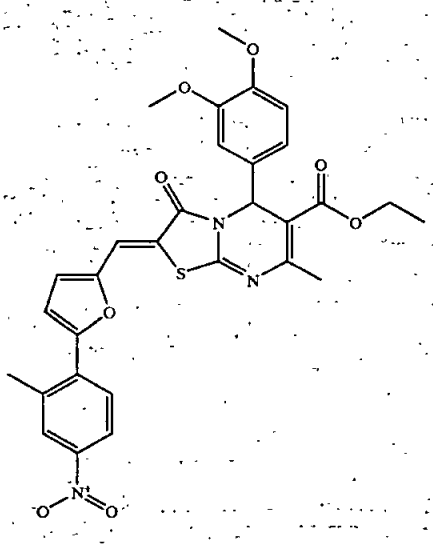
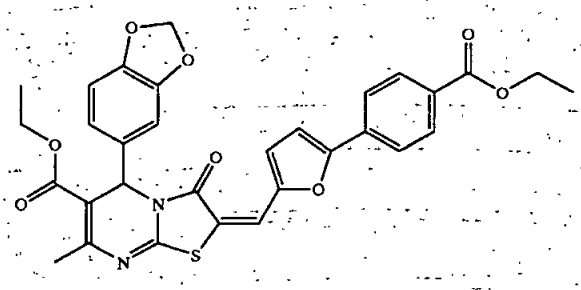
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SP |  | NT | ** | ** | ** | ND | ** |
| SQ |  | NT | ** | *** | ** | ND | ** |
| SR |  | NT | * | * | ** | ND | * |

TABLE 5-continued

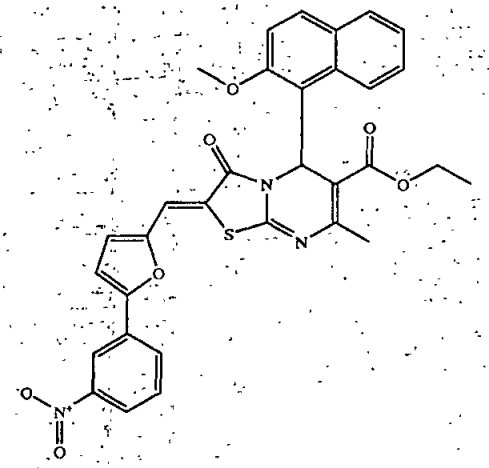
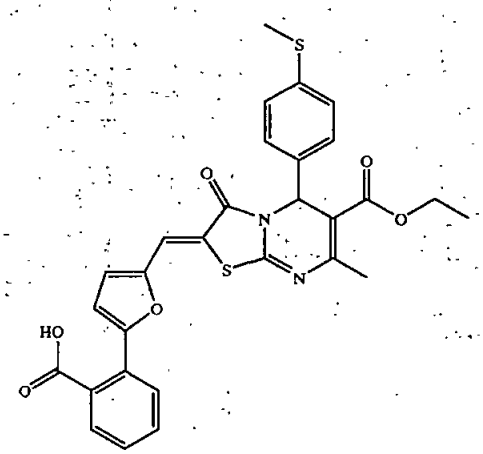
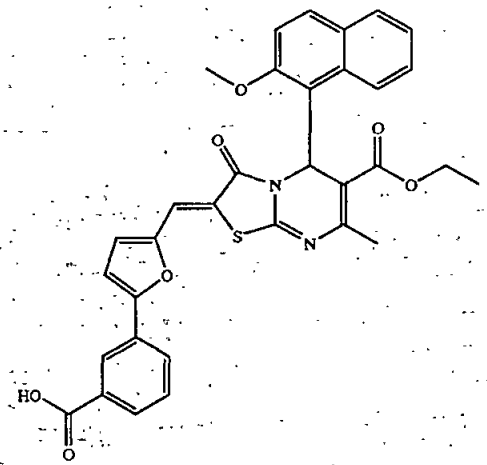
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SS |  | NT | * | * | ** | ND | ND |
| ST |  | NT | ** | * | ** | ND | ** |
| SU |  | NT | * | * | ** | ND | ** |

TABLE 5-continued

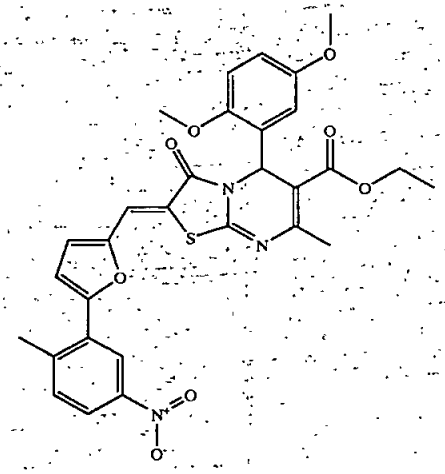
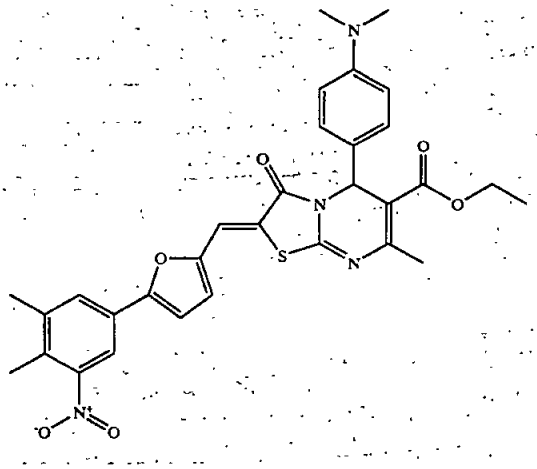
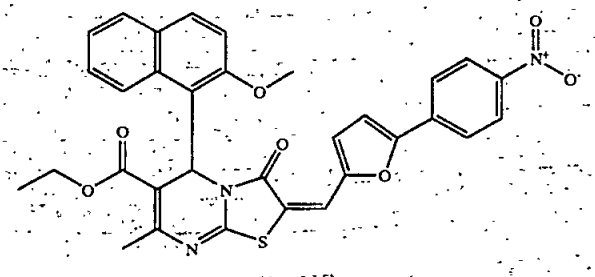
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SV |  | ** | ** | * | ** | ** | ** |
| SW |  | * | * | ** | ** | ** | * |
| SX |  | * | NT | NT | ** | ND | * |

TABLE 5-continued

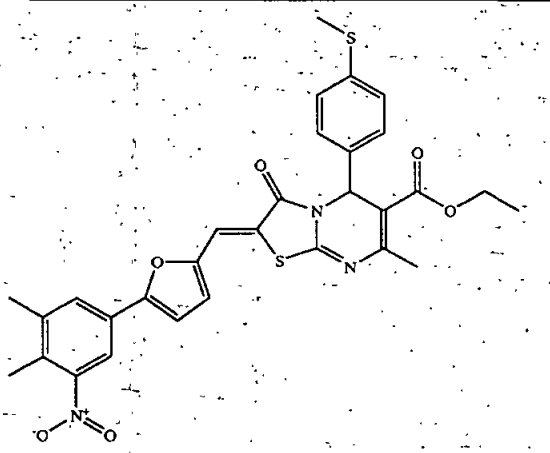
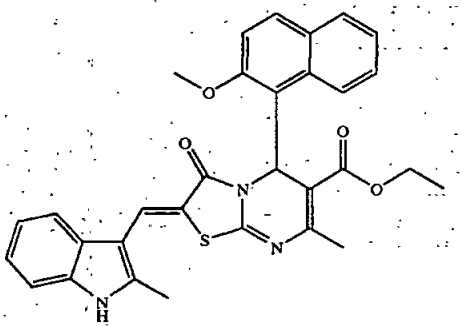
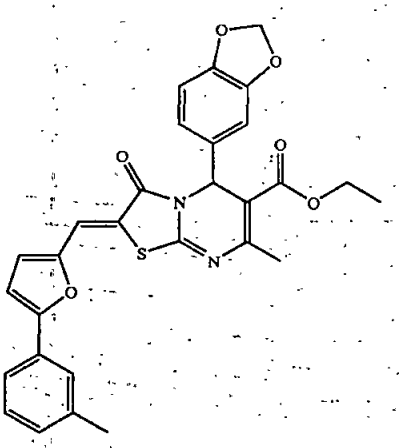
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| SY |  | *** | * | * | ** | ND | ND |
| SZ |  | * | * | * | ** | ND | ND |
| TA |  | * | * | * | ** | ND | * |

TABLE 5-continued

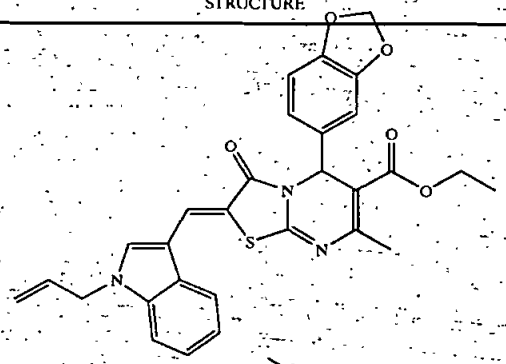
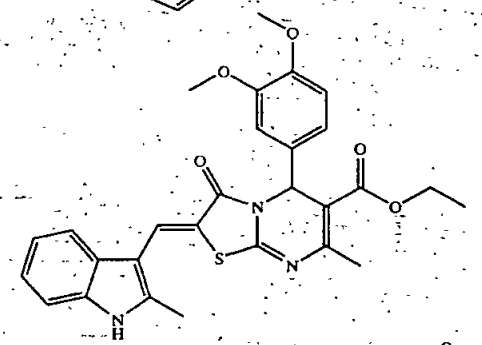
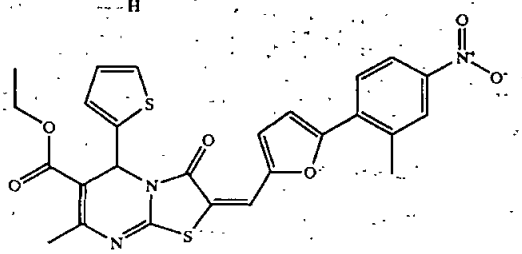
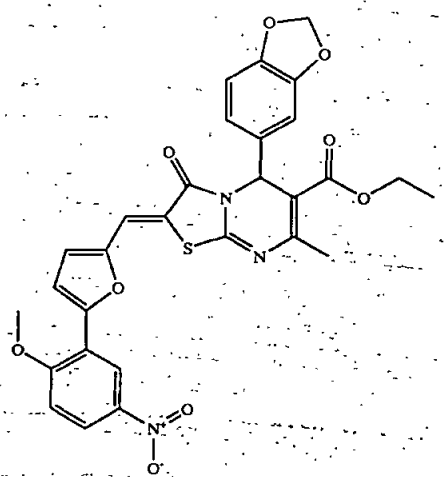
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TB |  | * | * | * | ** | ND | ** |
| TC |  | * | * | * | ** | ND | ND |
| TD |  | * | * | * | ** | ND | * |
| TE |  | * | * | * | ** | ND | ** |

TABLE 5-continued

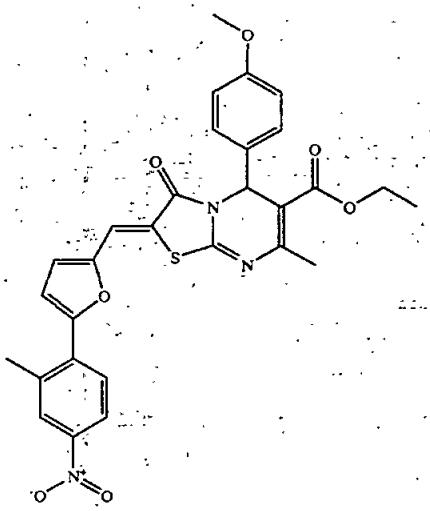
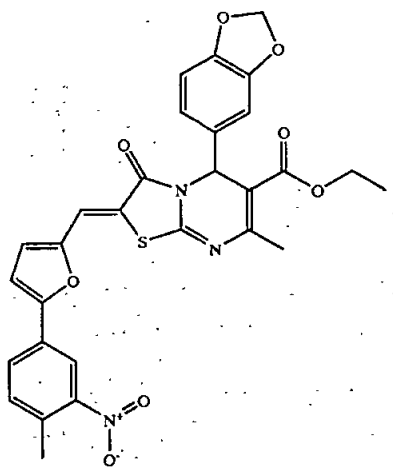
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TF |  | * | * | ** | ** | ND | ** |
| TG |  | *** | * | ** | ** | ND | ** |

TABLE 5-continued

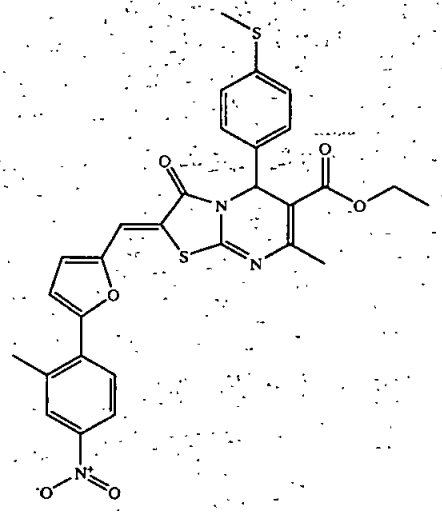
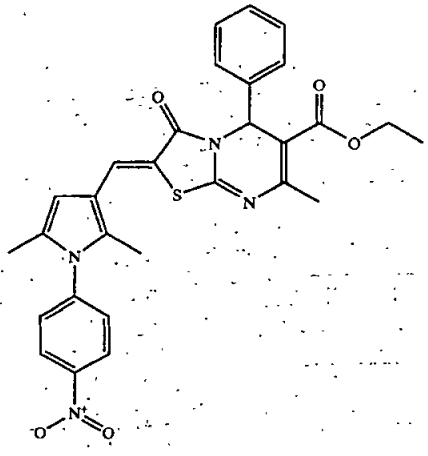
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TH |  | * | * | * | ** | ND | ** |
| TI |  | * | * | ** | ** | ND | * |

TABLE 5-continued

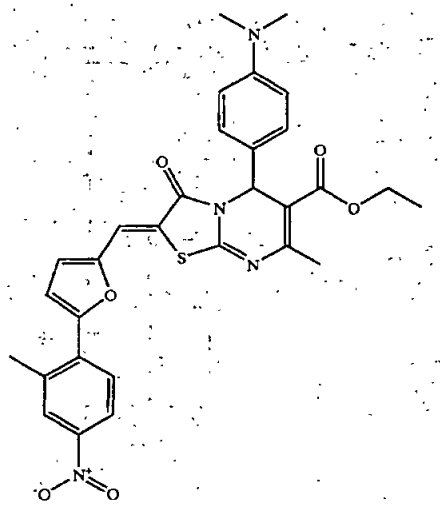
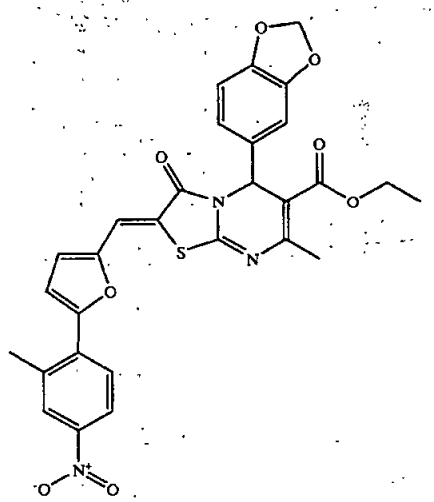
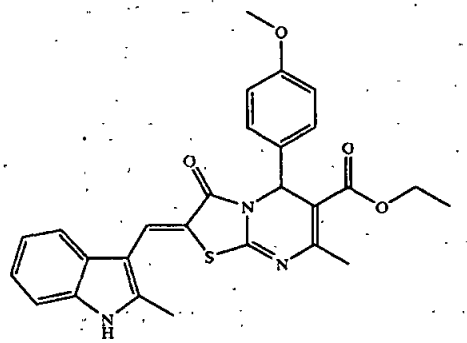
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TJ |  | * | * | * | ** | ND | ** |
| TK |  | * | ** | *** | ** | ND | ND |
| TL |  | * | * | * | ** | ND | ND |

TABLE 5-continued

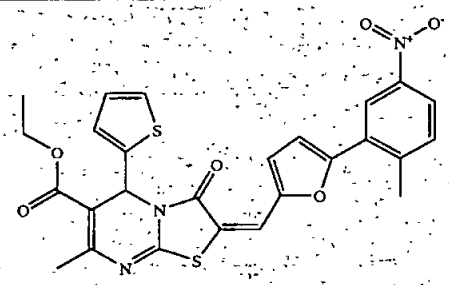
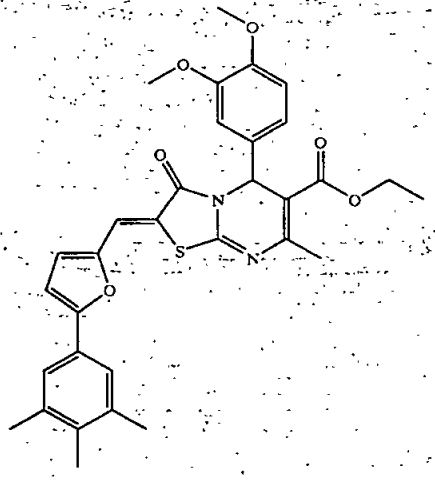
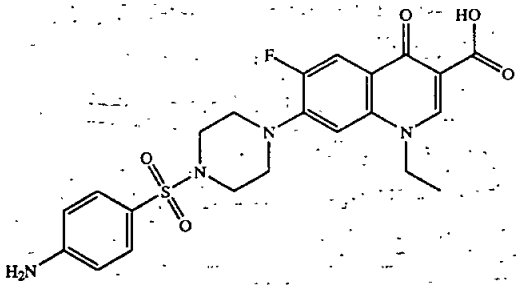
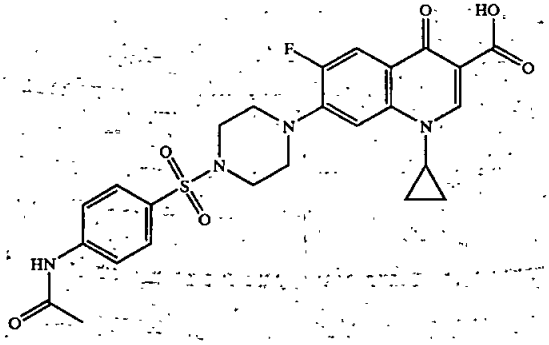
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TM |  | *** | *** | * | ** | ND | * |
| TN |  | * | * | * | ** | ND | ND |
| TO |  | * | * | *** | ** | ND | * |
| TP |  | * | * | ** | ** | ND | ND |

TABLE 5-continued

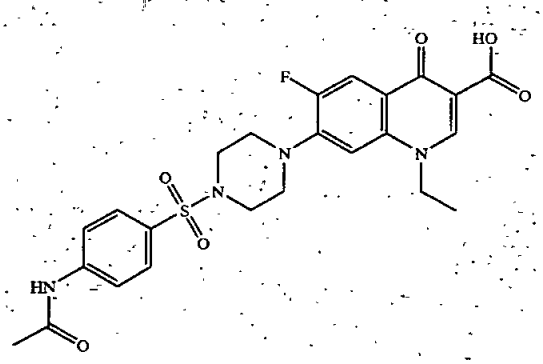
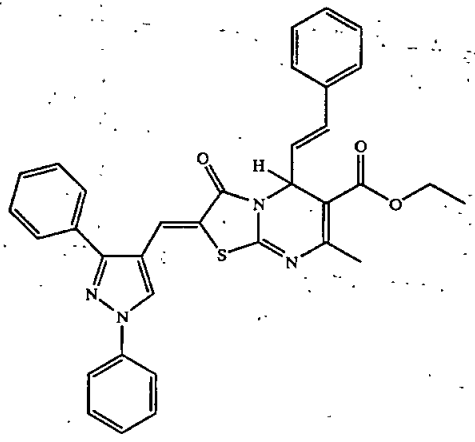
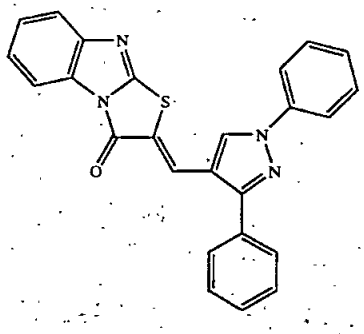
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TQ |  | * | ** | *** | ** | ND | * |
| TR |  | * | ** | ** | ** | ND | ND |
| TS |  | * | ** | * | ** | ND | ND |

TABLE 5-continued

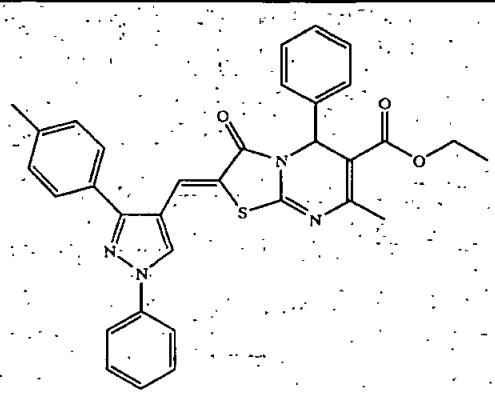
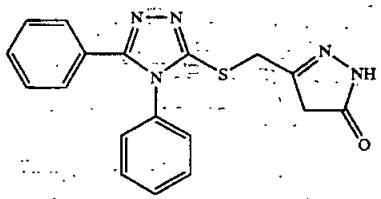
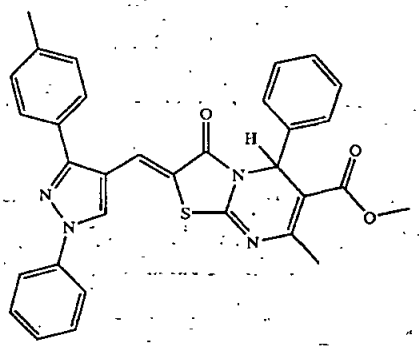
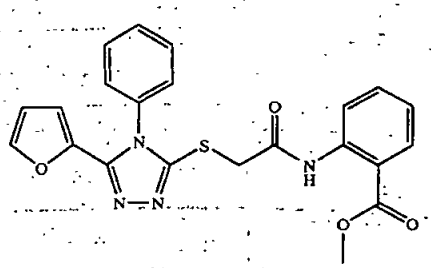
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TT |  | * | *** | *** | ** | ND | ND |
| TU |  | ** | * | * | ** | ND | * |
| TV |  | * | * | * | ** | ND | * |
| TW |  | * | * | * | ** | ND | * |

TABLE 5-continued

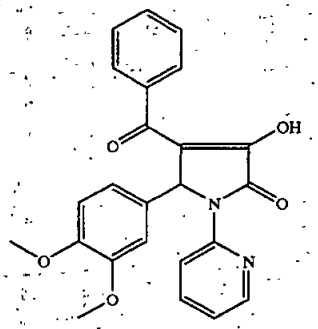
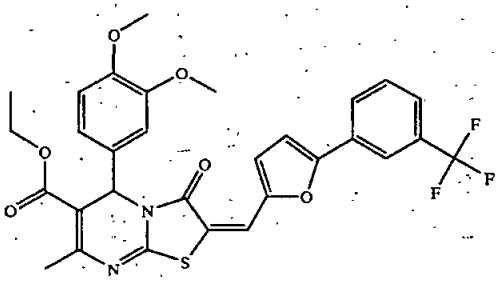
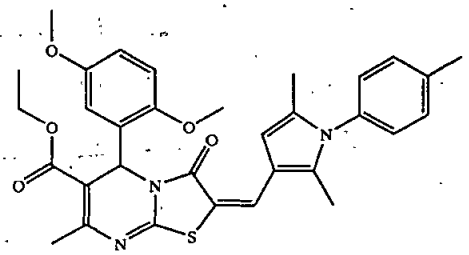
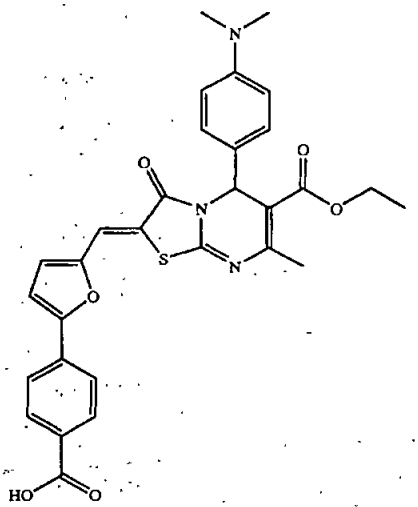
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| TX |  | *** | * | * | ** | ND | * |
| TY |  | * | * | * | ** | ND | ND |
| TZ |  | * | * | * | ** | ND | ND |
| UA |  | * | * | * | ** | ND | ND |

TABLE 5-continued

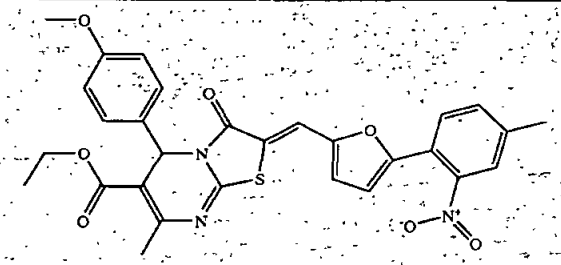
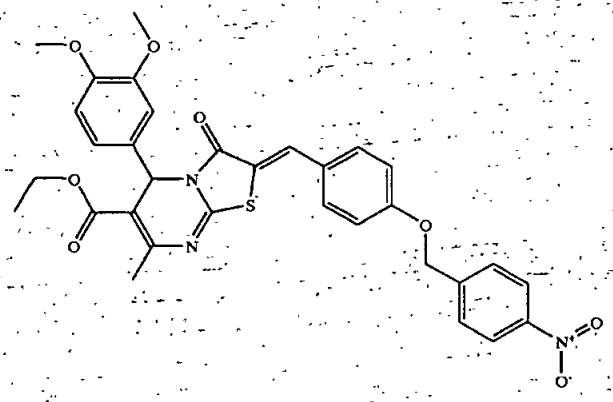
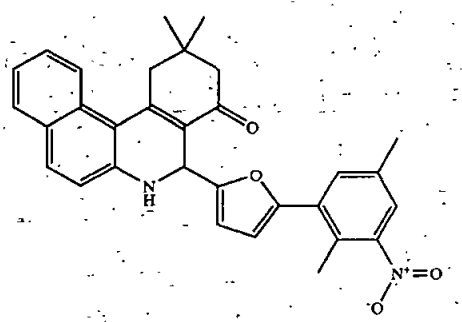
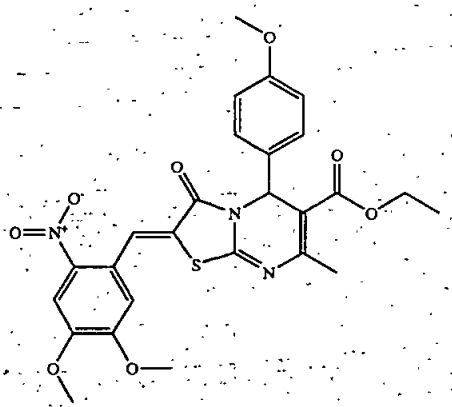
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UB |  | ** | * | * | ** | * | * |
| UC |  | ** | *** | *** | ** | ** | ** |
| UD |  | ** | **** | *** | ** | * | ** |
| UE |  | * | * | ** | ** | ND | ** |

TABLE 5-continued

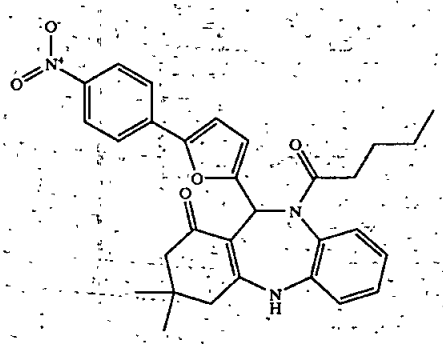
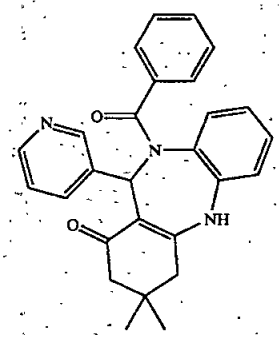
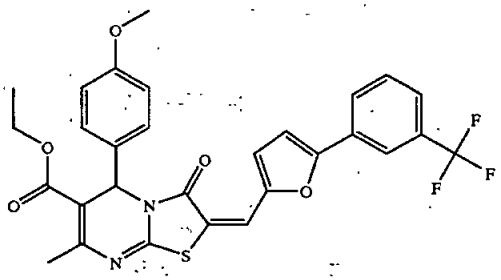
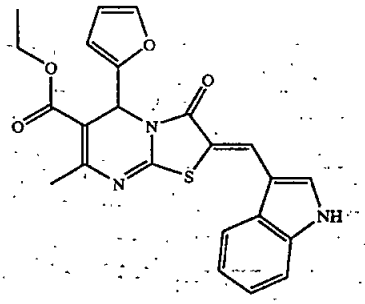
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UF |  | ** | ** | * | ** | ND | ** |
| UG |  | ** | ** | ** | ** | ND | ** |
| UH |  | *** | *** | *** | ** | ND | ** |
| UI |  | ** | * | ** | ** | ND | * |

TABLE 5-continued

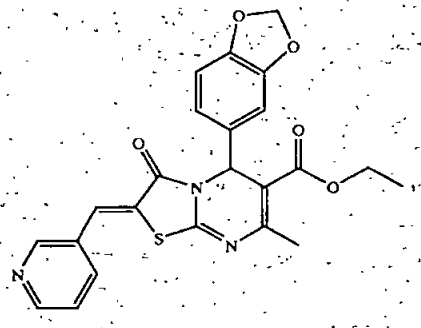
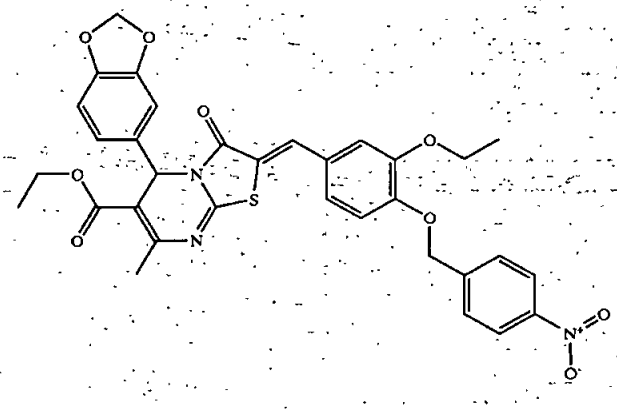
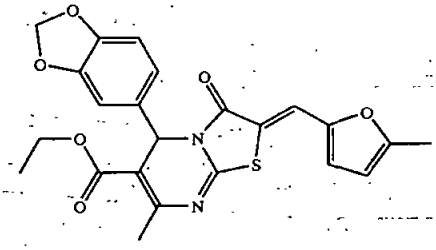
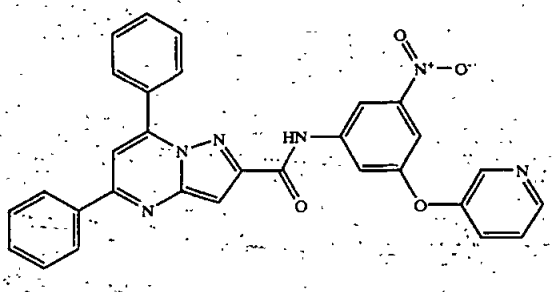
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UJ |  | ** | * | * | ** | ND | ND |
| UK |  | *** | *** | * | ** | ND | * |
| UL |  | ** | ** | ** | ** | ND | * |
| UM |  | ** | * | *** | ** | ND | ** |

TABLE 5-continued

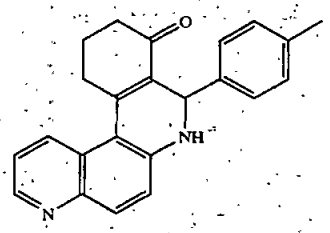
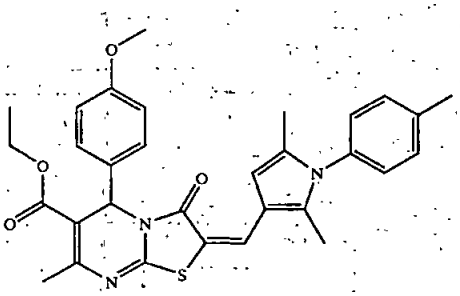
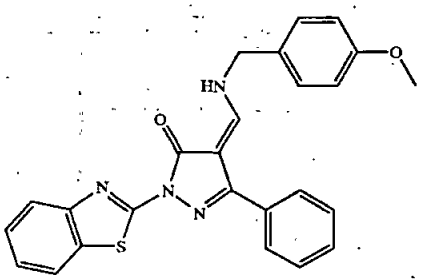
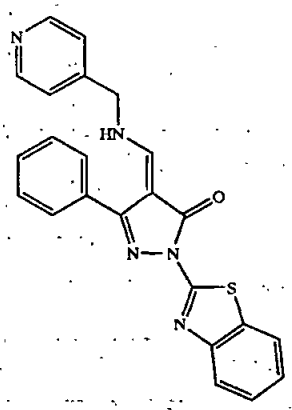
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UN |  | *** | * | ** | ** | ND | ND |
| UO |  | *** | * | * | ** | ND | ** |
| UP |  | *** | * | * | ** | ND | ND |
| UQ |  | ** | * | * | ** | ND | ** |

TABLE 5-continued

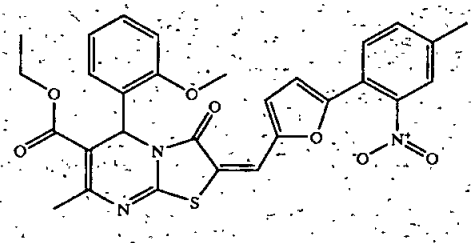
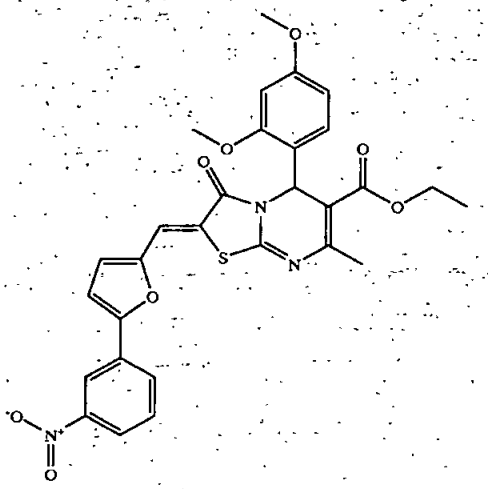
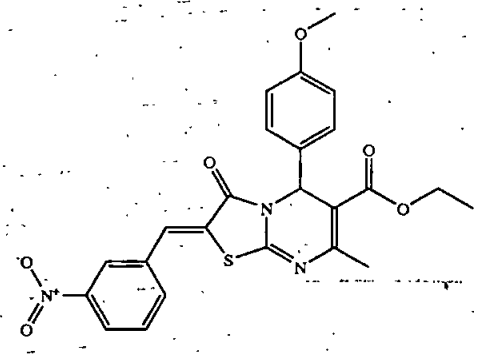
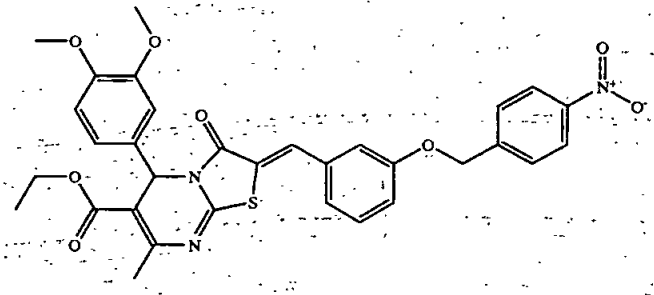
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UR |  | * | * | * | ** | ND | ** |
| US |  | ** | * | * | ** | ND | ** |
| UT |  | * | * | * | ** | ND | * |
| UU |  | * | ** | * | ** | ND | ND |

TABLE 5-continued

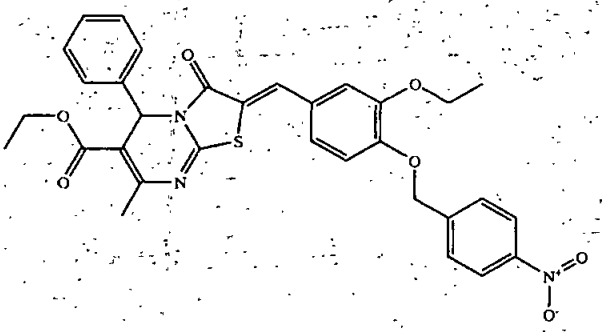
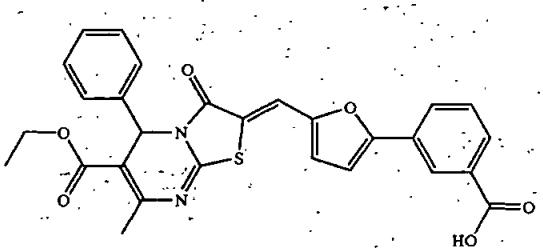
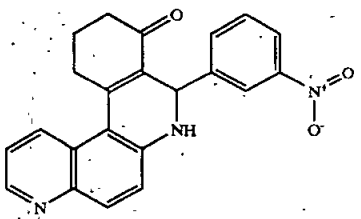
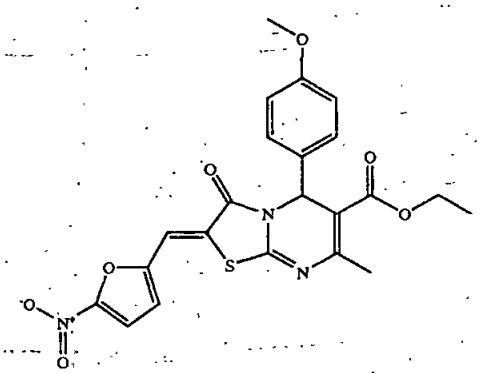
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UV |  | *** | *** | NT | ** | ND | ** |
| UW |  | *** | ** | NT | ** | ND | ** |
| UX |  | *** | *** | NT | ** | ND | * |
| UY |  | *** | * | NT | ** | ND | ** |

TABLE 5-continued

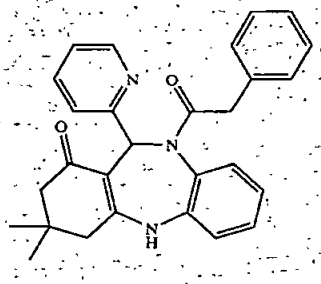
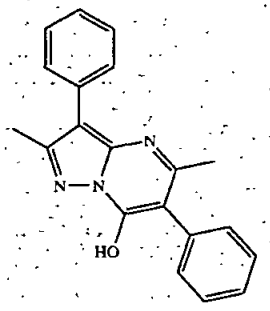
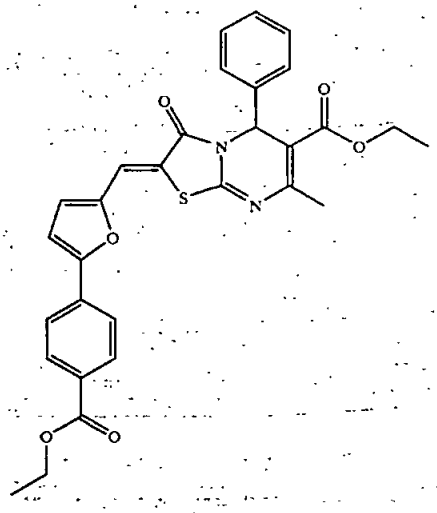
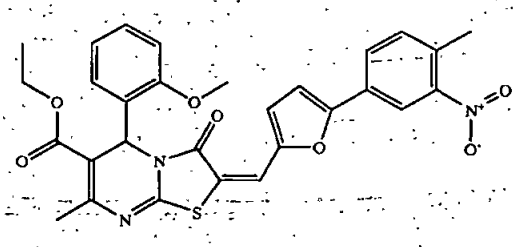
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| UZ |  | *** | ** | NT | ** | ND | ** |
| VA |  | * | * | * | ** | ** | ** |
| VB |  | * | * | * | ** | ** | ** |
| VC |  | * | * | * | ** | ** | ** |

TABLE 5-continued

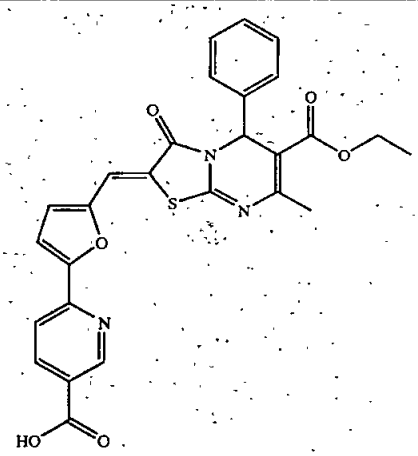
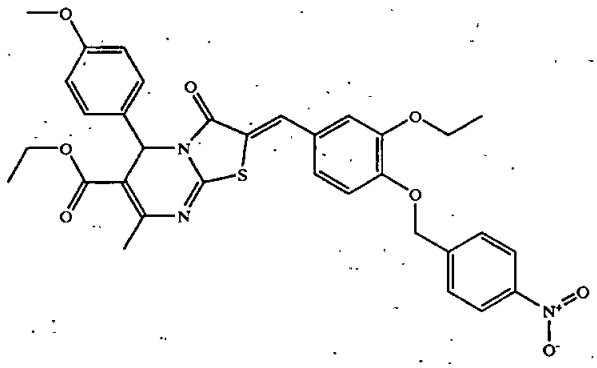
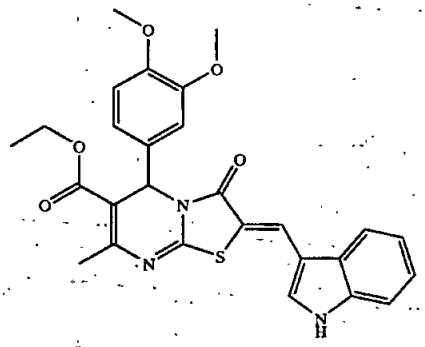
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VD |  | * | * | * | ** | ** | ** |
| VE |  | * | NT | NT | ** | ** | * |
| VF |  | * | NT | NT | ** | ** | * |

TABLE 5-continued

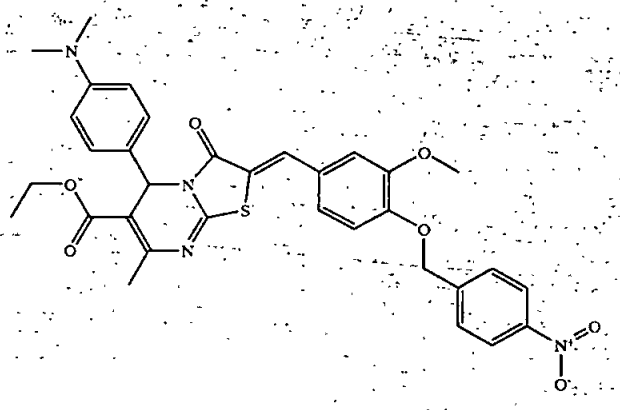
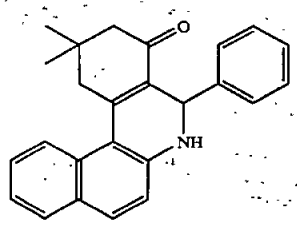
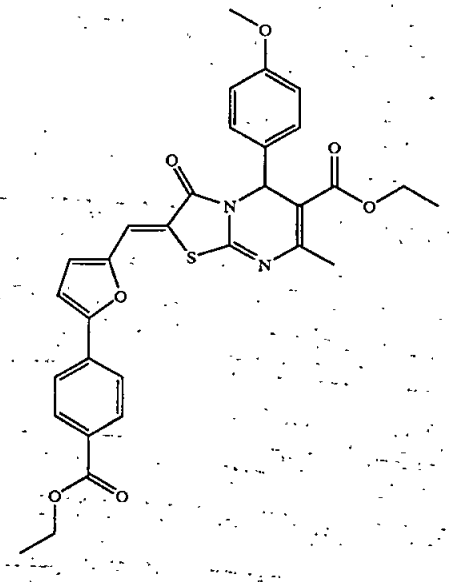
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VG |  | * | NT | NT | ** | * | ND |
| VH |  | ** | ** | *** | *** | ** | ** |
| VI |  | * | * | * | ** | * | * |

TABLE 5-continued

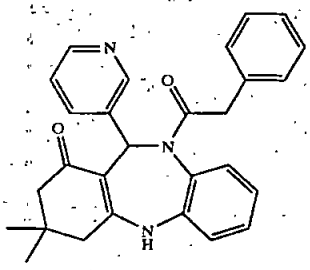
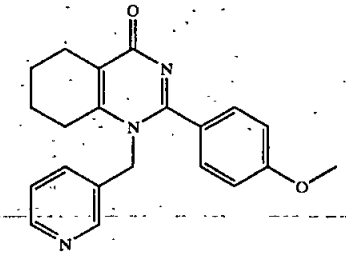
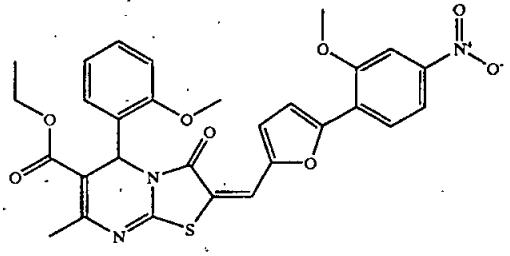
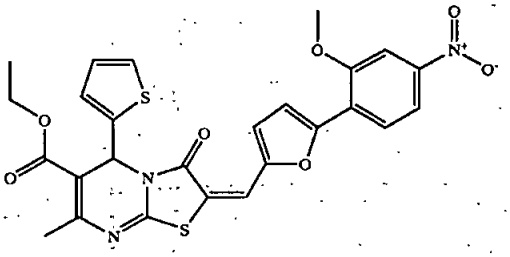
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VJ |  | • | •• | • | •• | •• | •• |
| VK |  | • | •• | •• | •• | •• | •• |
| VL |  | NT | •• | ••• | •• | ND | • |
| VM |  | NT | • | • | •• | ND | • |

TABLE 5-continued

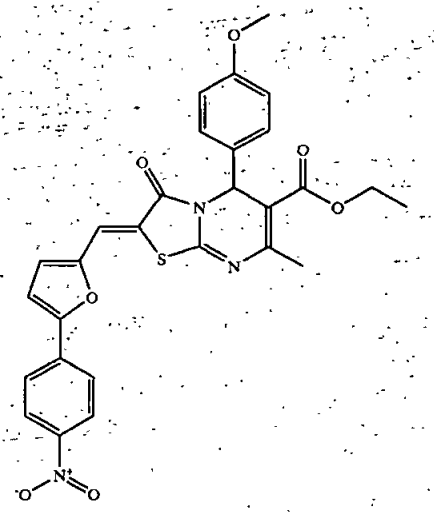
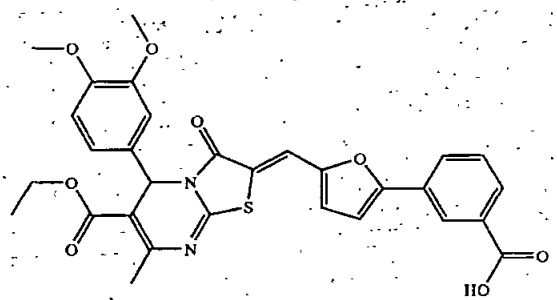
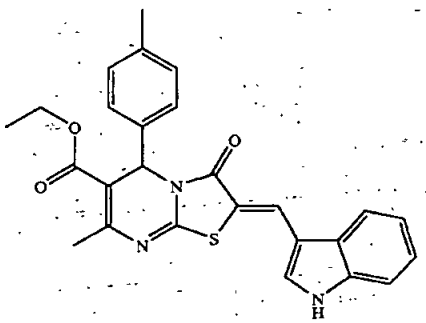
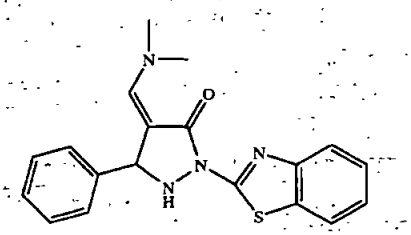
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VN |  | NT | ** | * | *** | ND | * |
| VO |  | * | *** | * | ** | ** | * |
| VP |  | *** | NT | NT | ** | ND | ND |
| VQ |  | ** | NT | NT | ** | ND | ND |

TABLE 5-continued

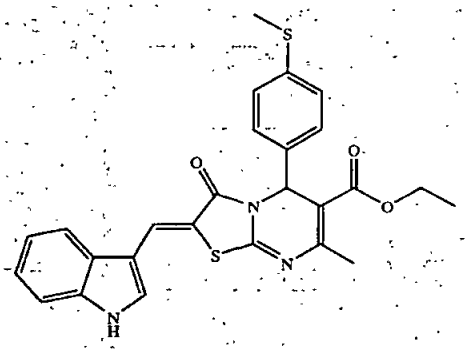
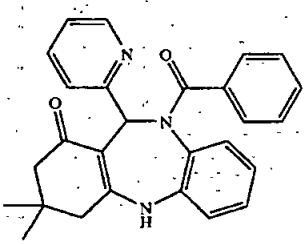
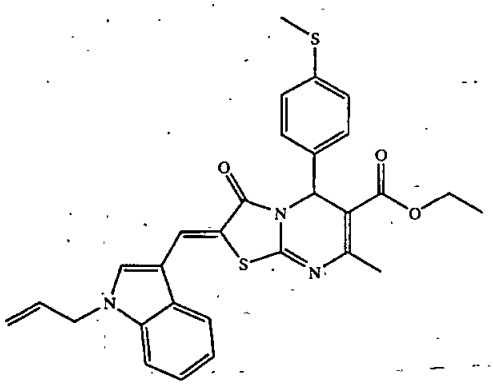
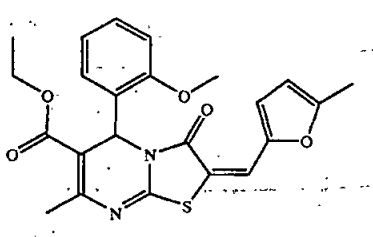
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VR |  | * | NT | NT | ** | ND | ND |
| VS |  | * | * | ** | ** | ND | ** |
| VT |  | * | * | *** | ** | ND | * |
| VU |  | *** | *** | *** | ** | ND | ** |

TABLE 5-continued

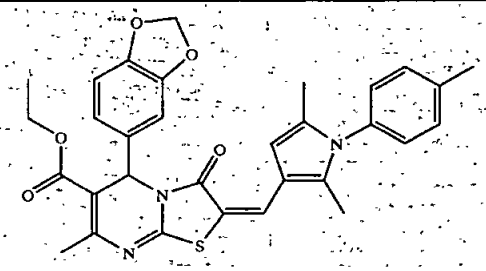
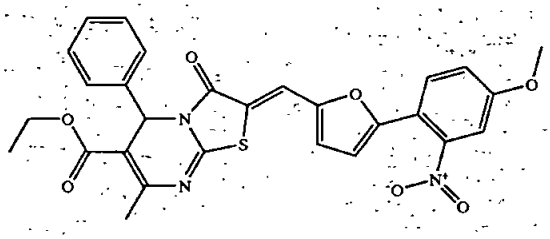
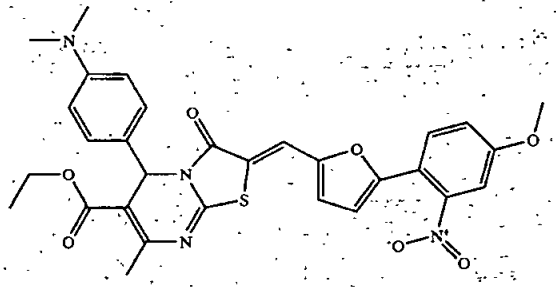
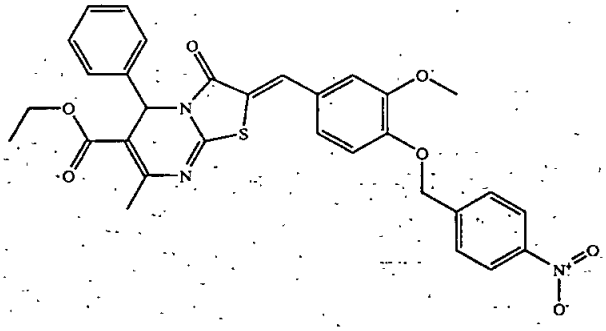
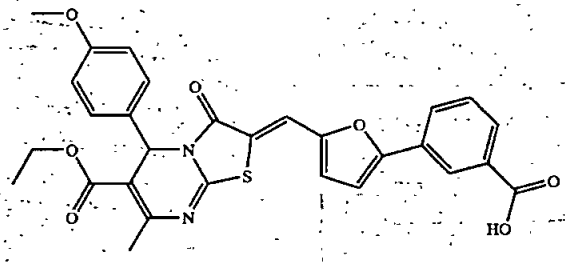
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| VV |  | * | * | *** | ** | ND | * |
| VW |  | * | * | * | ** | ND | ** |
| VX |  | * | * | ** | ** | ND | ** |
| VY |  | * | * | * | ** | ND | ND |
| VZ |  | * | * | * | ** | ND | ** |

TABLE 5-continued

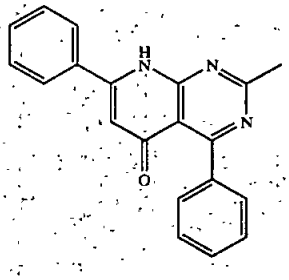
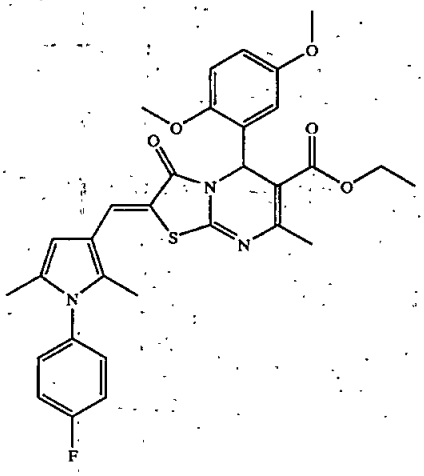
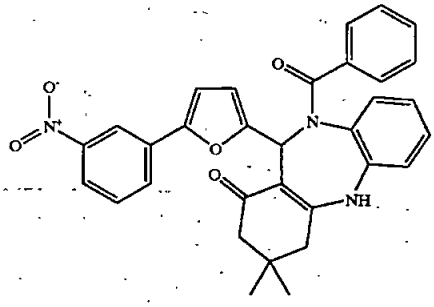
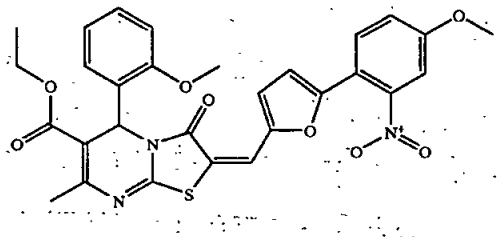
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WA |  | *** | * | * | ** | ND | ** |
| WB |  | ** | *** | * | ** | ND | ** |
| WC |  | * | ** | ** | ** | ND | ** |
| WD |  | *** | * | * | ** | ND | ** |

TABLE 5-continued

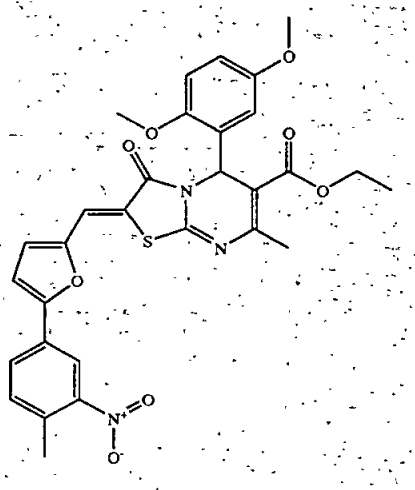
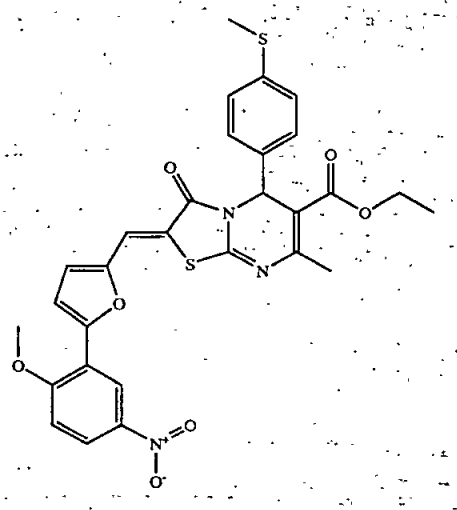
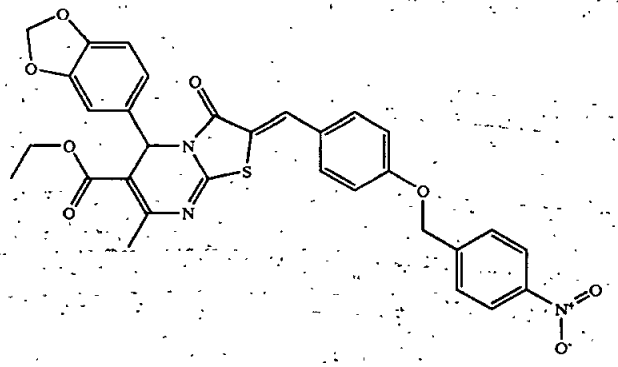
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD. | CV | Luc |
|---|---|---|---|---|---|---|---|
| WE |  | ** | ** | * | ** | ND | ND |
| WF |  | ** | *** | ** | ** | ND | ** |
| WG |  | * | * | * | ** | ND | ND |

TABLE 5-continued

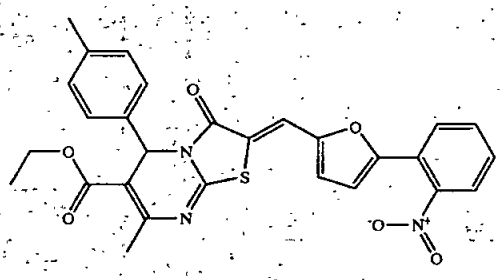
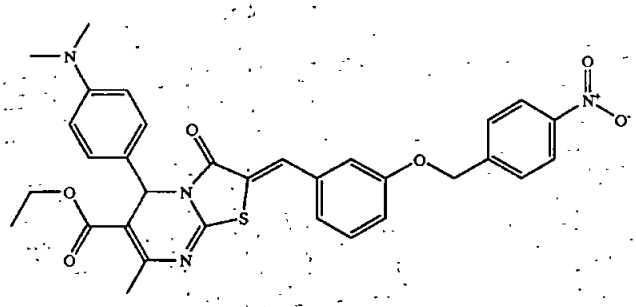
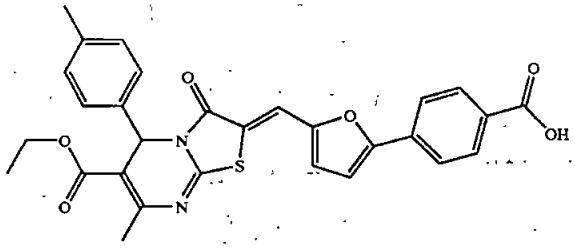
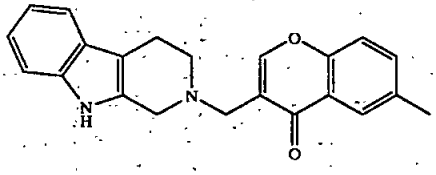
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WH |  | ** | * | *** | ** | ND | ** |
| WI |  | * | ** | *** | ** | ND | ** |
| WJ |  | * | * | * | ** | ND | * |
| WK |  | * | * | * | ** | ND | * |

TABLE 5-continued

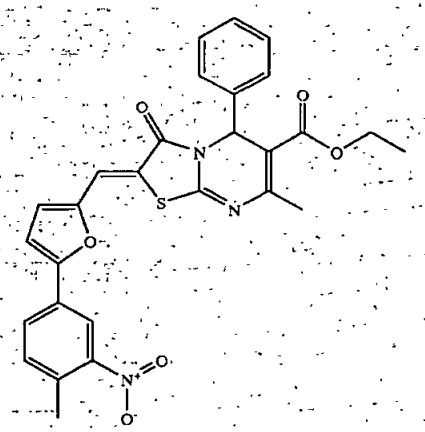
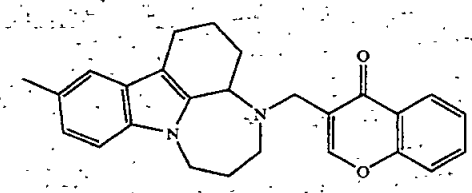
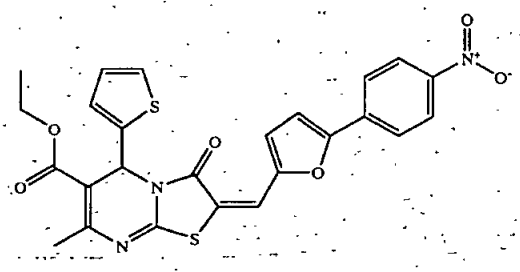
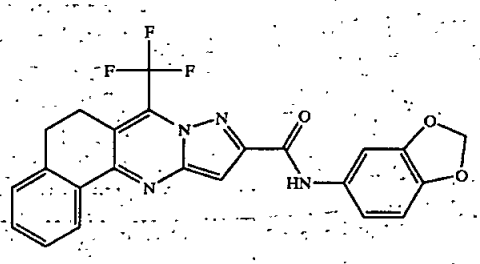
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | QD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WL |  | * | * | * | ** | ND | ** |
| WM |  | * | * | ** | ** | ND | * |
| WN |  | * | * | * | ** | ND | * |
| WO |  | * | ** | *** | ** | ND | ** |

TABLE 5-continued

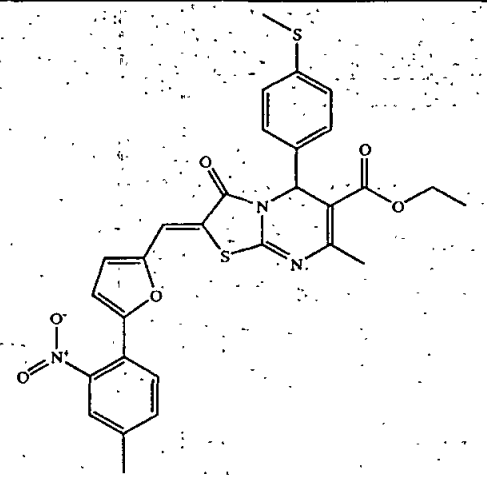
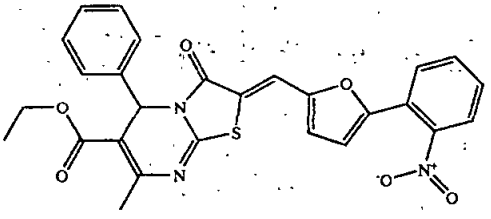
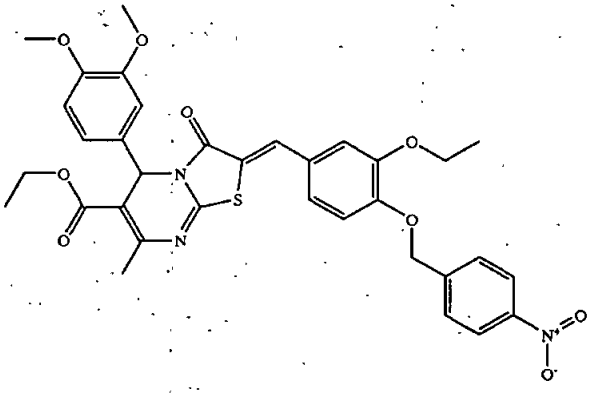
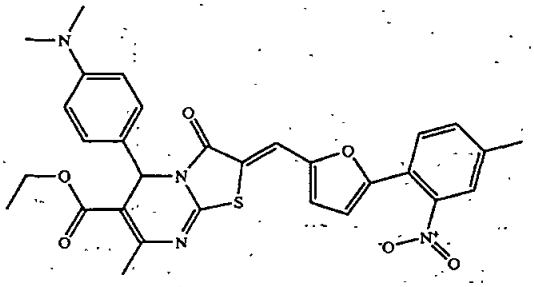
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WP |  | * | * | ** | ** | ND | * |
| WQ |  | * | *** | *** | ** | ND | ND |
| WR |  | * | * | * | ** | ND | ND |
| WS |  | * | * | * | ** | ND | ND |

TABLE 5-continued

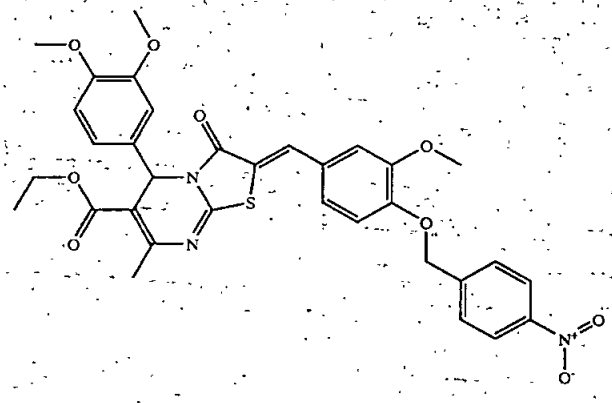
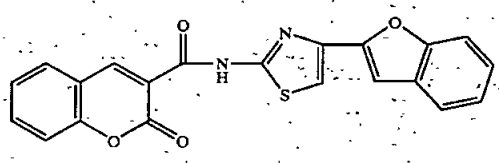
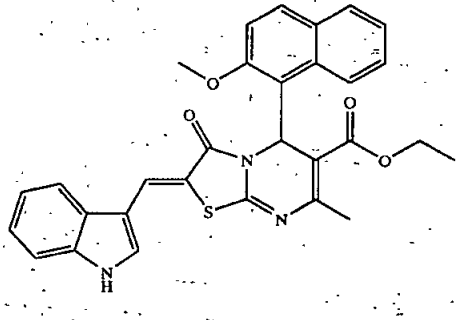
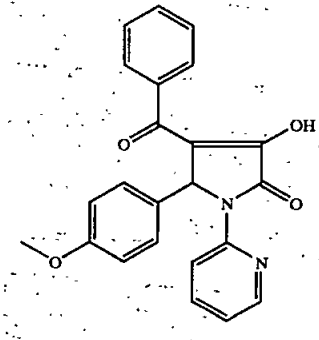
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WT |  | ** | * | * | ** | ND | ** |
| WU |  | * | * | * | ** | ND | * |
| WV |  | * | * | * | ** | ND | ** |
| WW |  | * | * | * | ** | ND | * |

TABLE 5-continued

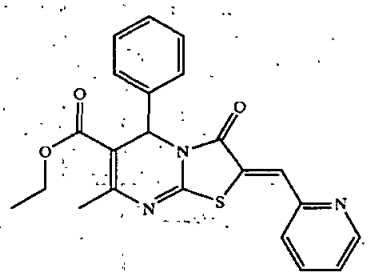
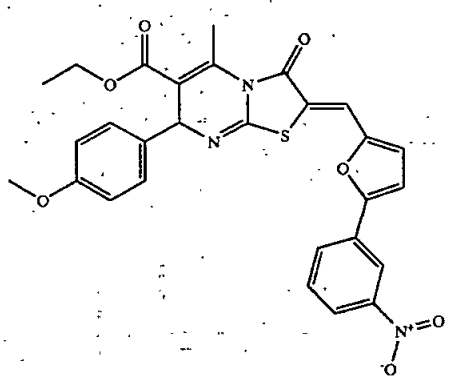
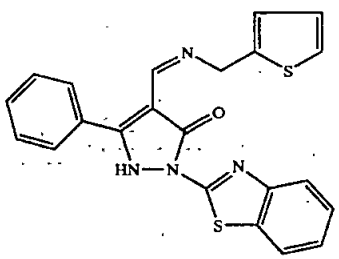
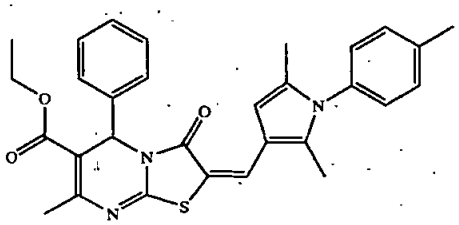
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| WX |  | ** | * | * | ** | ND | * |
| WY |  | * | * | * | ** | ND | ** |
| WZ |  | * | * | * | ** | ND | ND |
| XA |  | * | * | * | ** | ND | ND |

TABLE 5-continued

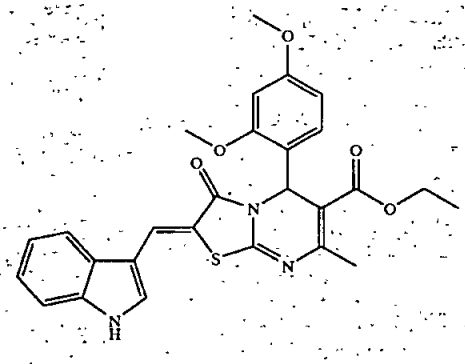
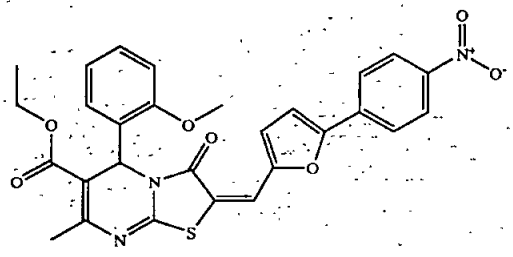
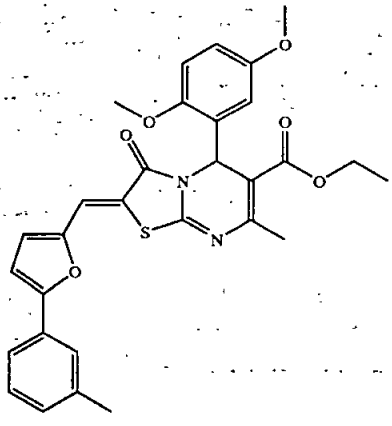
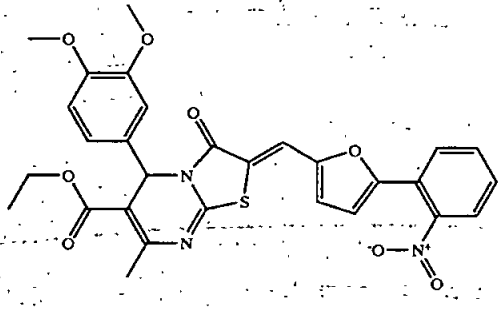
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XB |  | * | * | * | ** | ND | * |
| XC |  | * | * | * | ** | ND | ND |
| XD |  | *** | * | * | ** | ND | ND |
| XE |  | * | * | ** | ** | ND | ND |

TABLE 5-continued

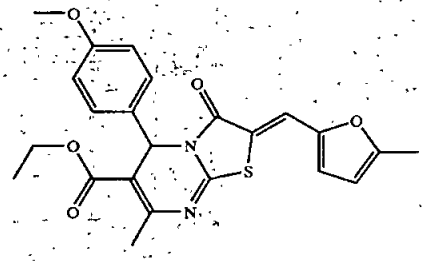
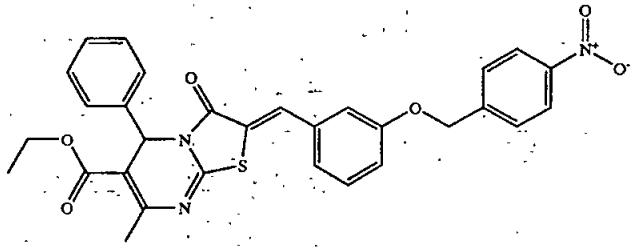
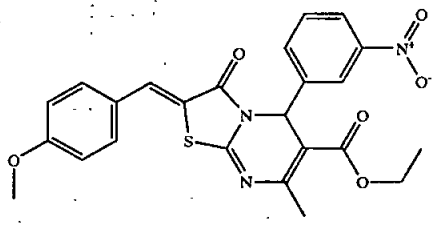
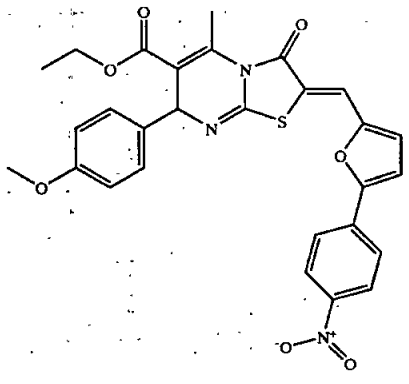
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XF |  | *** | *** | ** | ** | * | ND |
| XG |  | * | ** | * | ** | * | * |
| XH |  | * | ** | * | ** | ** | ** |
| XI |  | ** | ** | *** | ** | * | ** |

TABLE 5-continued

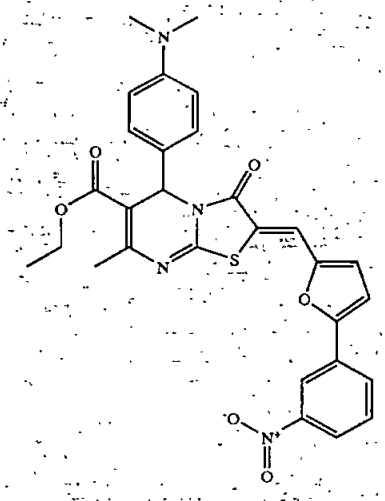
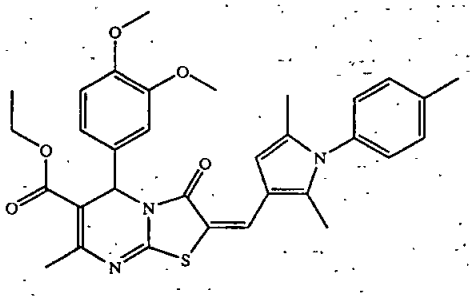
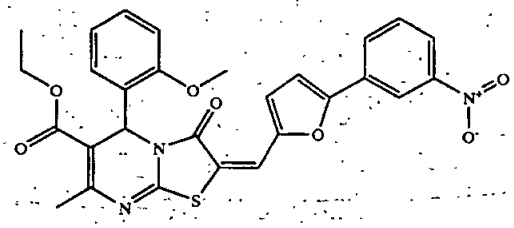
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XG |  | * | ** | * | ** | ** | * |
| XH |  | * | * | * | ** | * | ND |
| XI |  | * | * | * | ** | ** | * |

TABLE 5-continued

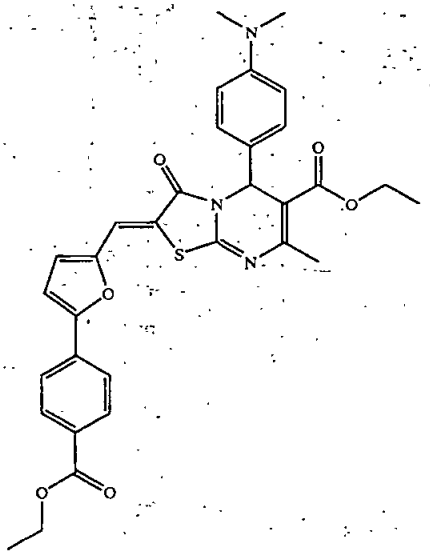
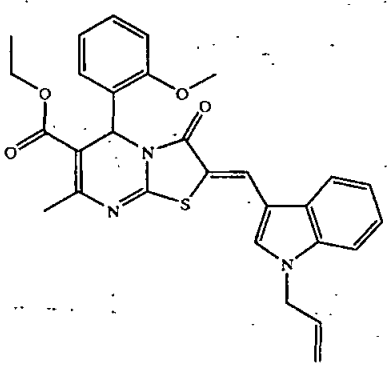
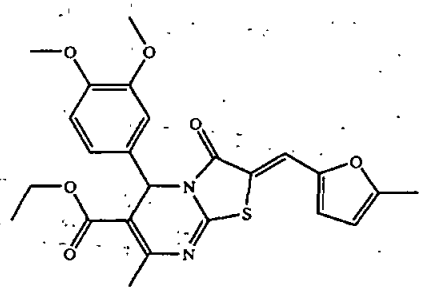
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XJ |  | * | ** | *** | ** | ** | ** |
| XK |  | * | * | * | ** | * | * |
| XL |  | ** | ** | ** | ** | ND | * |

TABLE 5-continued

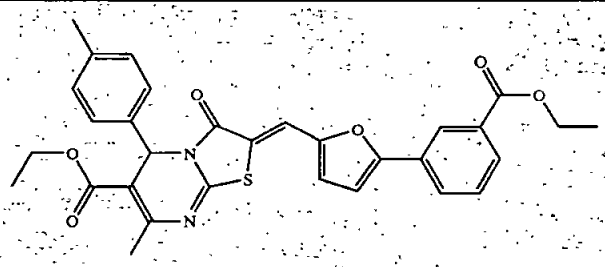
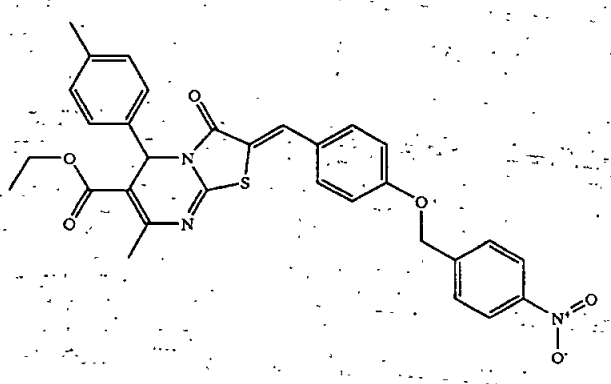
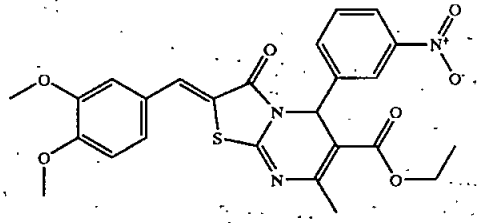
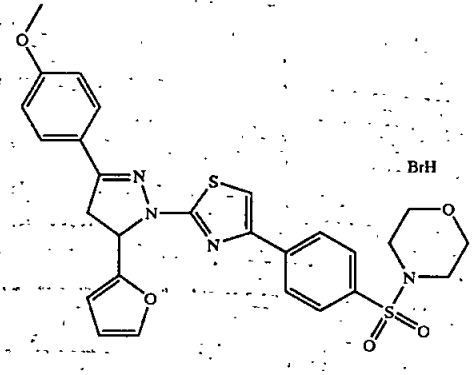
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XM |  | *** | * | * | ** | ND | ** |
| XN |  | ** | * | * | ** | ND | ND |
| XO |  | *** | *** | * | ** | ND | ** |
| XP |  | ** | * | ** | ** | ND | ND |

TABLE 5-continued

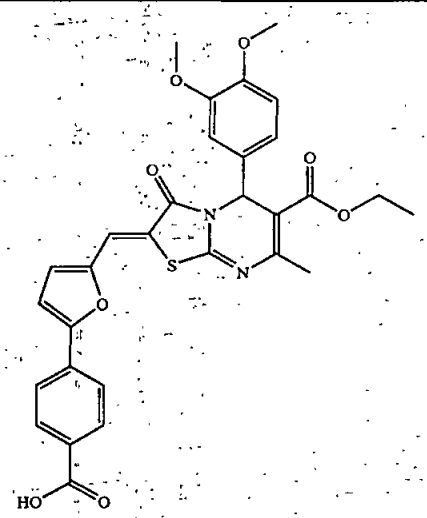
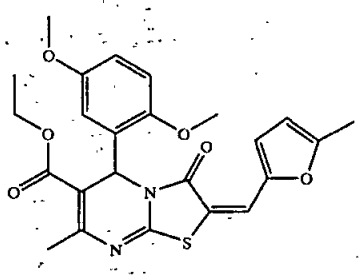
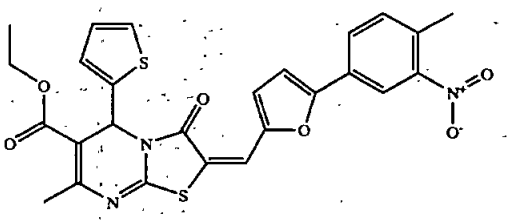
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XQ |  | ** | * | ** | ** | ND | * |
| XR |  | *** | * | * | ** | ND | ** |
| XS |  | ** | * | * | ** | ND | ** |

TABLE 5-continued

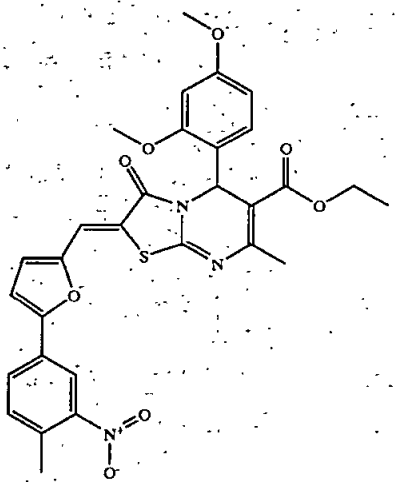
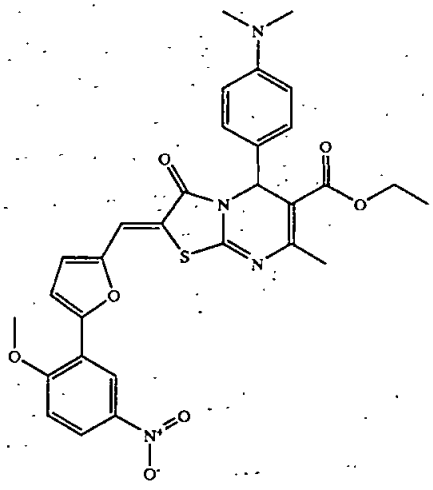
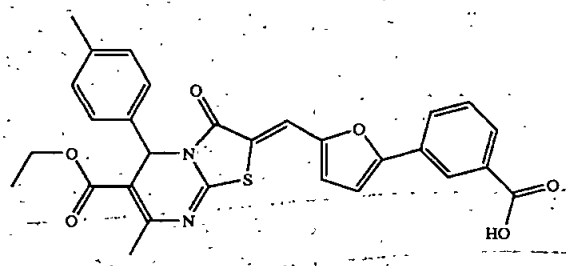
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XT |  | ** | * | * | ** | ND | ** |
| XU |  | * | * | * | ** | ND | * |
| XV |  | ** | * | * | ** | ND | * |

TABLE 5-continued

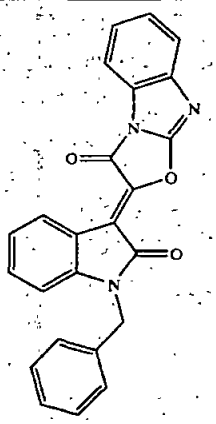
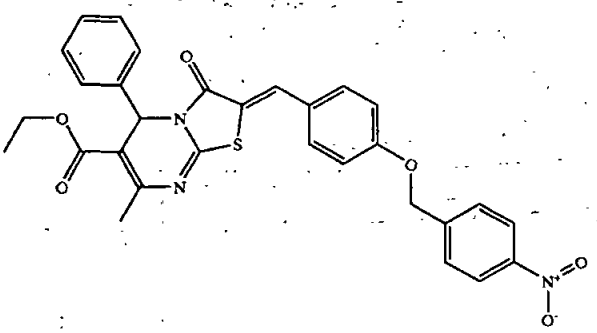
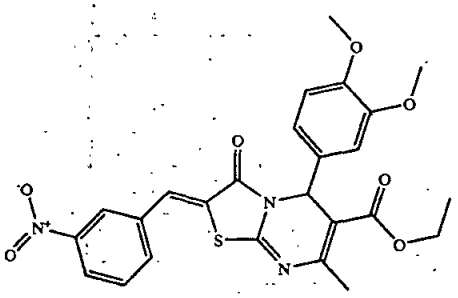
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| XW |  | ** | * | ** | ** | ND | ND |
| XY |  | * | *** | * | ** | ND | ** |
| XZ |  | *** | *** | NT | ** | ND | ** |

TABLE 5-continued

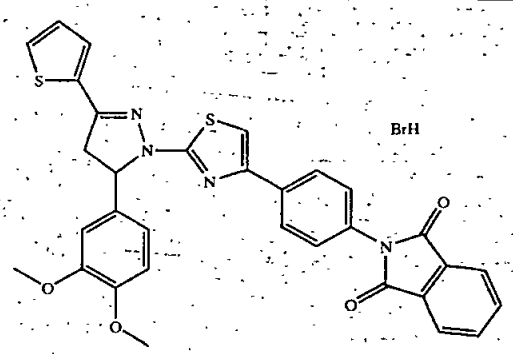
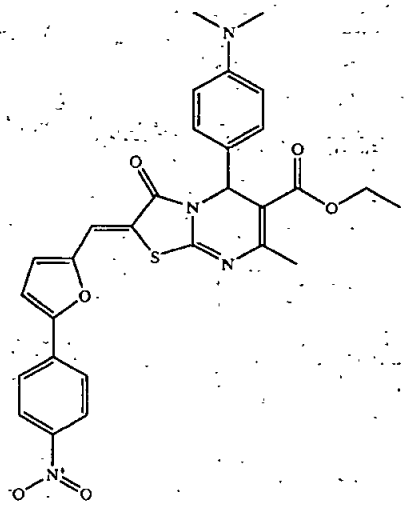
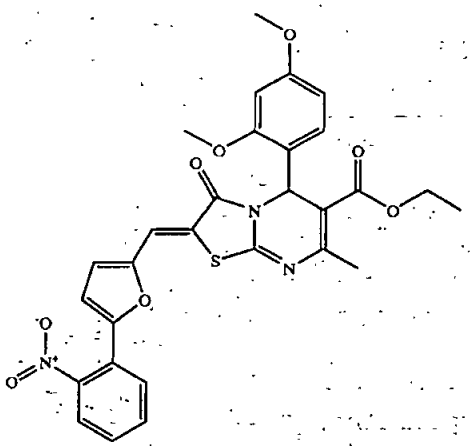
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YA |  | ** | * | * | ** | ** | * |
| YB |  | * | * | *** | ** | ** | ** |
| YC |  | * | * | ** | ** | ** | ** |

TABLE 5-continued

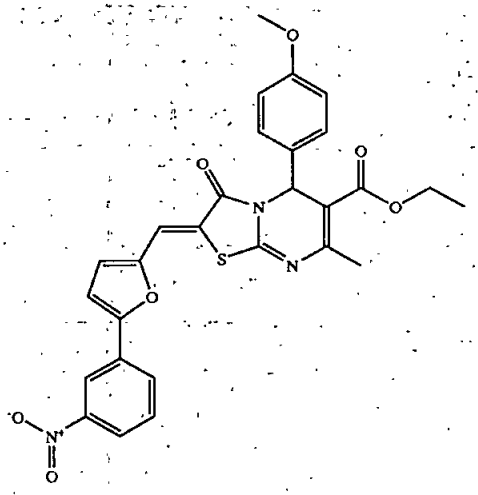
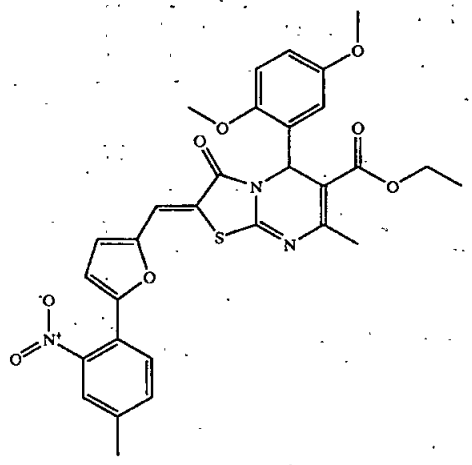
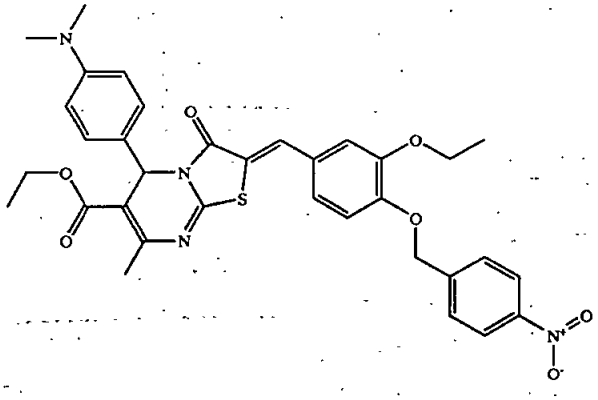
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YD |  | * | NT | NT | ** | ** | ** |
| YE |  | * | NT | NT | ** | ** | * |
| YF |  | *** | *** | *** | ** | ** | ** |

TABLE 5-continued

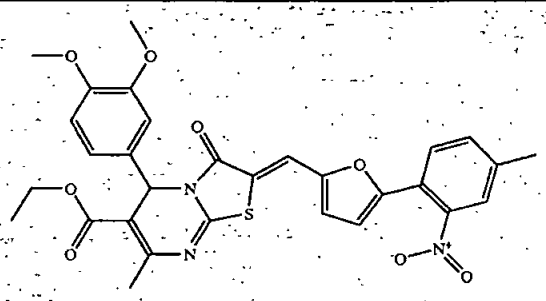
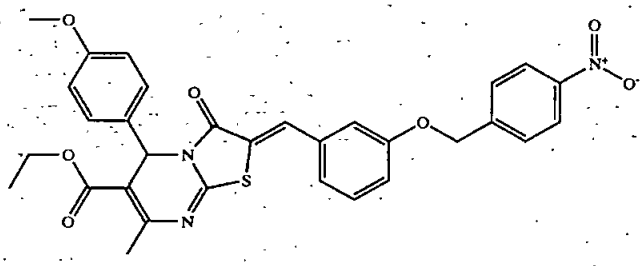
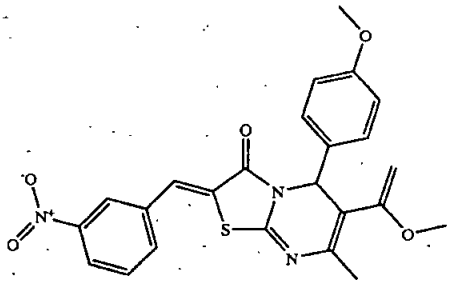
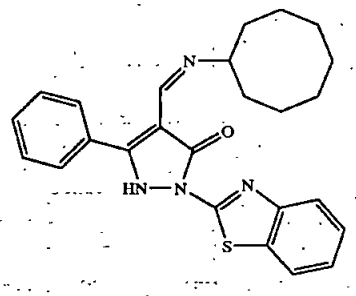
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YG |  | * | ** | *** | ** | ** | * |
| YH |  | *** | *** | * | ** | ** | ** |
| YI |  | *** | *** | * | ** | ** | ** |
| YJ |  | NT | ** | * | ** | ND | ** |

TABLE 5-continued

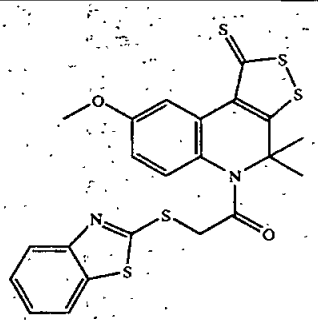
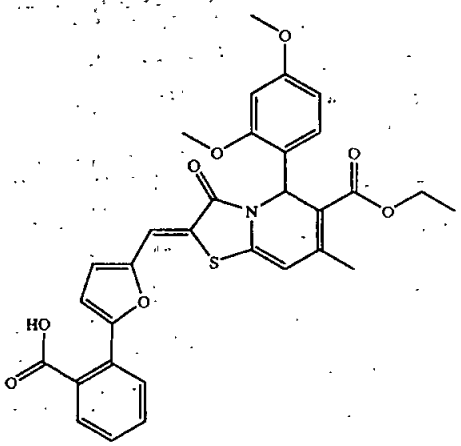
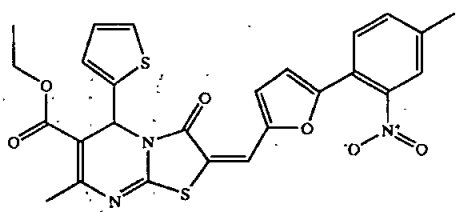
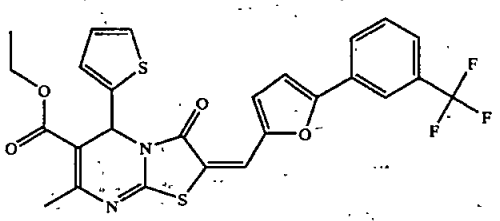
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YK |  | NT | *** | * | ** | ND | * |
| YL |  | NT | ** | * | ** | ND | ** |
| YM |  | NT | ** | * | ** | ND | * |
| YN |  | NT | ** | ** | ** | ND | ** |

TABLE 5-continued

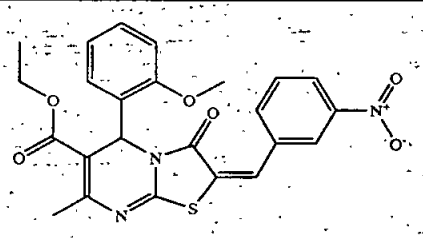
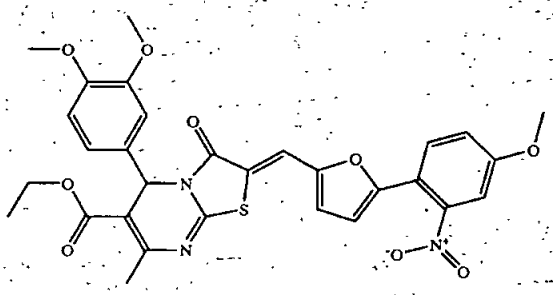
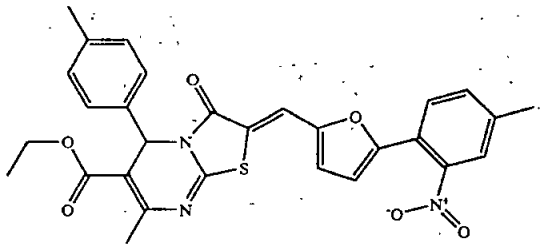
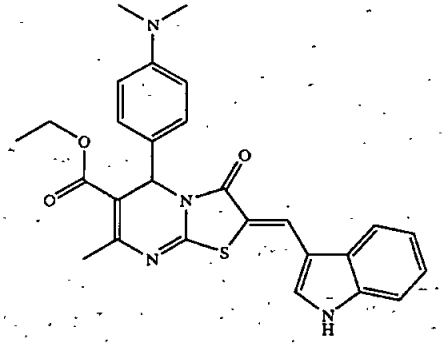
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YO |  | * | * | * | ** | ** | ND |
| YP |  | * | * | * | ** | ** | ** |
| YQ |  | * | ** | * | ** | ** | ** |
| YR |  | ** | NT | NT | ** | ND | ND |

TABLE 5-continued

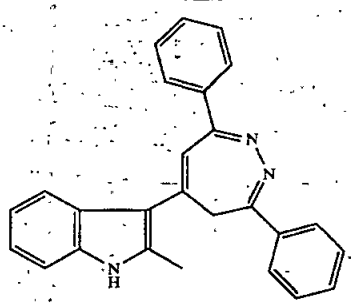
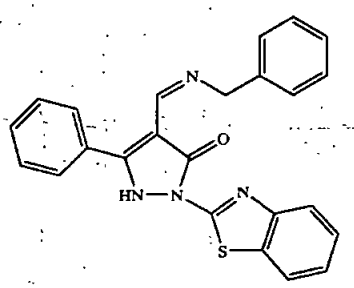
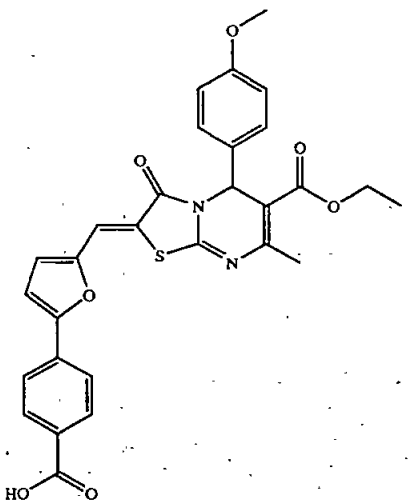
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YS |  | ** | * | *** | ** | ND | * |
| YT |  | *** | * | * | ** | ND | * |
| YU |  | ** | * | ** | ** | ND | ** |

TABLE 5-continued

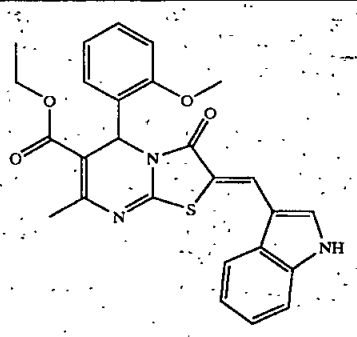
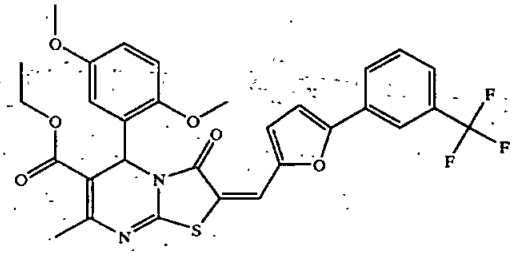
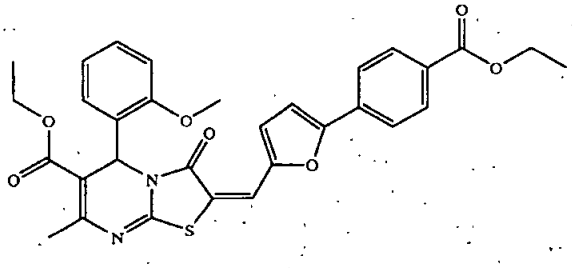
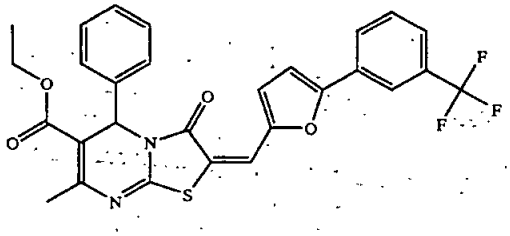
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | QD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YV |  | * | * | * | ** | ND | ** |
| YW |  | *** | *** | *** | ** | ND | ** |
| YX |  | * | * | ** | ** | ND | ** |
| YY |  | * | * | * | ** | ND | ** |

TABLE 5-continued

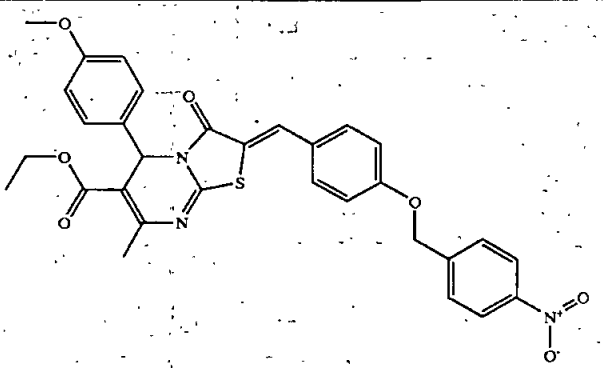
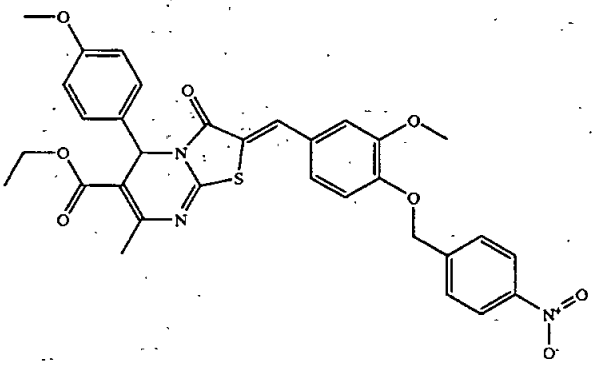
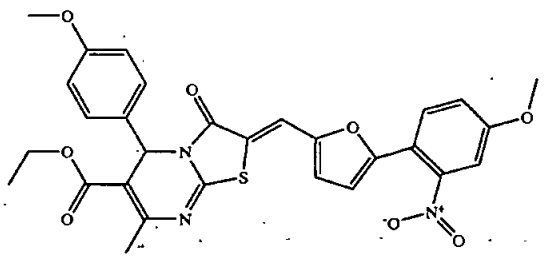
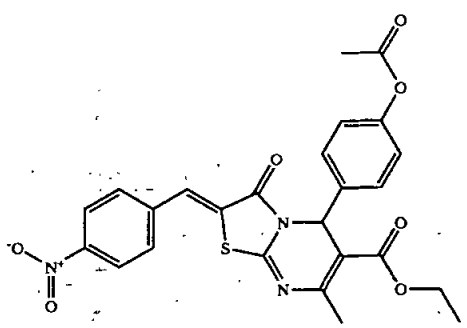
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YZ |  | ** | * | * | ** | ND | * |
| ZA |  | * | * | * | ** | ND | ** |
| ZB |  | * | * | * | ** | ND | ND |
| ZC |  | *** | *** | *** | ** | ND | ** |

TABLE 5-continued

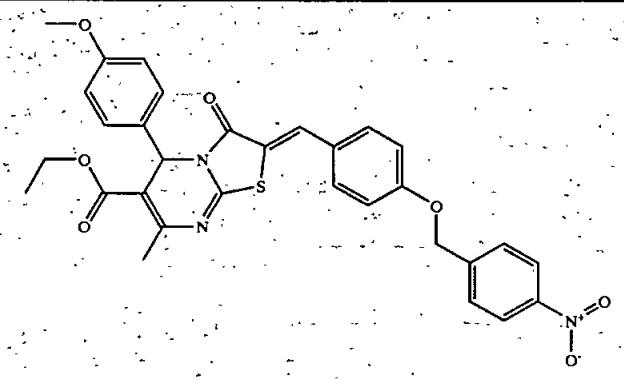
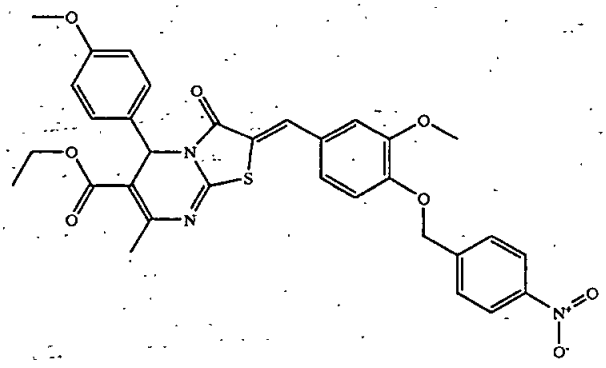
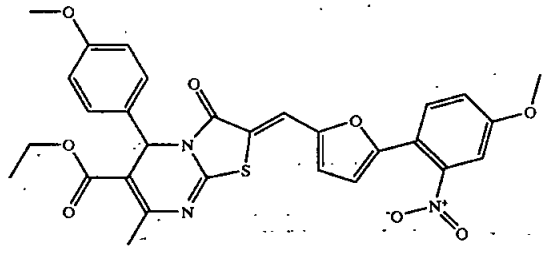
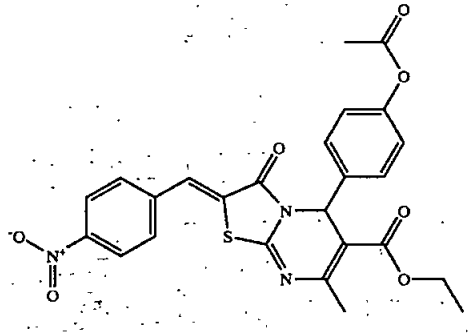
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YZ |  | ** | * | * | ** | ND | * |
| ZA |  | * | * | * | ** | ND | ** |
| ZB |  | * | * | * | ** | ND | ND |
| ZC |  | *** | *** | *** | ** | ND | ** |

TABLE 5-continued

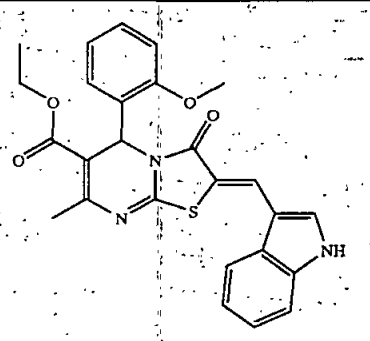
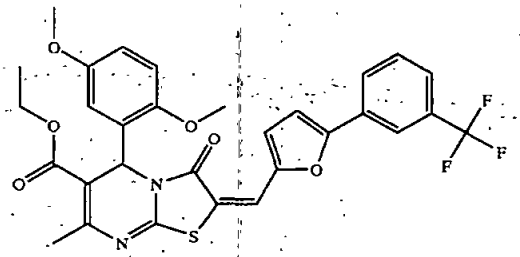
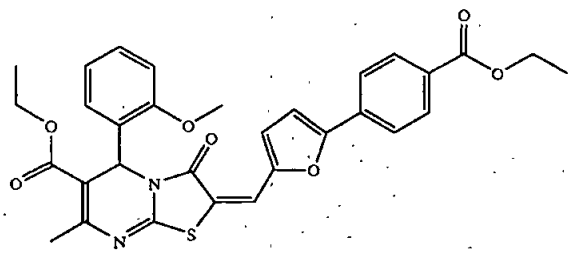
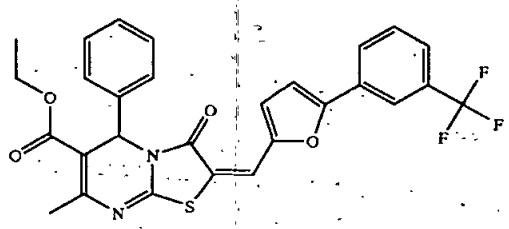
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | QD | CV | Luc |
|---|---|---|---|---|---|---|---|
| YV |  | • | • | • | •• | ND | •• |
| YW |  | ••• | ••• | ••• | •• | ND | •• |
| YX |  | • | • | •• | •• | ND | •• |
| YY |  | • | • | • | •• | ND | •• |

TABLE 5-continued

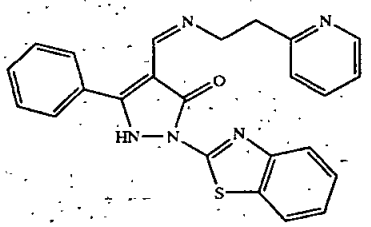
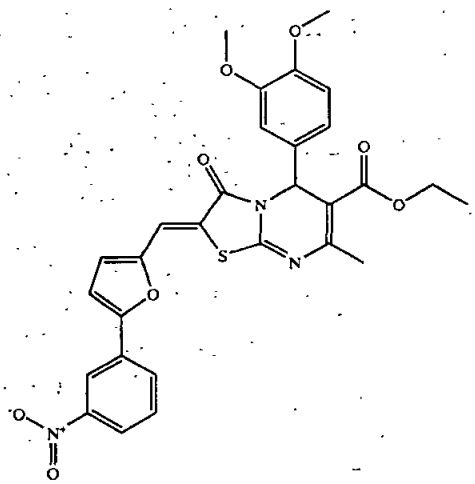
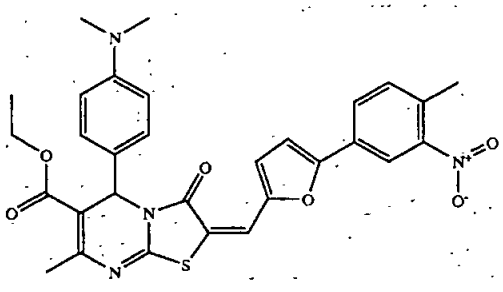
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZD |  | *** | *** | *** | ** | ND | ND |
| ZE |  | ** | * | *** | ** | ND | ND |
| ZF |  | ** | * | * | ** | ND | * |

TABLE 5-continued

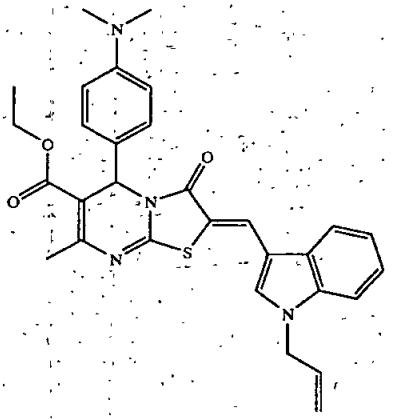
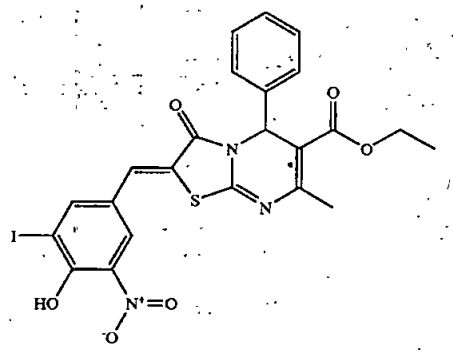
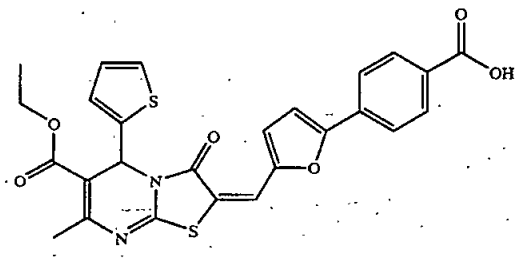
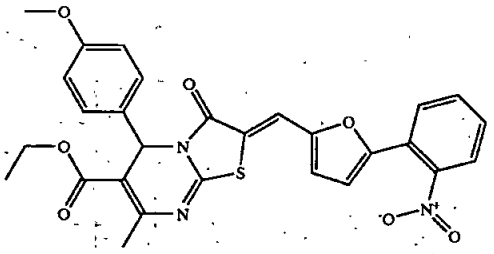
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZG |  | ** | * | *** | ** | ND | ND |
| ZH |  | * | ** | *** | ** | ND | * |
| ZI |  | * | * | * | ** | ND | ND |
| ZJ |  | * | * | * | ** | ND | * |

TABLE 5-continued

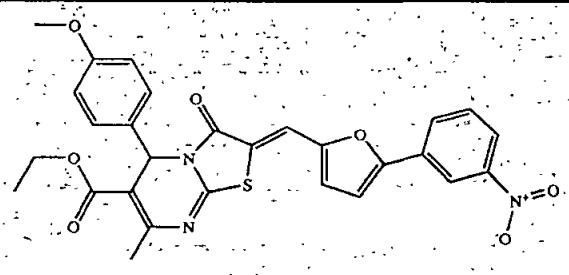
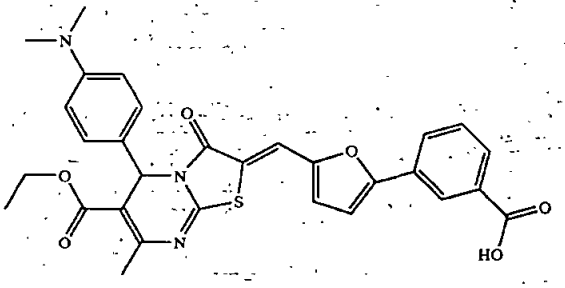
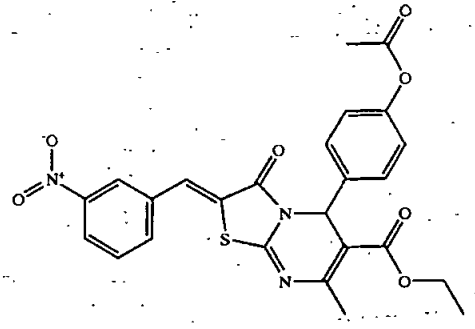
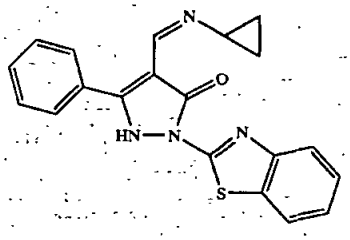
| ID | STRUCTURE | MurA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZK |  | * | * | * | ** | * | ND |
| ZL |  | * | * | * | ** | * | ND |
| ZM |  | * | ** | ** | ** | * | ND |
| ZN |  | * | ** | * | ** | * | ND |

TABLE 5-continued

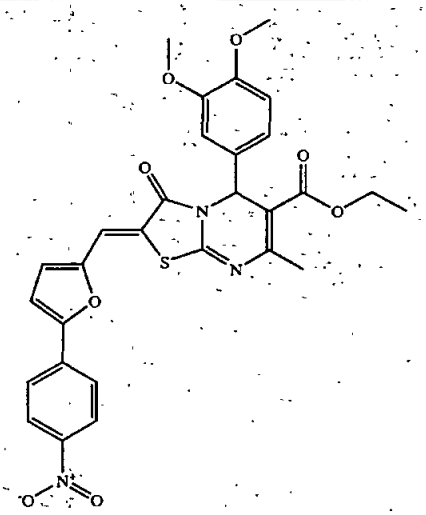
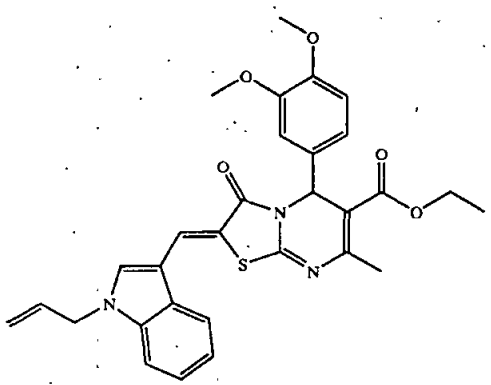
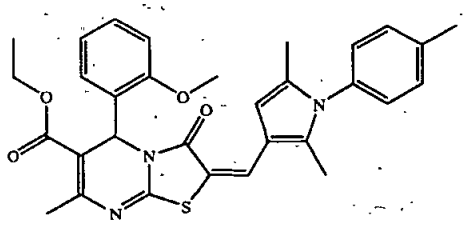
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZO |  | * | * | * | ** | * | ** |
| ZP |  | * | ** | *** | ** | ** | ** |
| ZQ |  | ** | *** | *** | ** | * | ND |

TABLE 5-continued

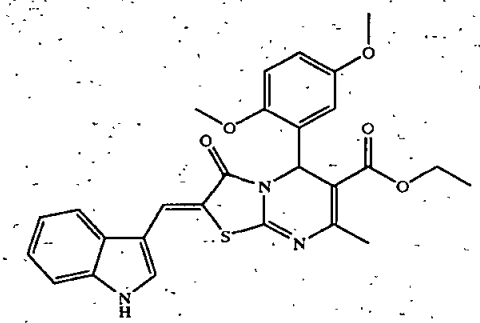
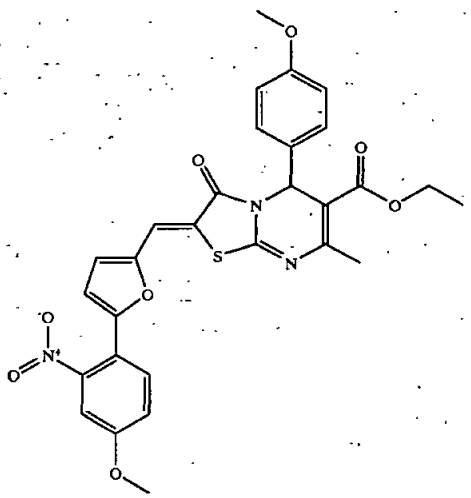
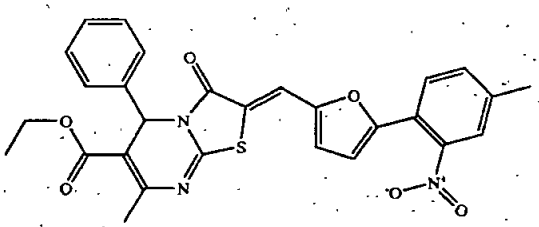
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZR |  | *** | *** | * | ** | ** | ** |
| ZS |  | * | *** | * | ** | ND | ** |
| ZT |  | ** | * | ** | ** | ND | ** |

TABLE 5-continued

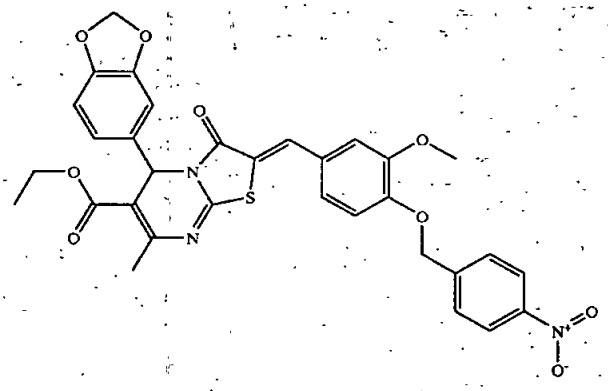
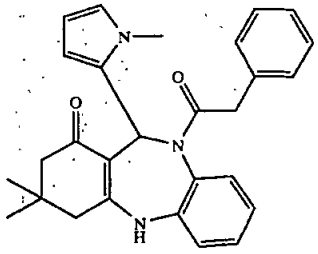
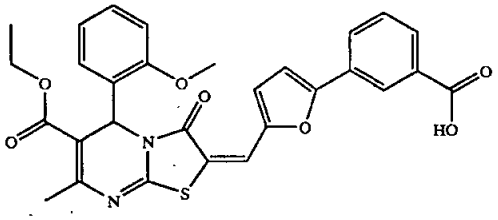
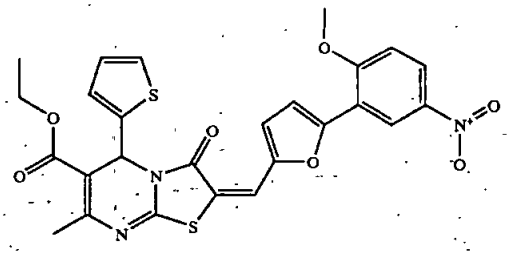
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ZU |  | * | * | * | ** | ND | ** |
| AAA |  | * | * | * | ** | ND | * |
| AAB |  | *** | *** | * | ** | ND | ND |
| AAC |  | ** | * | * | ** | ND | ND |

TABLE 5-continued

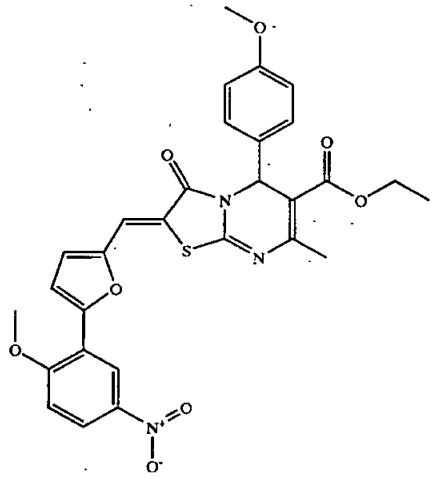
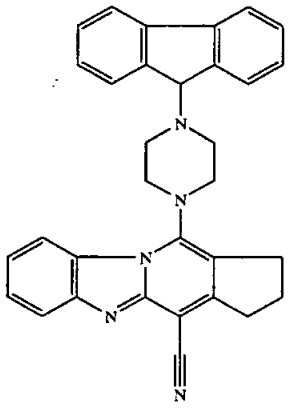
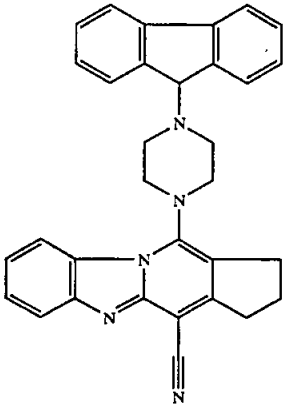
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAD |  | *** | - | - | ** | ND | - |
| AAE |  | ** | - | - | ** | ND | ** |
| AAF |  | *** | - | - | ** | ND | - |

TABLE 5-continued

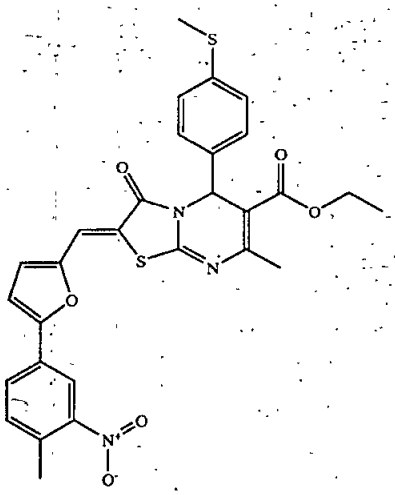
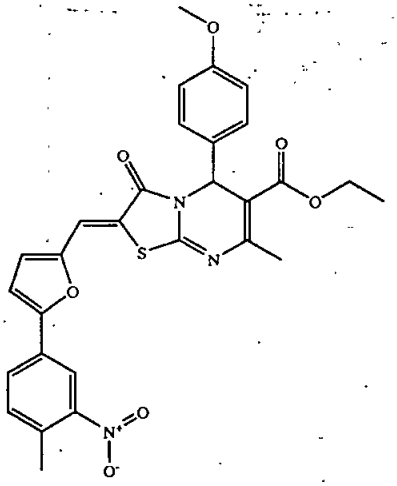
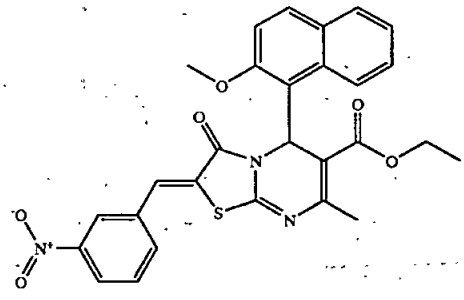
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAG |  | *** | * | * | ** | ND | ** |
| AAH |  | ** | * | * | ** | ND | ** |
| AAI |  | ** | * | *** | ** | ND | ND |

TABLE 5-continued

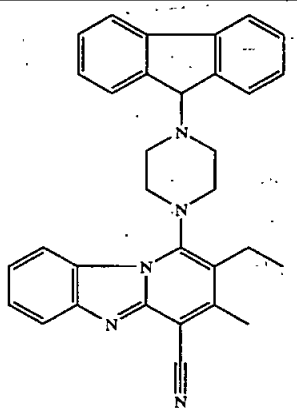
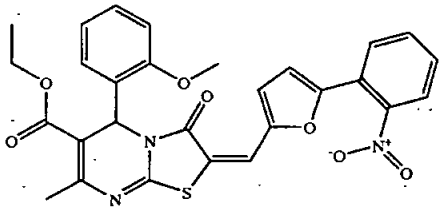
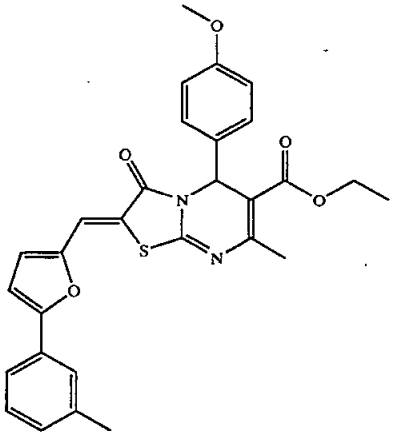
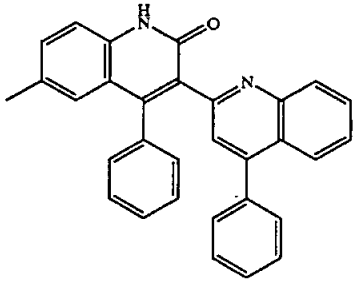
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAJ |  | *** | ** | NT | ** | ND | ND |
| AAK |  | *** | * | NT | ** | ND | * |
| AAL |  | * | * | * | ** | ND | ND |
| AAM |  | ** | *** | ** | ** | ** | ND |

TABLE 5-continued

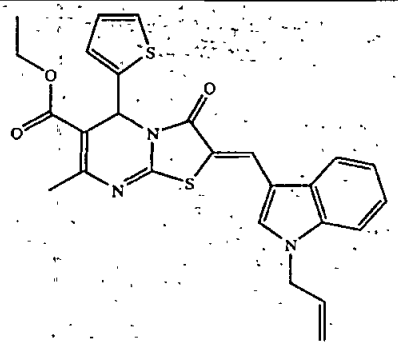
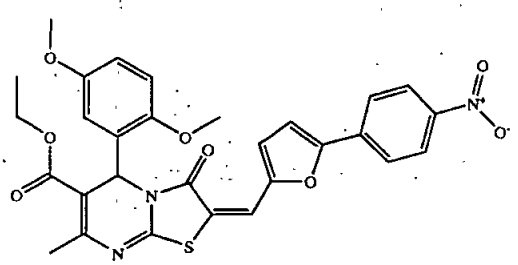
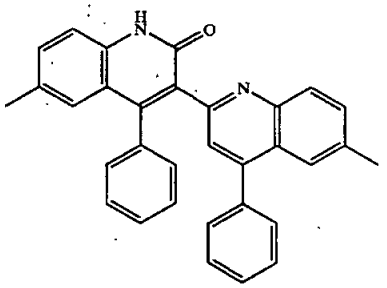
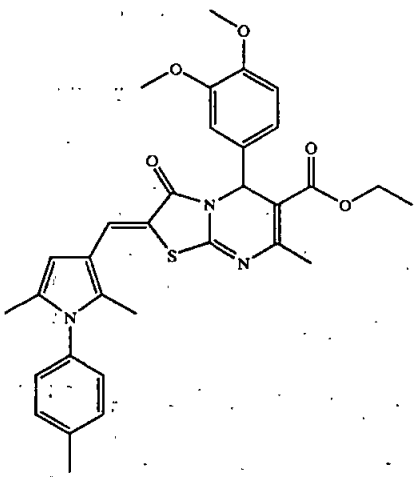
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAN |  | * | * | * | ** | ** | ND |
| AAO |  | * | * | * | ** | * | * |
| AAP |  | * | * | * | ** | * | * |
| AAQ |  | ** | * | * | ** | ** | ** |

TABLE 5-continued

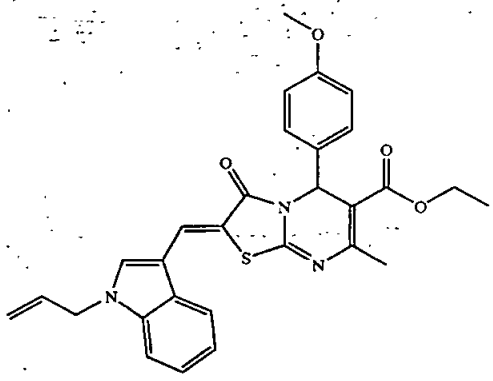
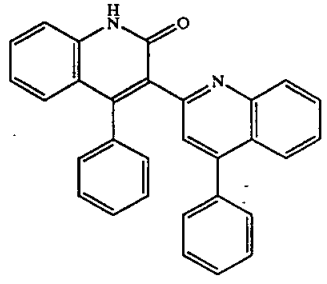
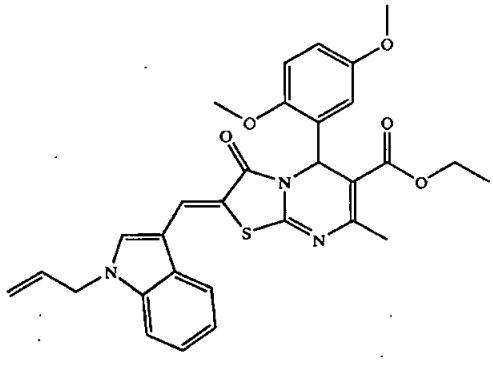
| ID | STRUCTURE | MarA Lance. | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAR |  | . | . | . | .. | .. | .. |
| AAS |  | . | .. | ... | .. | .. | . |
| AAT |  | . | ... | .. | .. | .. | . |

TABLE 5-continued

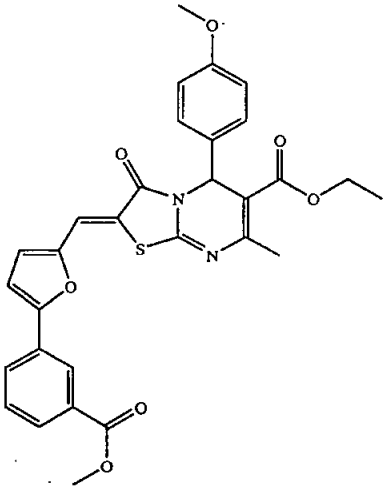
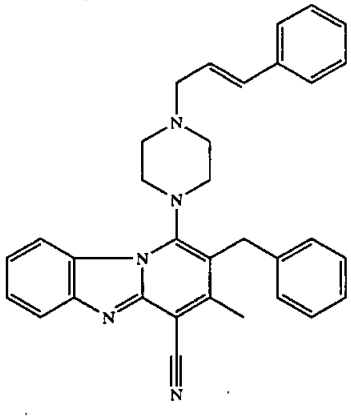
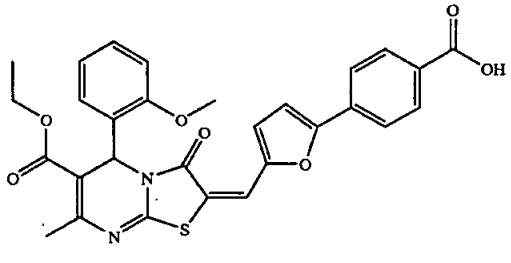
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAU |  | NT | * | * | ** | ND | * |
| AAV |  | NT | * | *** | ** | ND | ** |
| AAW |  | NT | *** | ** | ** | ND | ND |

TABLE 5-continued

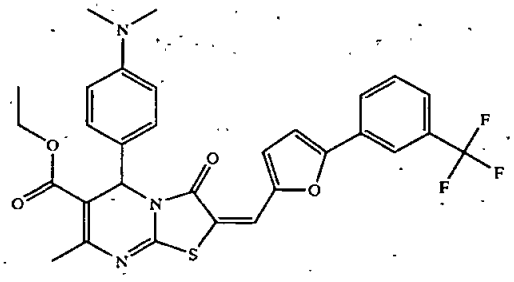
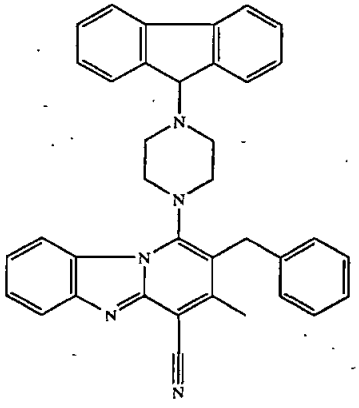
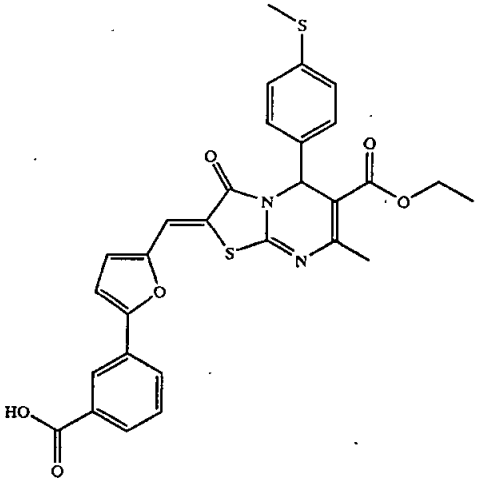
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AAX |  | NT | *** | ** | ** | ND | ** |
| AAZ |  | NT | * | * | ** | ND | ND |
| ABA |  | NT | ** | * | ** | ND | ** |

TABLE 5-continued

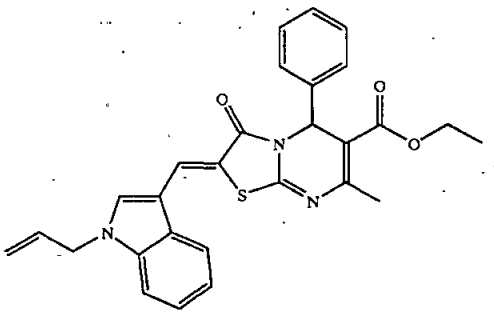
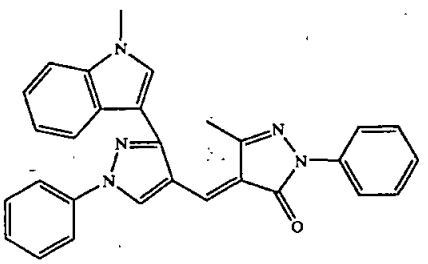
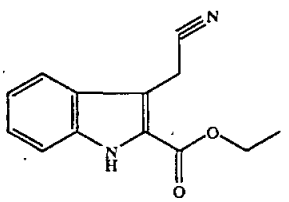
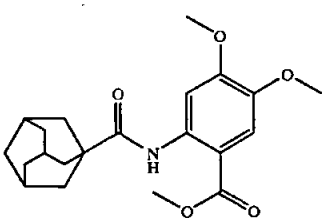
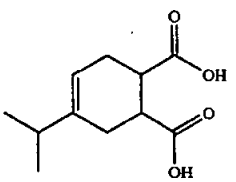
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ABB |  | NT | *** | *** | ** | ND | ** |
| ABC |  | *** | *** | * | ** | ** | ** |
| ABD |  | * | * | * | ** | * | ND |
| ABE |  | * | ** | * | ** | * | ** |
| ABF |  | * | * | * | ** | ** | ND |

TABLE 5-continued

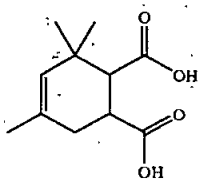
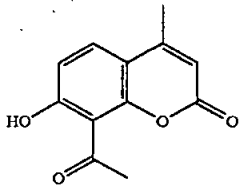
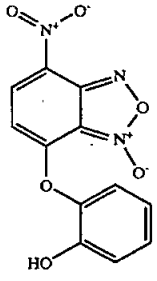
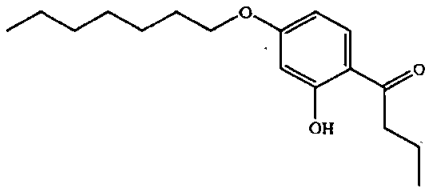
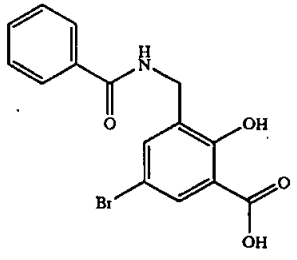
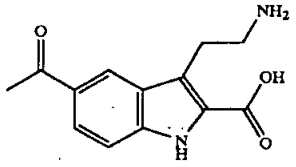
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ABG |  | * | ** | *** | ** | * | ND |
| ABH |  | * | * | * | ** | ** | * |
| ABI |  | ** | NT | NT | ** | ND | ** |
| ABJ |  | * | * | *** | ** | ND | * |
| ABK |  | * | * | ** | ** | ND | ** |
| ABL |  | * | * | * | ** | ND | ** |

TABLE 5-continued

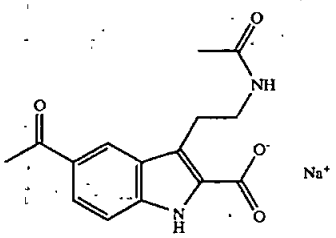
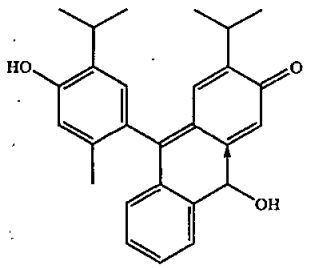
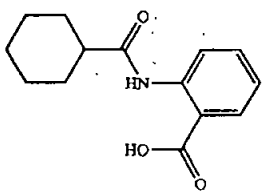
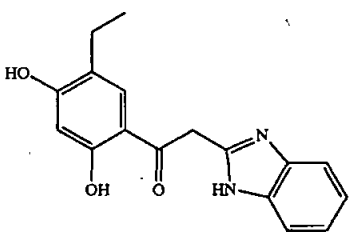
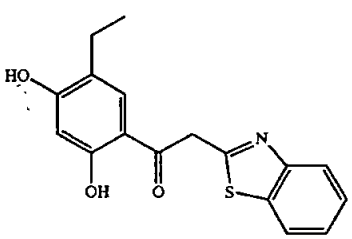
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ABN |  | * | * | * | ** | ND | ** |
| ABO |  | * | * | *** | ** | ND | ** |
| ABP |  | *** | * | * | ** | ND | ** |
| ABQ |  | * | * | * | ** | ND | ** |
| ABR |  | * | * | * | ** | ND | ** |

TABLE 5-continued

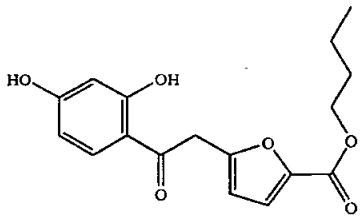
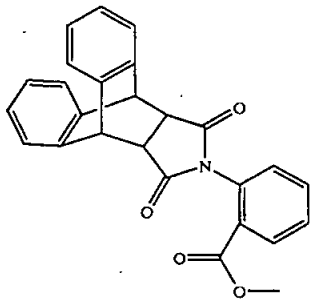
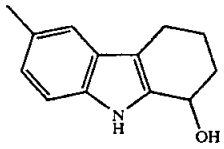
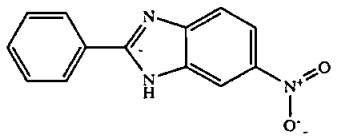
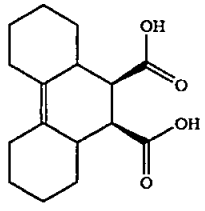
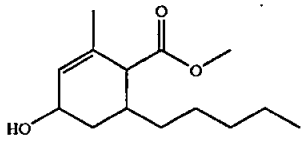
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ABS |  | * | * | * | ** | ND | ** |
| ABT |  | * | * | * | ** | ND | * |
| ABU |  | * | * | * | ** | ND | ** |
| ABV |  | * | ** | *** | ** | ND | ** |
| ABW |  | * | * | * | ** | ND | ** |
| ABX |  | *** | *** | ** | ** | ND | ** |

TABLE 5-continued

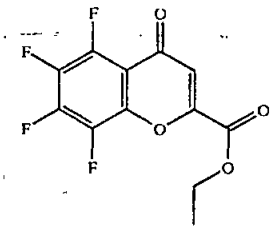
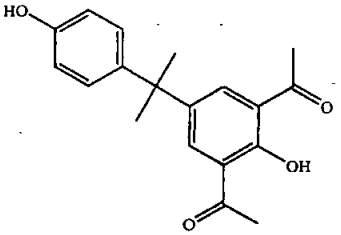
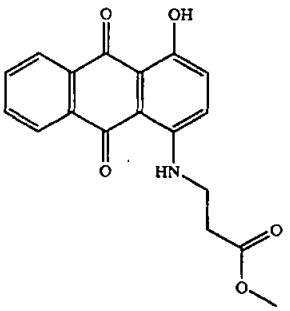
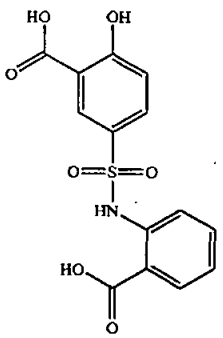
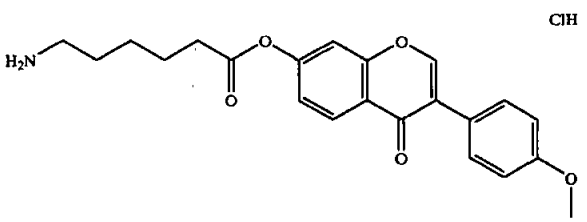
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ABY |  | * | * | * | ** | ND | ** |
| ABZ |  | * | ** | *** | ** | ND | * |
| ACA |  | * | ** | *** | ** | ND | * |
| ACB |  | * | * | * | ** | ND | ND |
| ACC |  | CIH | * | * | * | ** | ND | ND |

TABLE 5-continued

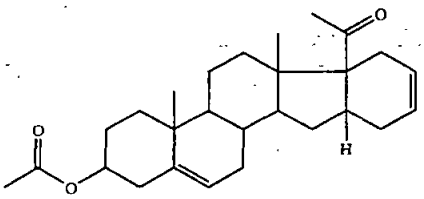
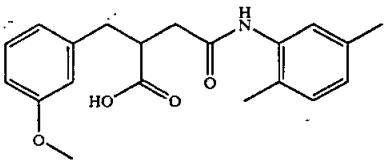
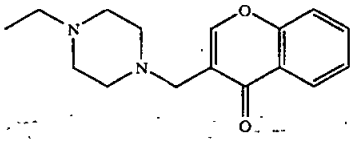
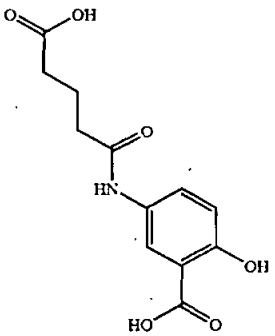
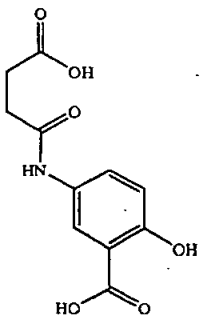
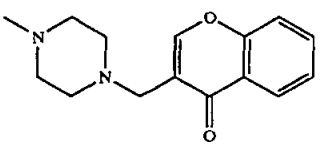
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ACD |  | * | * | * | ** | ND | * |
| ACE |  | * | * | * | ** | ND | ND |
| ACF |  | * | * | * | ** | ND | * |
| ACG |  | * | * | * | ** | ND | ** |
| ACH |  | ** | * | * | ** | ND | ND |
| ACI |  | * | ** | *** | ** | * | ** |

TABLE 5-continued

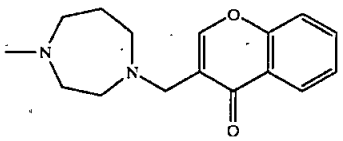
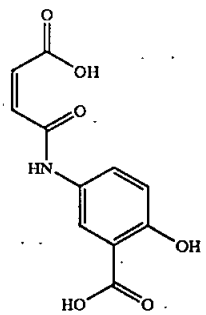
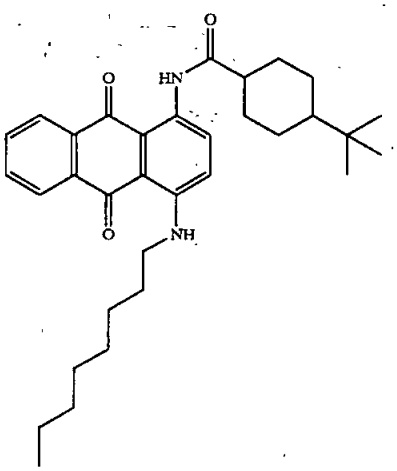
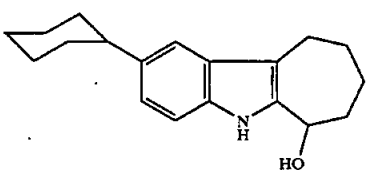
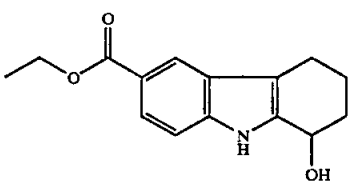
| ID | STRUCTURE | MurA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ACJ |  | * | ** | * | ** | * | ND |
| ACK |  | * | * | * | ** | * | ND |
| ACL |  | * | * | * | ** | * | ND |
| ACM |  | *** | *** | *** | ** | ND | ** |
| ACN |  | *** | *** | ** | ** | * | ** |

TABLE 5-continued

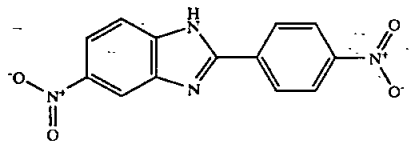
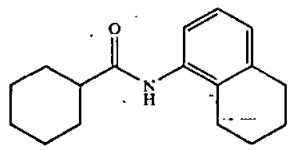
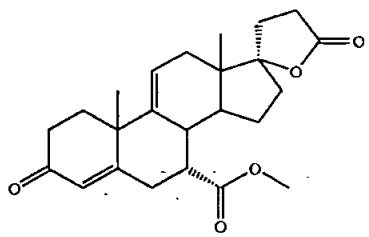
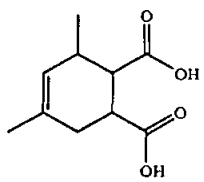
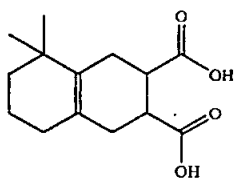
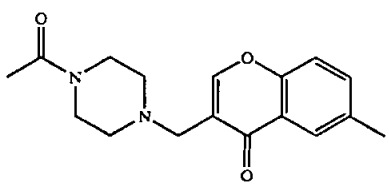
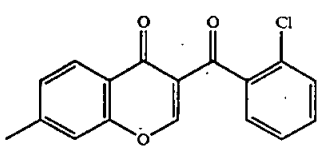
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ACO |  | *** | ** | * | ** | * | ** |
| ACP |  | *** | *** | ** | ** | ** | ** |
| ACQ |  | ** | *** | ** | ** | ** | ** |
| ACR |  | *** | *** | * | ** | ** | ** |
| ACS |  | * | * | * | ** | ND | ** |
| ACT |  | ** | * | ** | ** | ND | ** |
| ACU |  | ** | * | *** | ** | ND | ** |

TABLE 5-continued

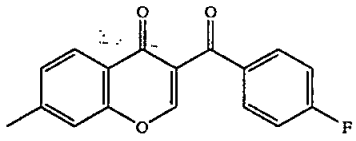
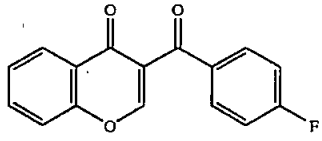
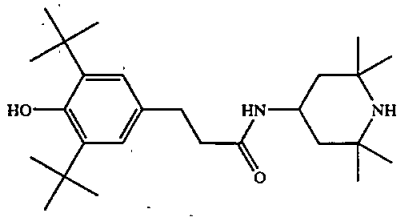
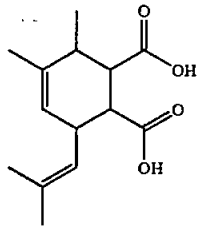
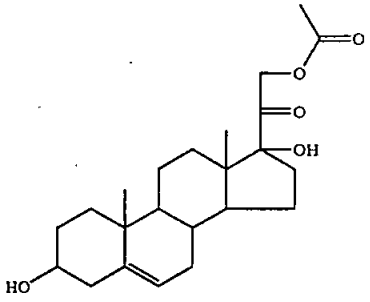
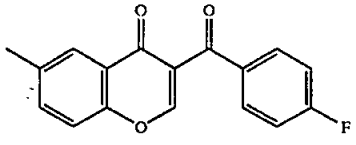
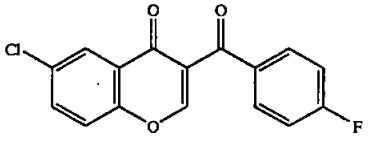
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ACV |  | * | ** | * | ** | ND | ** |
| ACW |  | ** | ** | ** | ** | ND | * |
| ACX |  | ** | ** | ** | ** | ND | * |
| ACY |  | ** | * | ** | ** | ND | ND |
| ACZ |  | ** | * | * | ** | ND | * |
| ADA |  | *** | *** | *** | ** | ND | ND |
| ADB |  | ** | * | *** | ** | ND | ND |

TABLE 5-continued

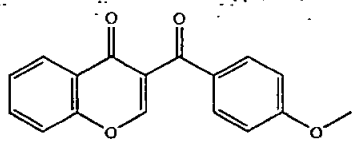
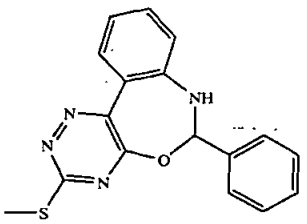
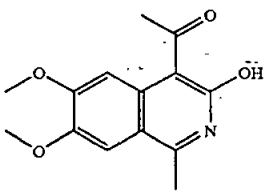
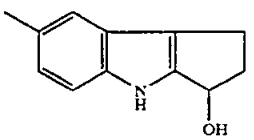
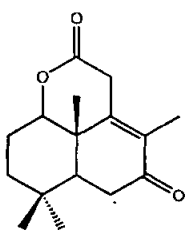
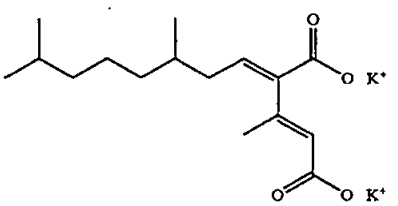
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ADC |  | *** | ** | *** | ** | ND | * |
| ADD |  | *** | *** | *** | ** | ND | ** |
| ADE |  | ** | * | * | ** | ND | * |
| ADF |  | ** | * | * | ** | ND | ** |
| ADG |  | * | * | * | ** | ND | ** |
| ADH |  | ** | * | * | ** | ND | ND |

TABLE 5-continued

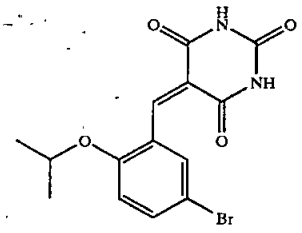
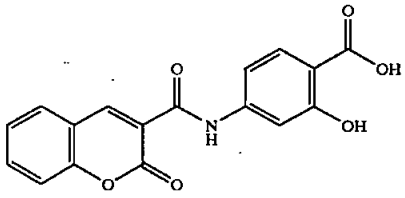
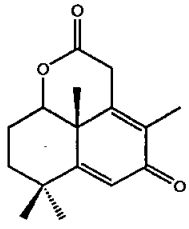
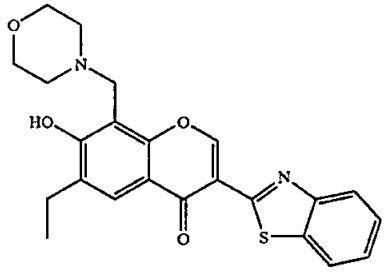
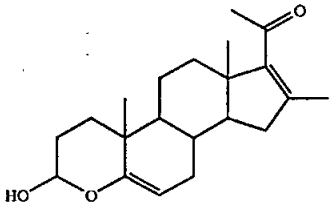
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ADI |  | ** | * | * | ** | ND | * |
| ADJ |  | ** | * | * | ** | ND | ** |
| ADK |  | ** | * | * | ** | ND | ** |
| ADL |  | * | * | * | ** | ND | ** |
| ADM |  | ** | * | * | ** | ND | * |

TABLE 5-continued

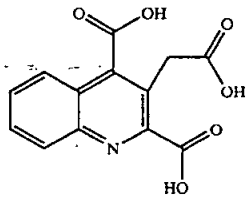
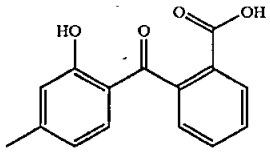
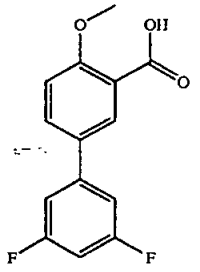
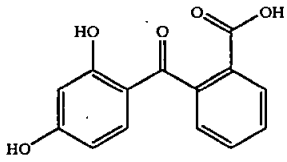
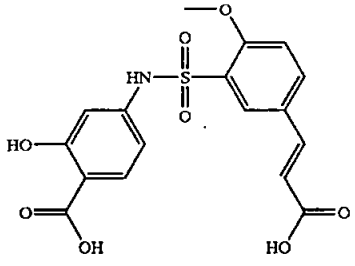
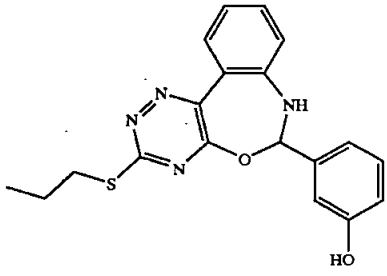
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ADN |  | ** | * | * | ** | ND | * |
| ADO |  | ** | * | * | ** | ND | ND |
| ADP |  | ** | ** | *** | ** | ND | * |
| ADQ |  | ** | *** | * | ** | ND | ND |
| ADR |  | *** | ** | NT | ** | ND | ** |
| ADS |  | * | ** | ** | * | ** | ** |

TABLE 5-continued

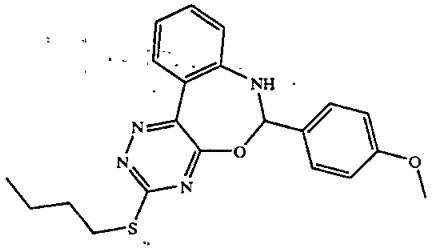
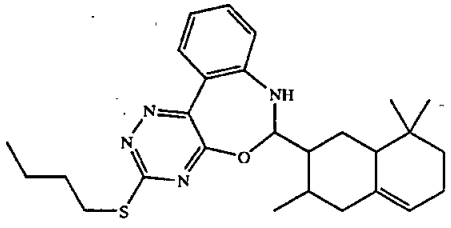
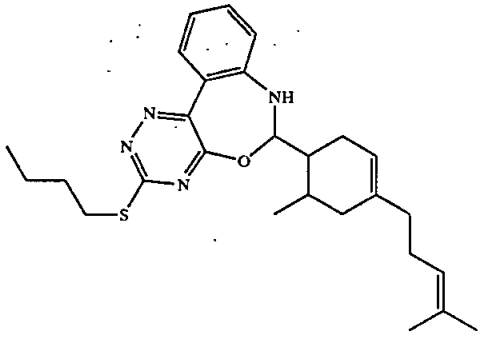
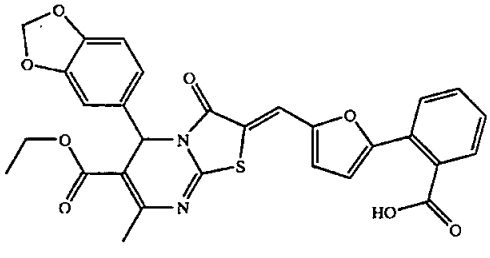
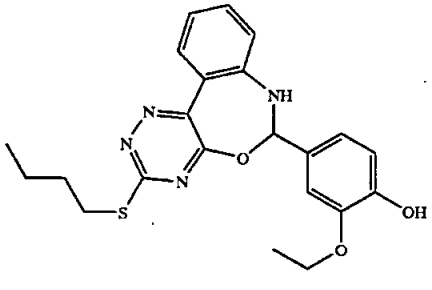
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ADT |  | * | * | * | * | ** | ** |
| ADU |  | NT | * | ** | * | * | ** |
| ADV |  | NT | ** | * | * | * | ND |
| ADW |  | NT | * | * | * | * | ND |
| ADX |  | *** | *** | * | * | * | * |

TABLE 5-continued

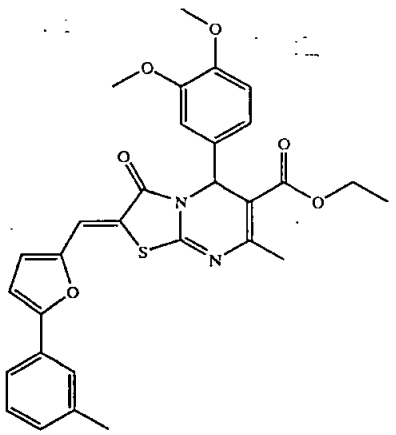
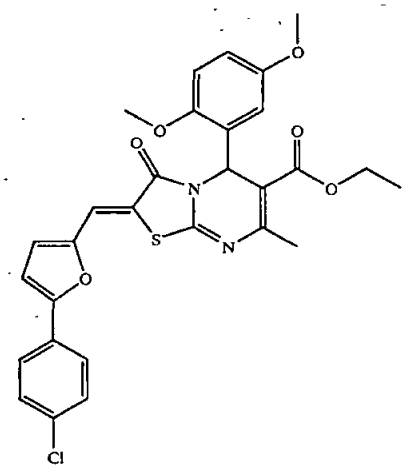
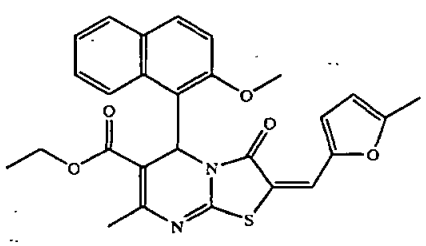
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ADY |  | • | • | • | • | •• | •• |
| ADZ |  | • | •• | •• | • | •• | •• |
| AEA |  | • | • | • | • | • | •• |

TABLE 5-continued

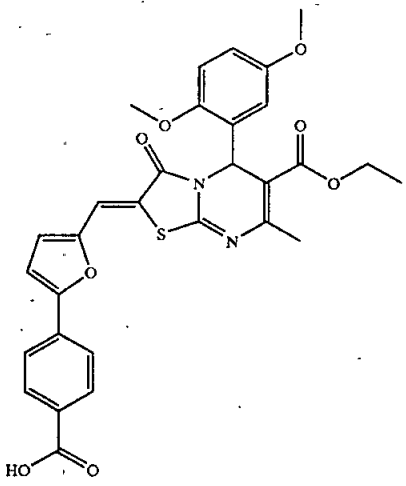
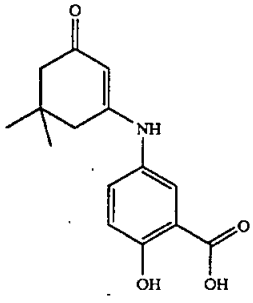
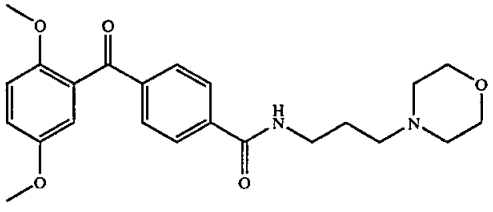
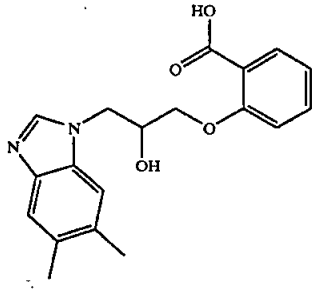
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AEB |  | * | * | *** | * | * | ** |
| AEC |  | * | * | ** | * | * | ** |
| AED |  | *** | * | * | * | * | ** |
| AEF |  | * | * | * | * | * | ND |

TABLE 5-continued

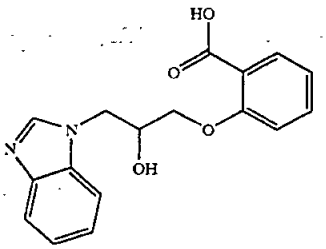
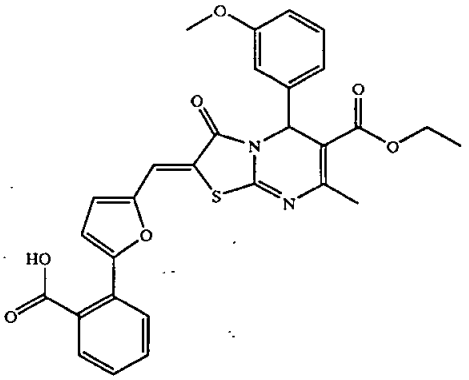
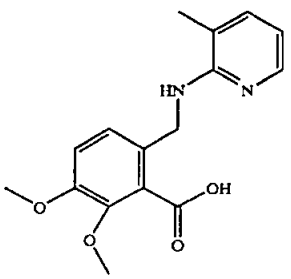
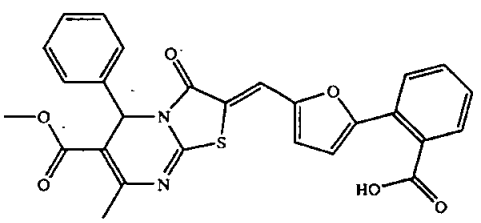
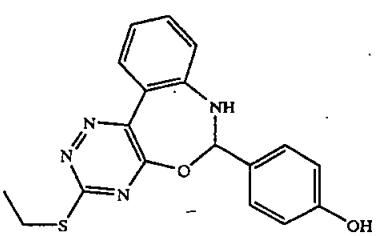
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AEG |  | • | •• | ••• | • | • | •• |
| AEH |  | • | • | • | • | • | •• |
| AEI |  | • | • | • | • | • | • |
| AEJ |  | • | •• | ••• | • | • | • |
| AEK |  | • | •• | •• | • | • | ND |

TABLE 5-continued

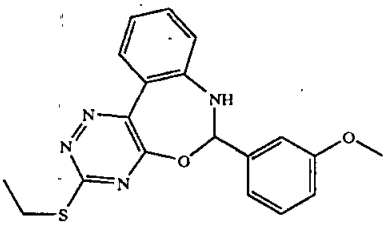
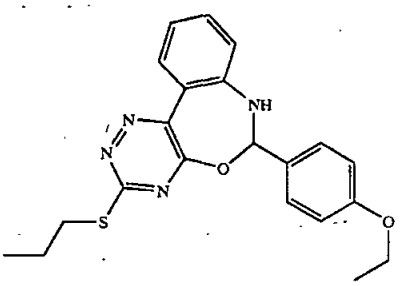
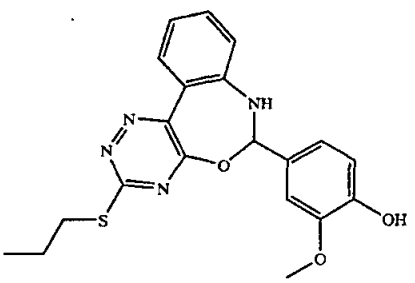
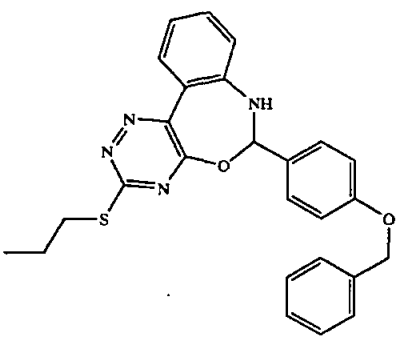
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AEL |  | * | * | * | * | * | ND |
| AEM |  | * | * | ** | * | * | ** |
| EN |  | * | * | * | * | ** | ND |
| AEO |  | * | * | * | * | * | ** |

TABLE 5-continued

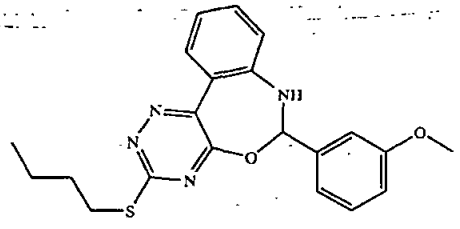
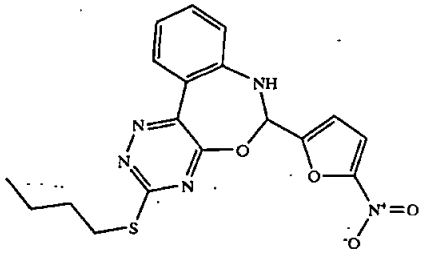
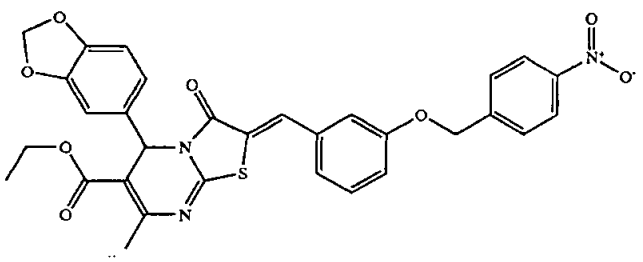
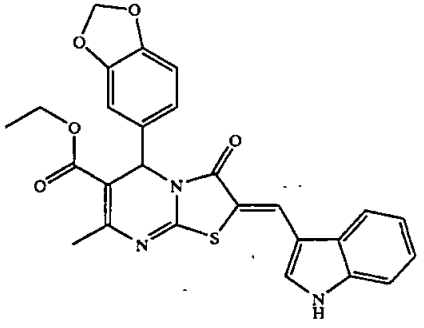
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AEQ |  | * | * | * | * | ** | ** |
| ER |  | * | *** | *** | * | ** | ** |
| AES |  | *** | * | * | * | * | ** |
| AET |  | *** | *** | * | * | * | ** |

TABLE 5-continued

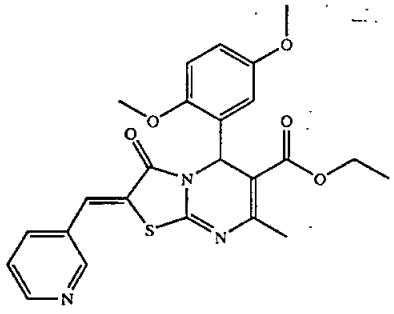
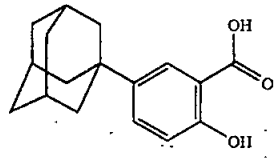
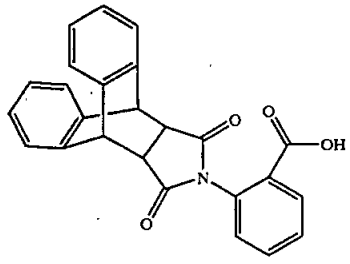
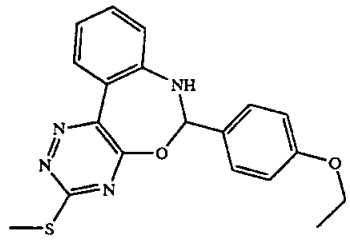
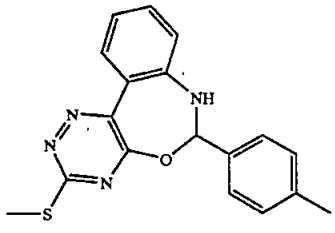
| ID | STRUCTURE | MurA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| EU |  | *** | *** | * | * | ** | ** |
| AEV |  | ** | ** | * | * | * | * |
| AEW |  | ** | * | * | * | * | * |
| EX |  | ** | ** | * | * | * | ND |
| AEY |  | ** | ** | ** | * | * | ** |

TABLE 5-continued

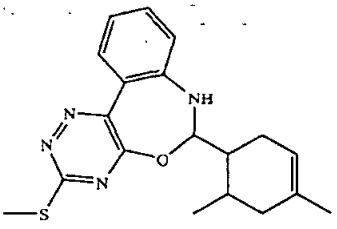
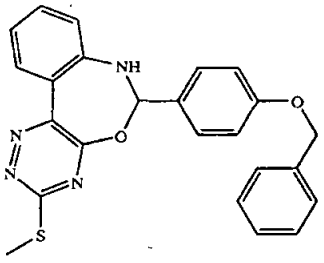
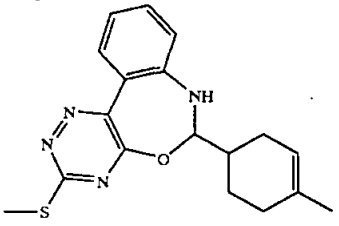
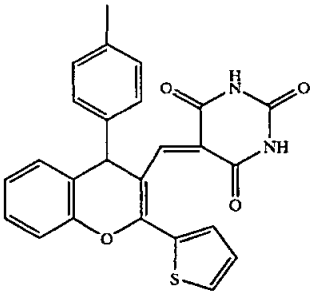
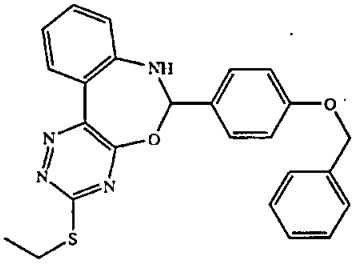
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AEZ |  | ** | * | * | * | * | ** |
| AFA |  | ** | * | ** | * | * | * |
| FB |  | ** | * | ** | * | * | ** |
| AFC |  | ** | * | * | * | * | ** |
| AFD |  | * | * | * | * | * | ** |

TABLE 5-continued

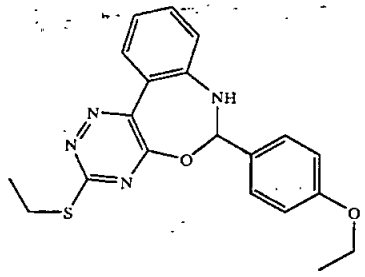
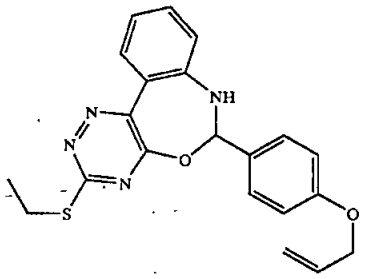
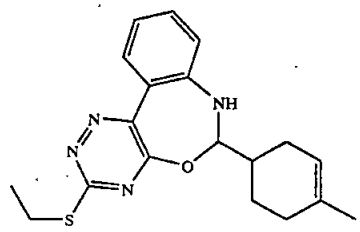
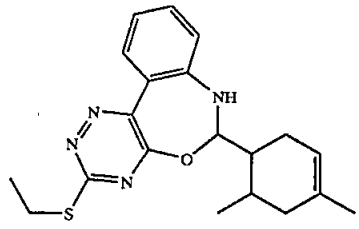
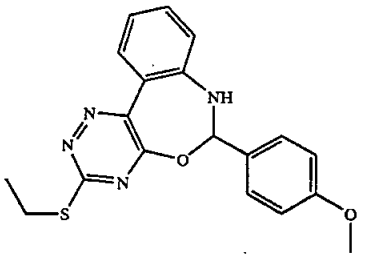
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AFE |  | * | * | * | * | * | ** |
| AFG |  | ** | * | * | * | * | ** |
| AFH |  | * | * | * | * | * | ND |
| AFI |  | ** | ** | * | * | * | ** |
| AFJ |  | * | ** | ** | * | ** | * |

TABLE 5-continued

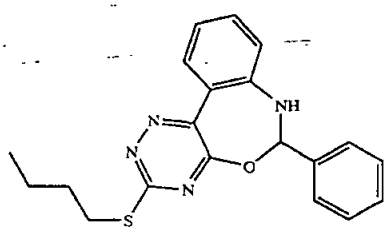
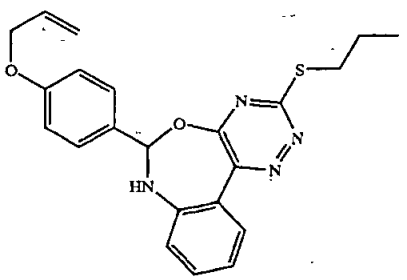
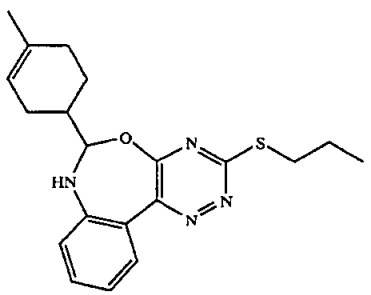
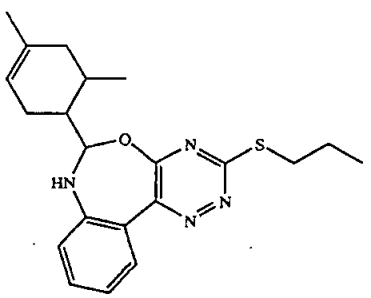
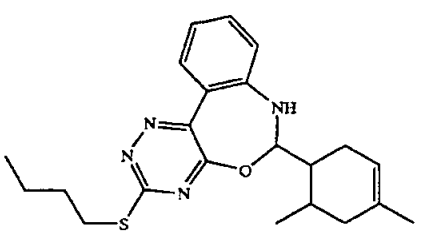
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AFK |  | * | * | ** | * | ** | ** |
| AFL |  | * | * | * | * | ** | * |
| AFM |  | * | * | * | * | ** | ** |
| AFN |  | * | NT | NT | * | ** | * |
| AFO |  | * | * | *** | * | ** | ND |

TABLE 5-continued

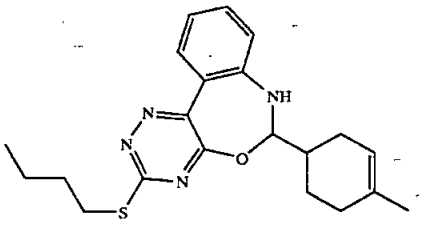
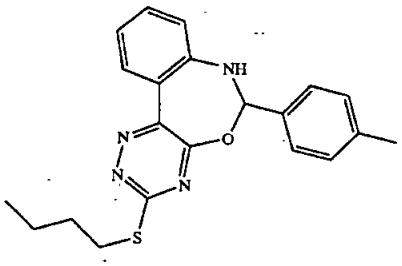
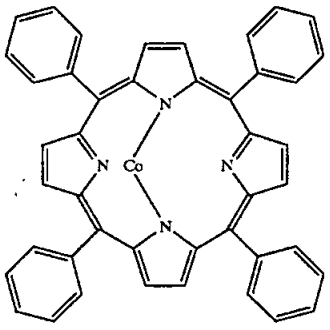
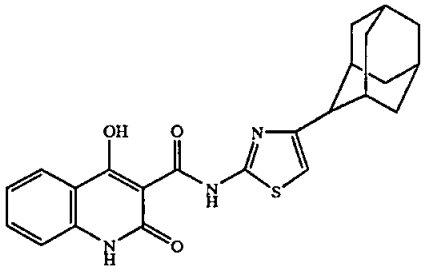
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AFP |  | * | * | * | * | ** | ** |
| AFQ |  | * | * | * | * | ** | ** |
| AFR |  | NT | *** | *** | * | * | * |
| AFS |  | NT | ** | ** | * | * | * |

TABLE 5-continued

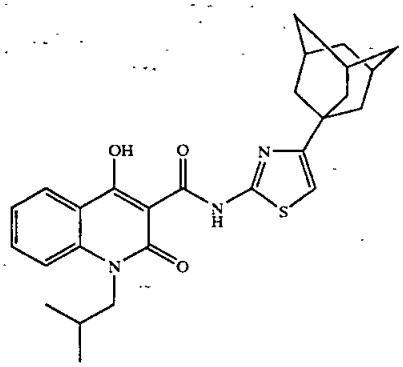
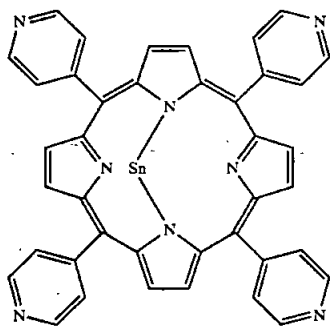
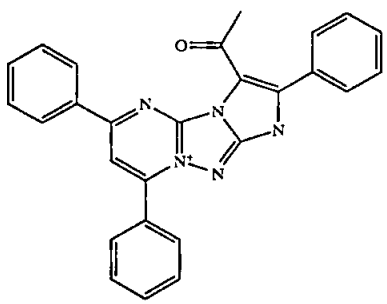
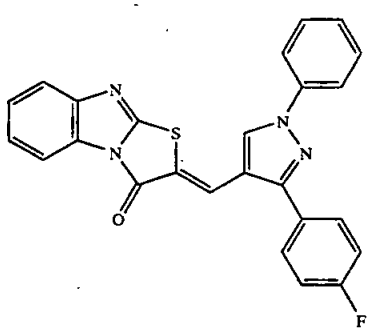
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AFT |  | NT | * | * | * | * | * |
| AFU |  | * | NT | NT | * | * | ND |
| AFV |  | * | * | *** | * | * | ** |
| AFW |  | * | * | * | * | * | * |

TABLE 5-continued

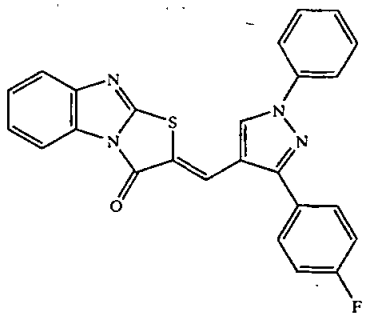
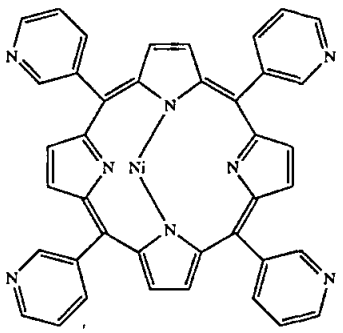
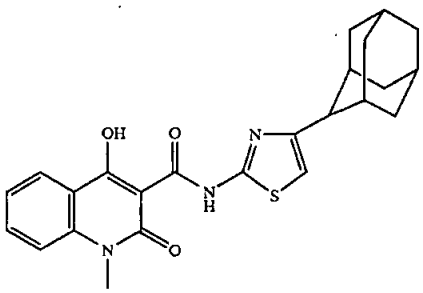
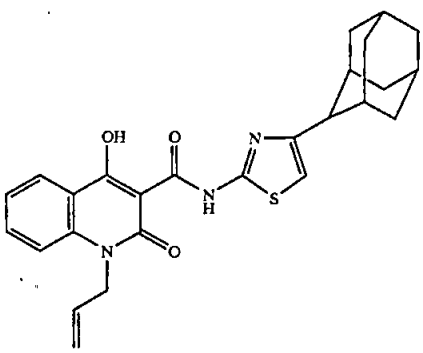
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AFX |  | * | - | * | * | * | ** |
| AFY |  | *** | *** | * | * | * | ND |
| AFZ |  | * | * | *** | * | * | ** |
| AGA |  | * | * | * | * | * | ND |

TABLE 5-continued

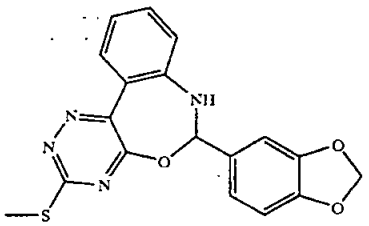
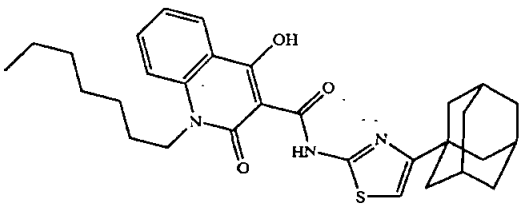
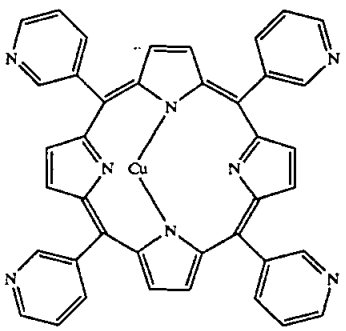
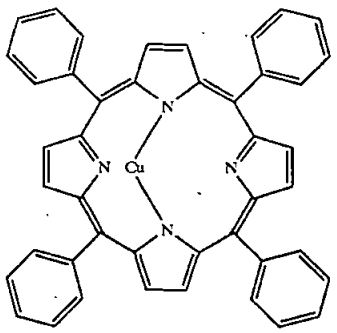
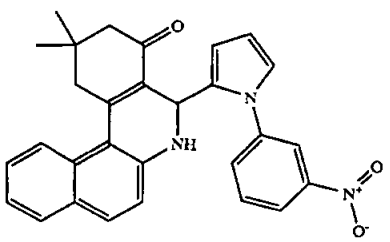
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGB |  | * | ** | *** | - | * | * |
| AGC |  | * | ** | *** | - | * | * |
| AGD |  | * | * | * | - | * | ND |
| AGE |  | ** | ** | *** | - | * | * |
| AGF |  | * | * | * | - | * | ** |

TABLE 5-continued

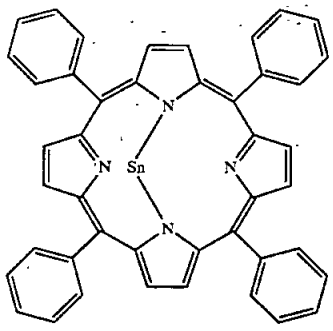
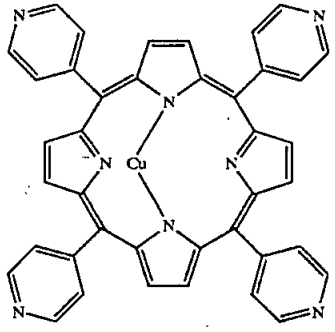
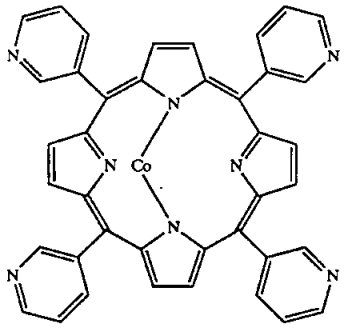
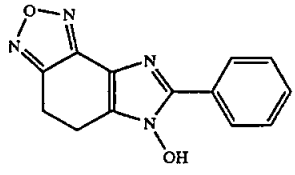
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGG |  | * | ** | ** | * | * | ** |
| AGH |  | * | * | ** | * | * | ** |
| AGI |  | * | * | * | * | * | ** |
| AGJ |  | * | * | * | * | * | ** |

TABLE 5-continued

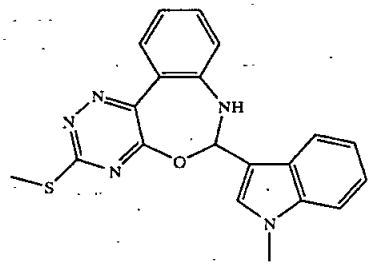
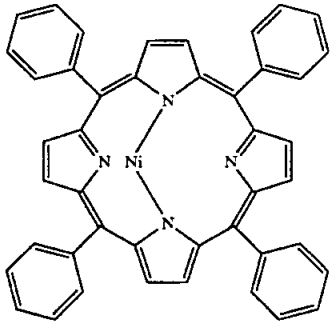
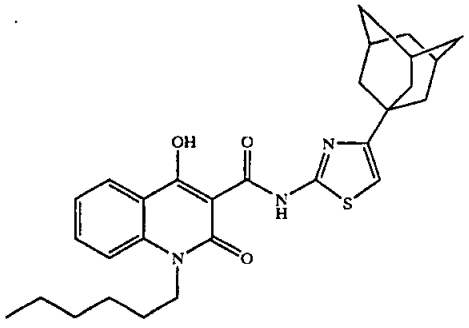
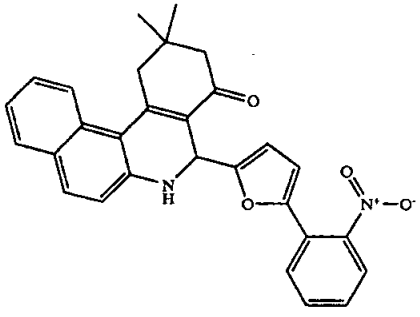
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGK |  | * | ** | * | * | ** | ** |
| AGL |  | *** | *** | * | * | * | ** |
| AGM |  | *** | *** | ** | * | * | ** |
| AGN |  | ** | * | ** | * | * | * |

TABLE 5-continued

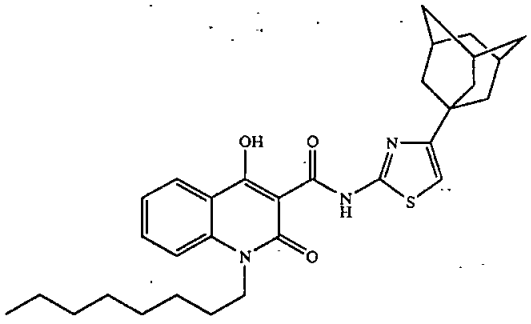
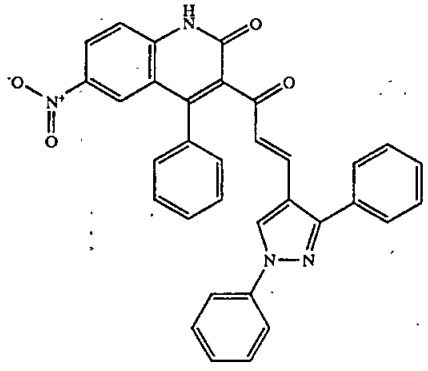
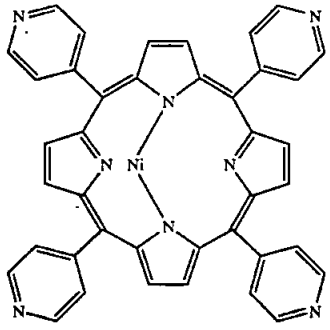
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGO |  | ** | * | * | * | * | * |
| AGP |  | ** | ** | ** | * | * | ** |
| AGQ |  | ** | * | * | * | * | ** |

TABLE 5-continued

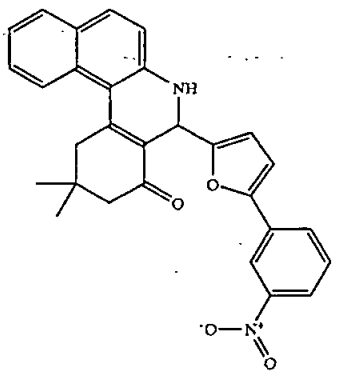
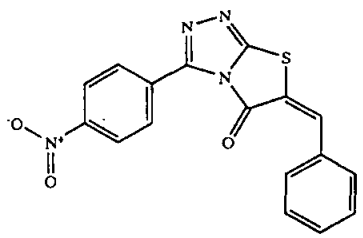
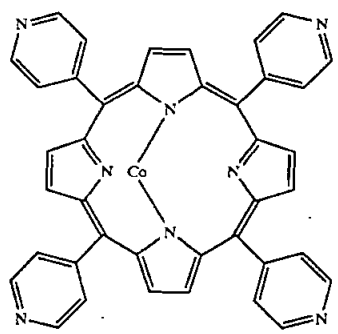
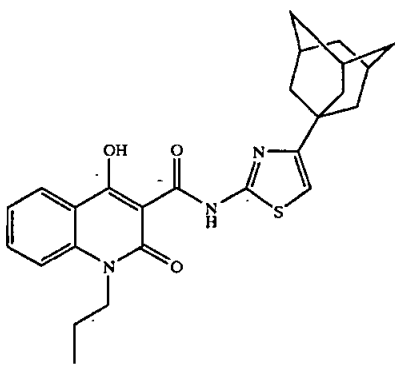
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGR |  | *** | - | ** | - | - | ND |
| AGS |  | *** | - | ** | - | - | ** |
| AGT |  | - | - | - | - | - | - |
| AGU |  | ** | - | - | - | - | ND |

TABLE 5-continued

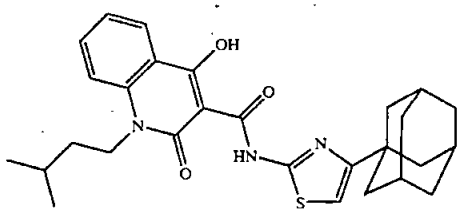
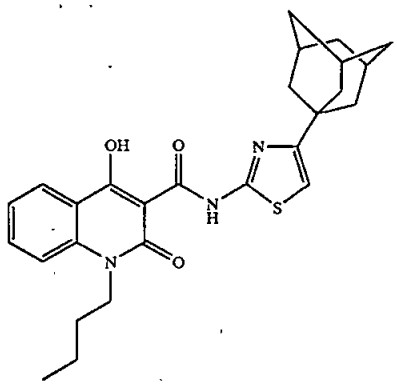
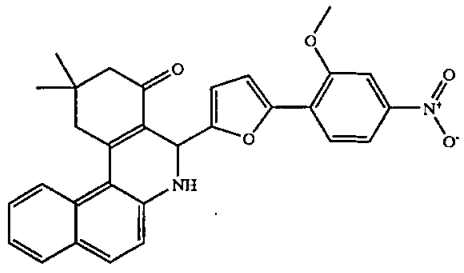
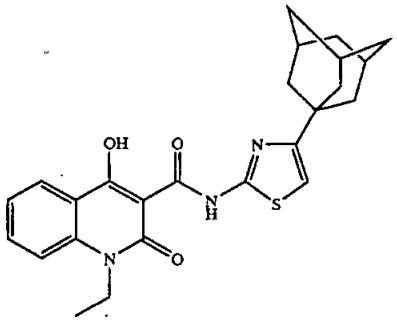
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGV |  | *** | *** | ** | * | * | ** |
| AGW |  | *** | ** | NT | * | * | * |
| AGX |  | ** | ** | *** | * | ** | ** |
| AGY |  | * | ** | * | * | ** | * |

TABLE 5-continued

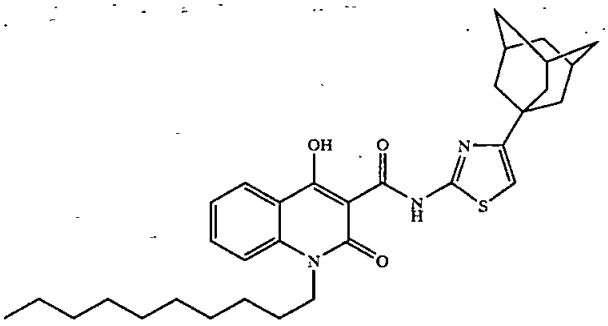
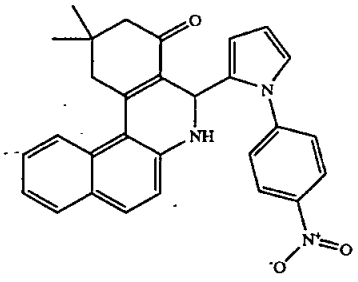
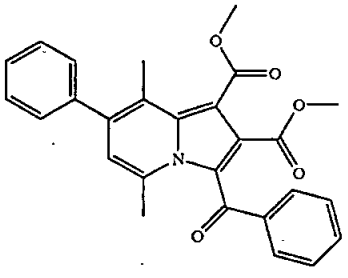
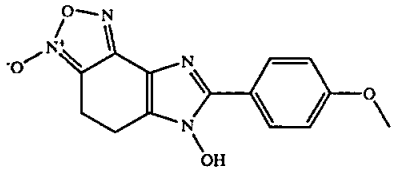
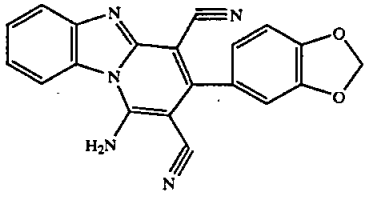
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AGZ |  | *** | *** | * | * | ** | ** |
| AHA |  | * | ** | * | * | ** | ** |
| AHB |  | NT | ** | * | * | * | ** |
| AHC |  | * | ** | * | * | * | * |
| AHD |  | * | * | * | * | ** | * |

TABLE 5-continued

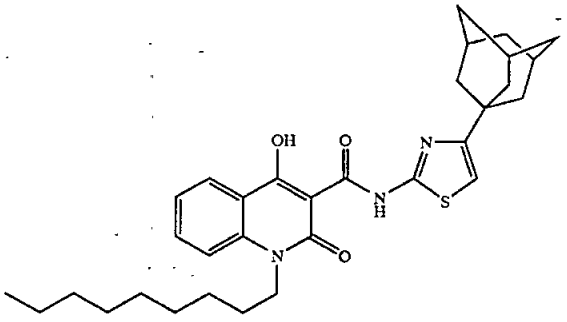
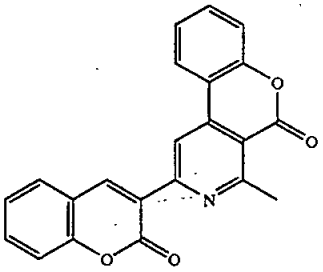
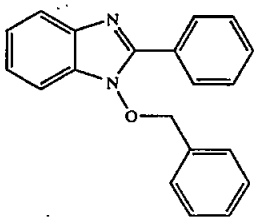
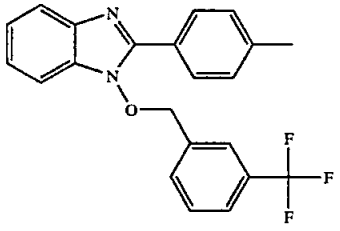
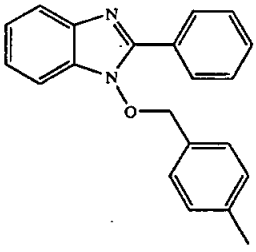
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AHE |  | * | * | * | * | * | * |
| AHF |  | * | * | * | * | * | ** |
| AHG |  | * | * | * | * | * | ** |
| AHH |  | * | * | * | * | * | ** |
| AHI |  | * | * | * | * | * | ND |

TABLE 5-continued

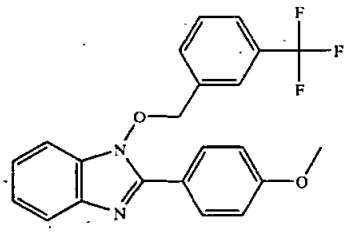
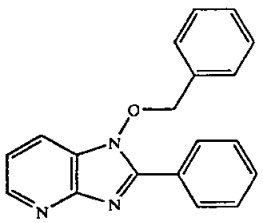
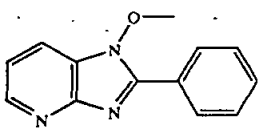
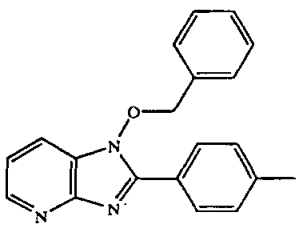
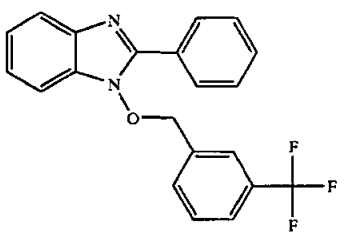
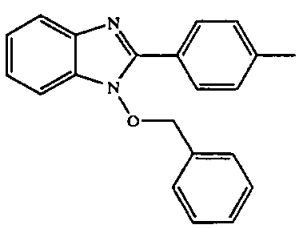
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AHJ |  | * | * | * | * | * | ND |
| HK |  | ** | ** | *** | * | * | ** |
| AHL |  | * | * | * | * | * | ND |
| AHM |  | * | * | * | * | * | ND |
| AHN |  | ** | ** | *** | * | * | ND |
| AHO |  | * | * | * | * | * | ND |

TABLE 5-continued

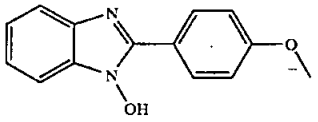
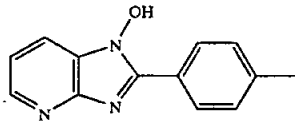
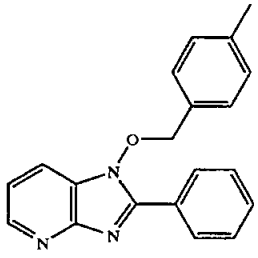
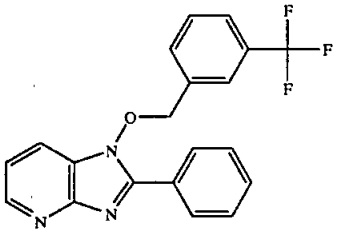
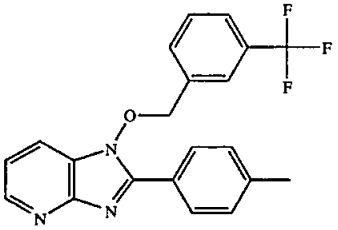
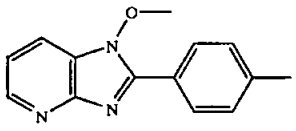
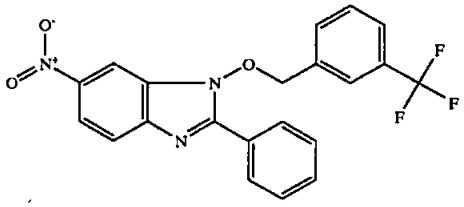
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AHP |  | *** | *** | ** | * | ** | ** |
| AHQ |  | * | * | * | * | * | * |
| AHR |  | *** | *** | *** | * | * | * |
| AHS |  | ** | * | ** | * | * | * |
| AHT |  | ** | * | * | * | * | ** |
| AHU |  | ** | * | * | * | * | ND |
| AHV |  | ** | * | * | * | * | ** |

TABLE 5-continued

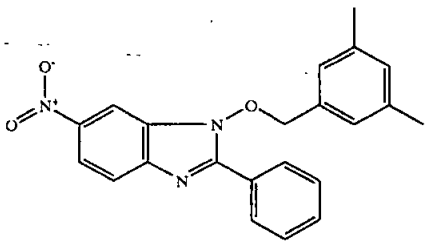
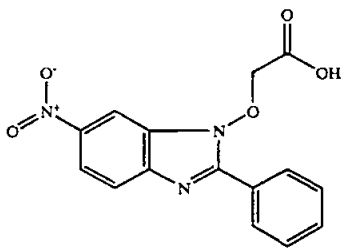
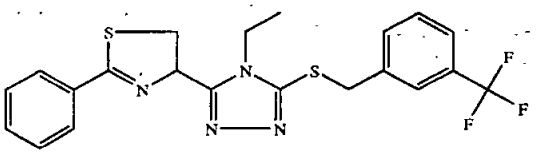
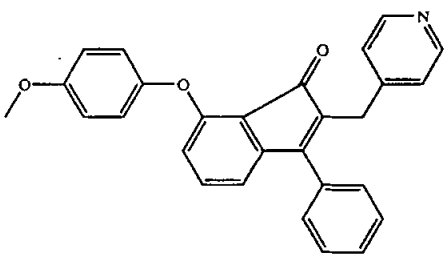
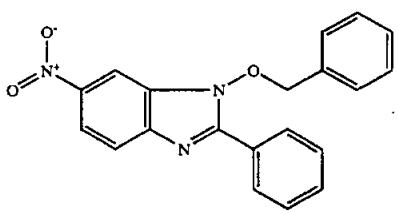
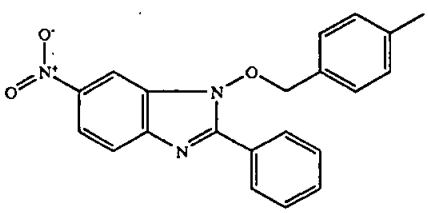
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AHW |  | ** | *** | ** | * | * | ND |
| AHX |  | *** | *** | NT | * | * | ND |
| AHY |  | * | ** | *** | * | ** | ** |
| AHZ |  | * | NT | NT | * | ** | ND |
| AIA |  | ** | ** | * | * | ** | ND |
| AIB |  | * | * | * | * | * | ** |

TABLE 5-continued

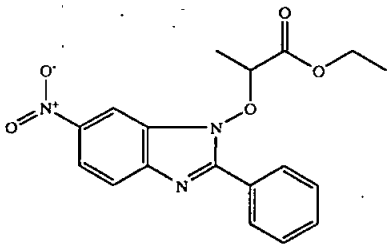
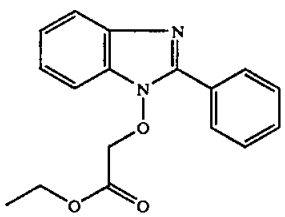
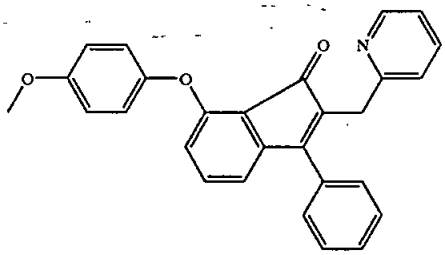
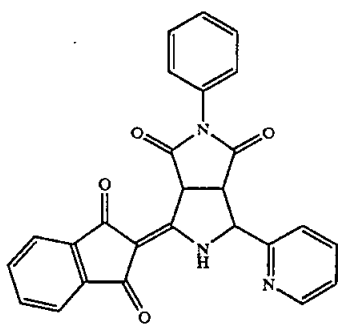
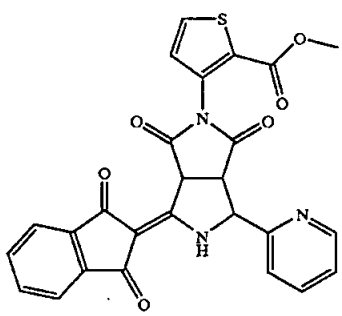
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AIC |  | *** | *** | *** | * | * | ** |
| AID |  | * | * | * | * | * | ND |
| AIE |  | * | * | * | * | * | * |
| AIF |  | * | * | * | * | * | ND |
| AIG |  | ** | * | * | * | * | ND |

TABLE 5-continued

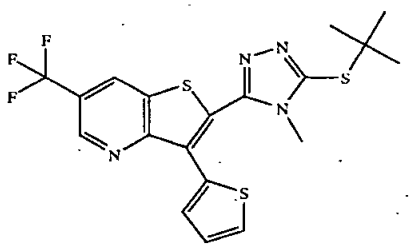
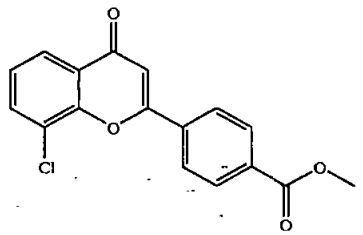
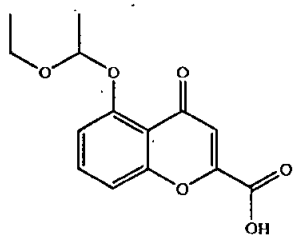
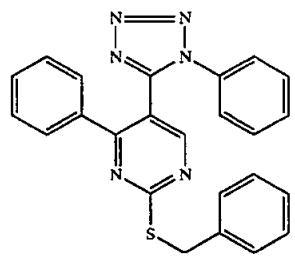
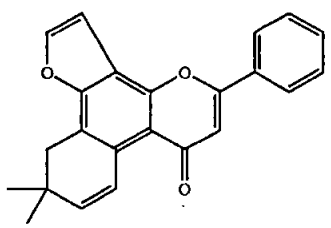
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AIH |  | * | * | * | * | * | ** |
| AII |  | * | ** | * | * | * | ND |
| AIJ |  | *** | *** | * | * | ** | ND |
| AIK |  | ** | ** | * | * | * | ND |
| AIL |  | *** | ** | *** | * | * | * |

TABLE 5-continued

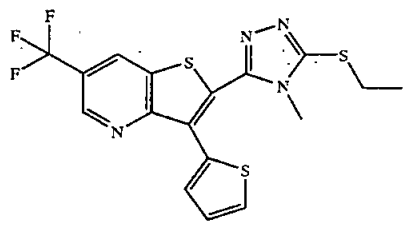
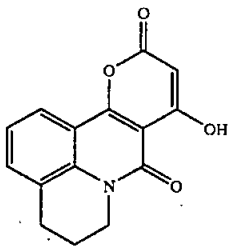
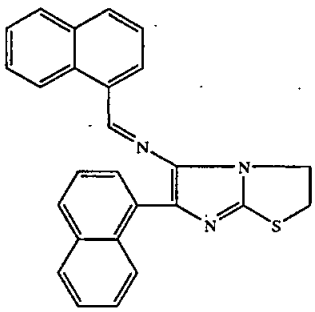
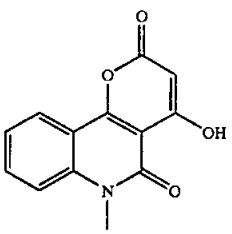
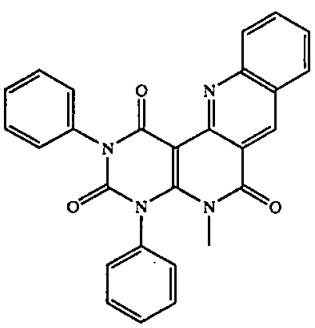
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AIM |  | * | * | * | * | * | ** |
| AIN |  | * | * | * | * | * | ** |
| AIO |  | * | ** | ** | * | * | ** |
| AIP |  | ** | * | * | * | * | ND |
| AIQ |  | ** | ** | ** | * | * | ND |

TABLE 5-continued

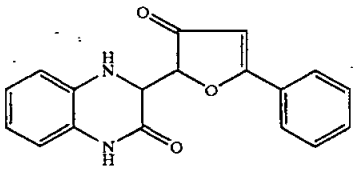
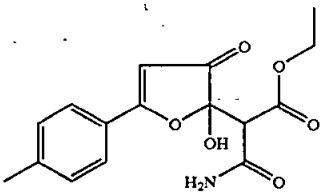
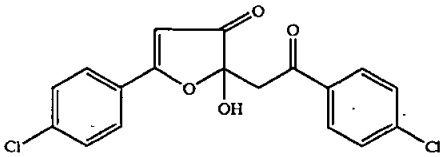
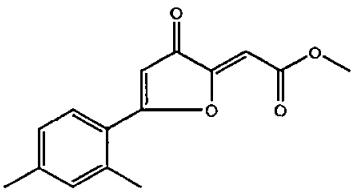
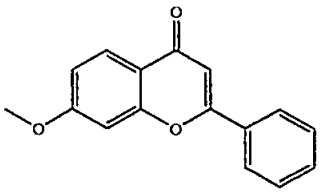
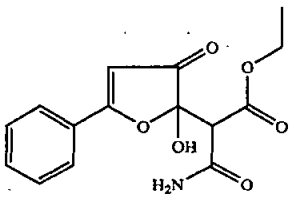
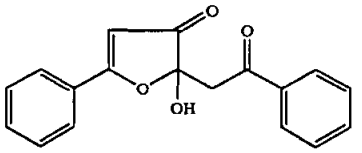
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AIR |  | • | • | • | • | •• | •• |
| AIS |  | • | • | • | • | •• | •• |
| AIT |  | • | • | • | • | •• | •• |
| AIU |  | • | NT | NT | • | • | • |
| AIV |  | ••• | ••• | •• | • | •• | •• |
| AIW |  | NT | ••• | • | • | • | •• |
| AIX |  | • | • | • | • | • | •• |

TABLE 5-continued

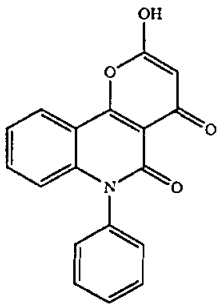
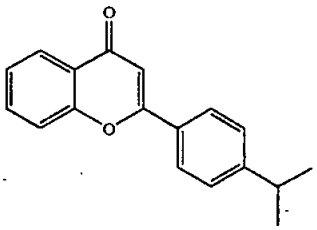
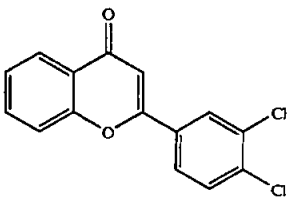
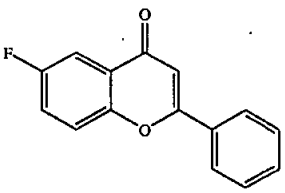
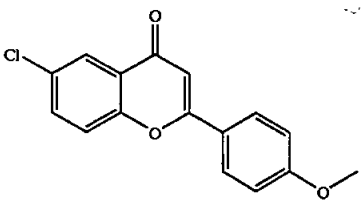
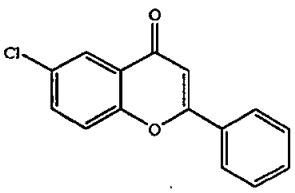
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AIY |  | * | * | * | * | * | ** |
| AIZ |  | * | * | * | * | * | ND |
| AJA |  | * | * | * | * | * | ND |
| AJB |  | * | * | * | * | * | ND |
| AJD |  | ** | * | * | * | * | * |
| AJE |  | * | ** | * | * | * | ND |

TABLE 5-continued

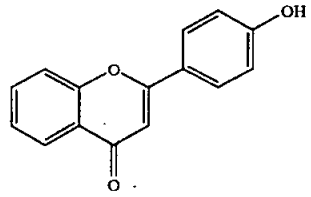
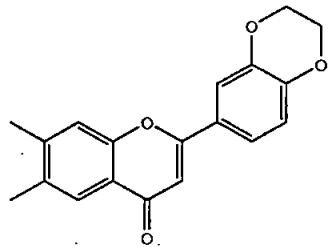
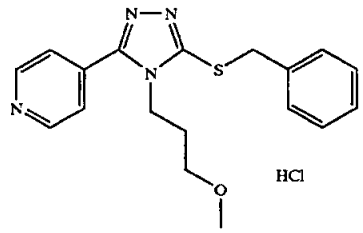
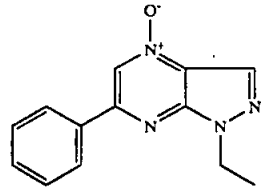
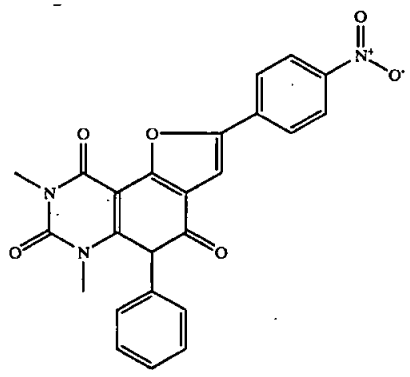
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AJF |  | * | * | * | * | * | * |
| AJG |  | *** | *** | *** | * | * | ** |
| AJH |  | *** | * | * | * | * | ** |
| AJI |  | ** | * | ** | * | * | * |
| AJJ |  | ** | ** | * | * | * | * |

TABLE 5-continued

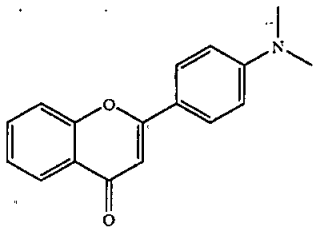
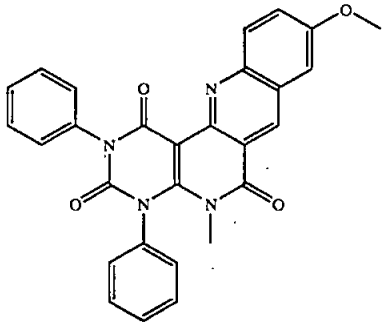
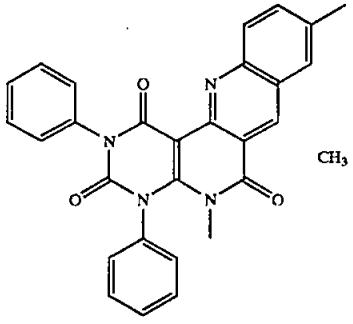
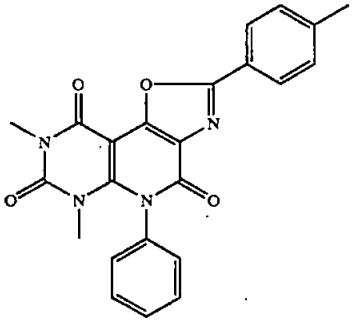
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AJK |  | * | * | * | * | * | ND |
| AJL |  | ** | * | * | * | * | * |
| AJM |  | ** | ** | *** | * | * | ND |
| AJN |  | *** | ** | NT | * | * | ** |

TABLE 5-continued

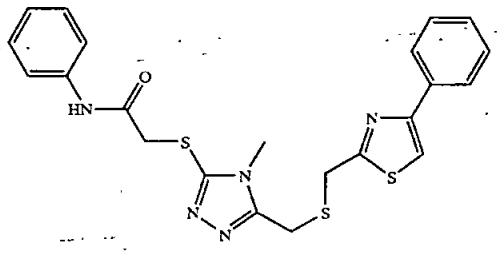
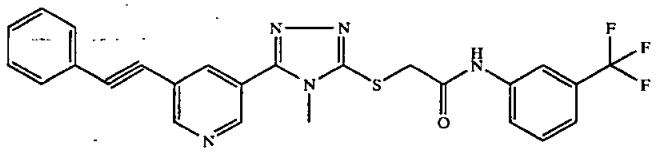
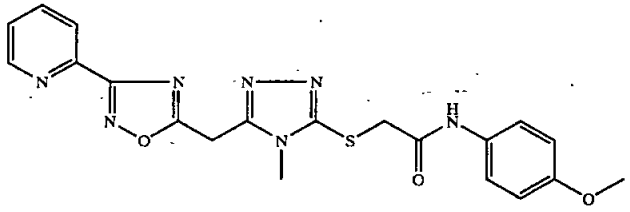
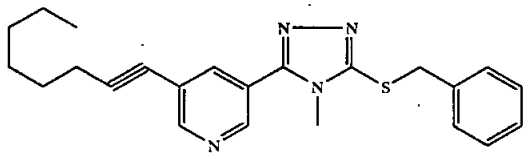
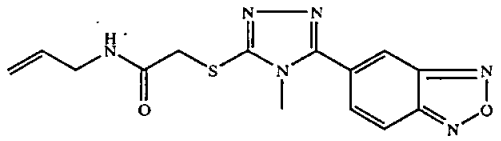
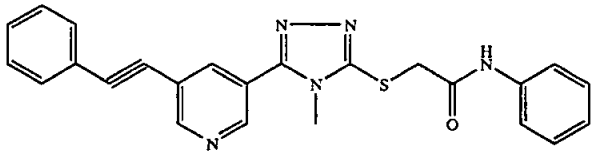
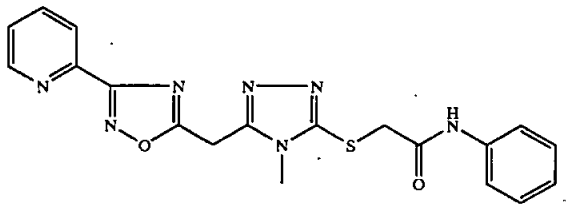
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AJO |  | NT | * | NT | * | * | ** |
| AJP |  | *** | * | NT | * | * | * |
| AJQ |  | * | * | * | * | * | * |
| AJR |  | * | ** | *** | * | * | ** |
| AJZ |  | * | NT | NT | * | * | ** |
| AKA |  | * | * | *** | * | * | ** |
| AKB |  | ** | ** | *** | * | * | ** |

TABLE 5-continued

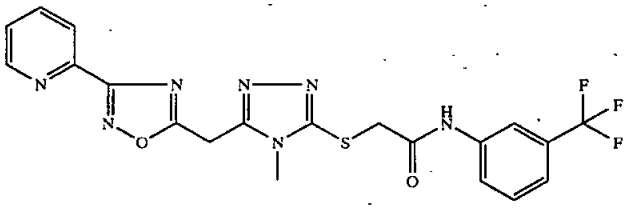
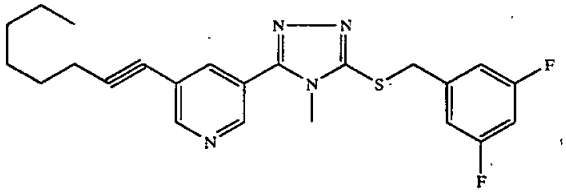
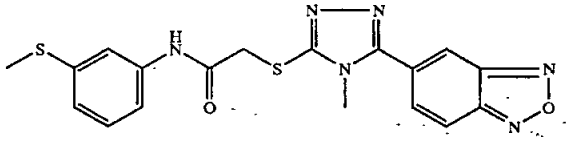
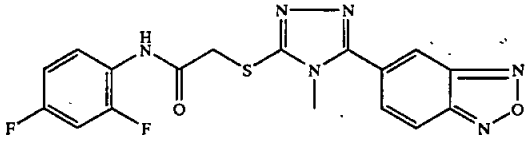
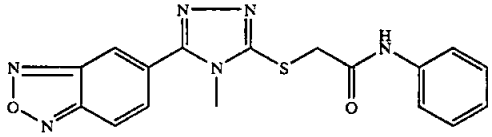
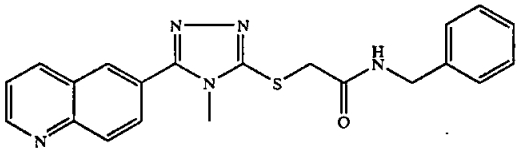
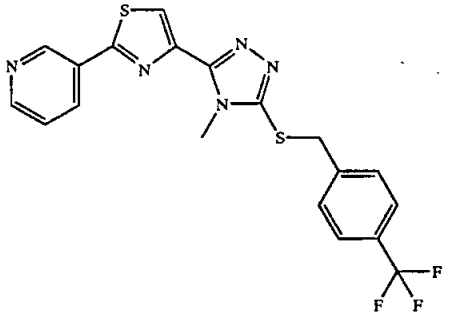
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AKC |  | * | ** | *** | * | * | ND |
| AKD |  | * | * | * | * | * | ND |
| AKE |  | * | ** | *** | * | * | ** |
| AKF |  | * | ** | *** | * | * | * |
| AKG |  | * | * | ** | * | * | ND |
| AKH |  | *** | *** | *** | * | * | ** |
| AKI |  | * | ** | *** | * | * | ** |

TABLE 5-continued

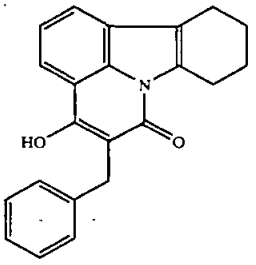
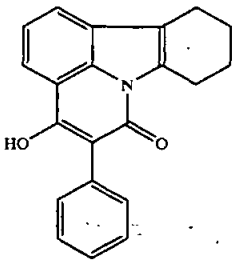
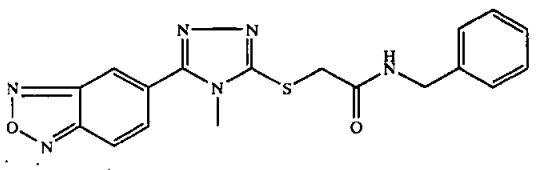
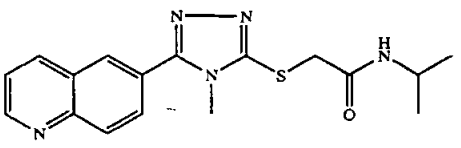
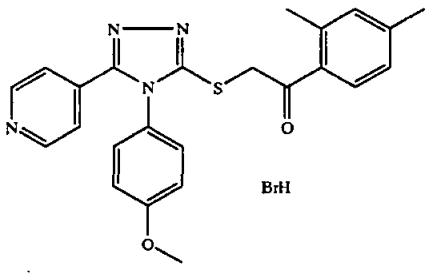
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AKJ |  | NT | •• | • | • | • | ND |
| AKK |  | NT | •• | •• | • | • | •• |
| AKL |  | • | •• | • | • | • | • |
| AKM |  | • | ••• | ••• | • | •• | •• |
| AKN |  BrH | •• | •• | • | • | •• | • |

TABLE 5-continued

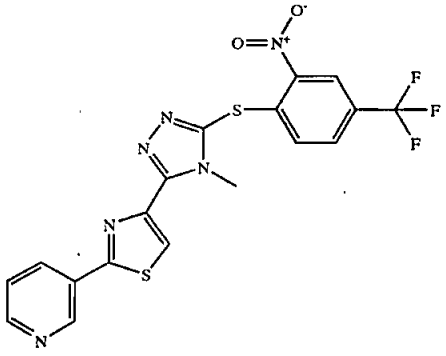
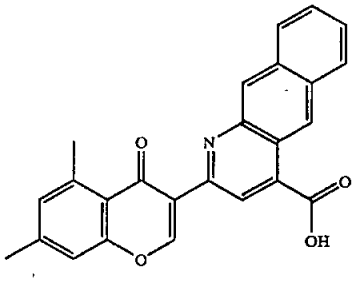
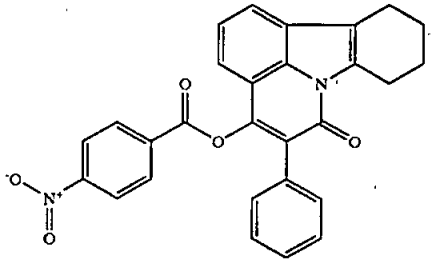
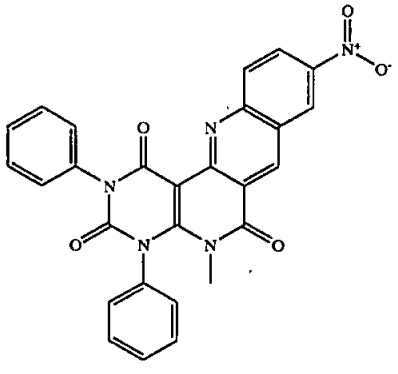
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AKO |  | * | * | *** | * | * | ** |
| AKP |  | NT | ** | *** | * | * | ND |
| AKQ |  | ** | ** | * | * | * | ** |
| AKR |  | *** | * | * | * | * | * |

TABLE 5-continued

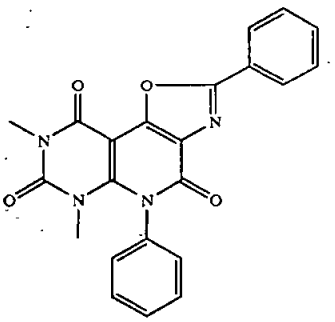
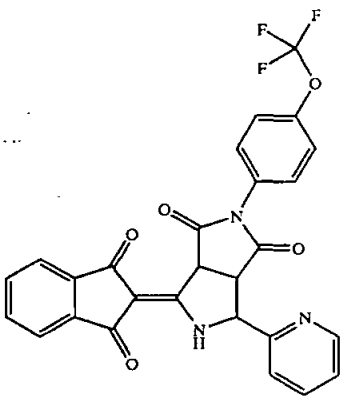
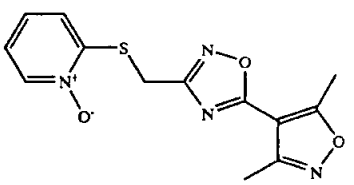
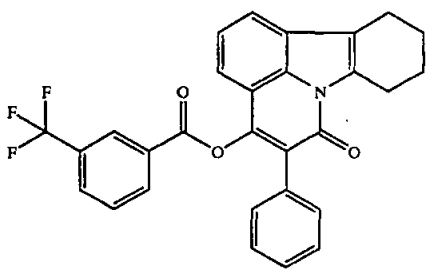
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AKS |  | *** | * | ** | * | * | ** |
| AKT |  | ** | *** | *** | * | * | ** |
| AKU |  | ** | *** | *** | * | * | ** |
| AKV |  | ** | ** | ** | * | ** | * |

TABLE 5-continued

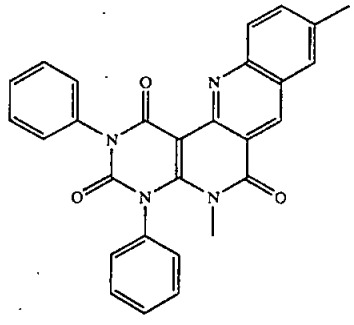
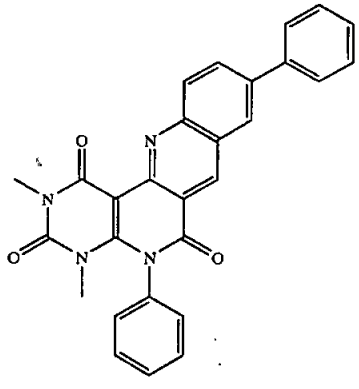
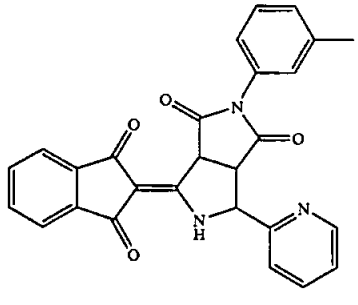
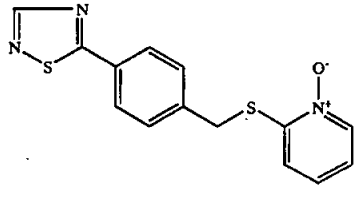
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AKW |  | *** | *** | * | * | ** | ** |
| AKX |  | *** | *** | *** | * | ** | ** |
| AKY |  | * | * | * | * | ** | ** |
| AKZ |  | ** | ** | ** | * | ** | ** |

TABLE 5-continued

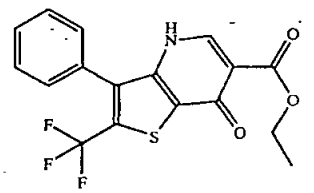
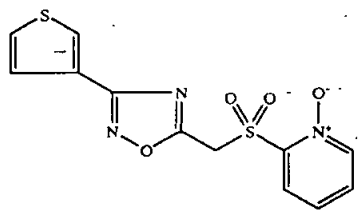
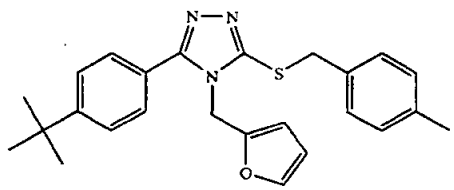
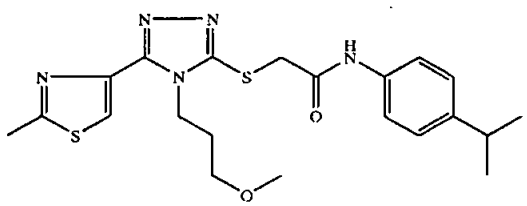
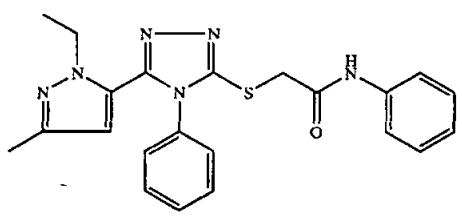
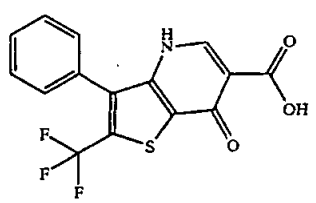
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ALA |  | * | * | * | * | * | ** |
| ALB |  | NT | * | * | * | * | ND |
| ALC |  | ** | * | * | * | * | ** |
| ALD |  | ** | NT | NT | * | * | ND |
| ALE |  | * | * | * | * | * | ** |
| ALF |  | ** | ** | *** | * | * | ND |

TABLE 5-continued

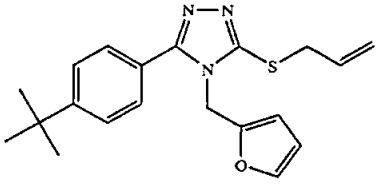
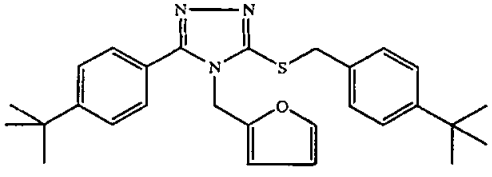
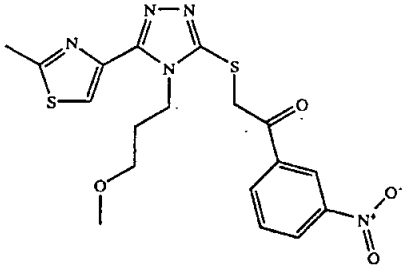
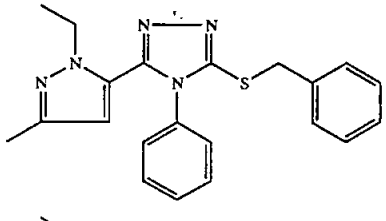
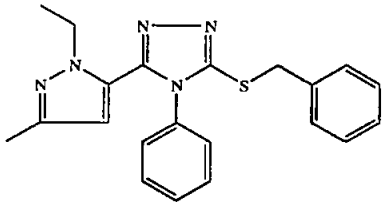
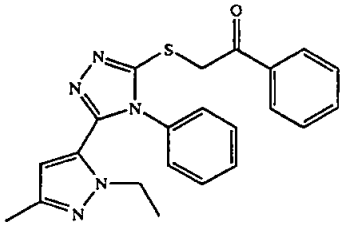
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ALG |  | *** | ** | *** | * | * | * |
| ALH |  | * | * | * | * | * | * |
| ALI |  | ** | * | * | * | * | ** |
| ALJ |  CH$_2$  | ** | * | * | * | * | ** |
| ALK |  | NT | ** | *** | * | * | ** |

TABLE 5-continued

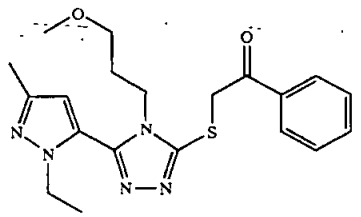
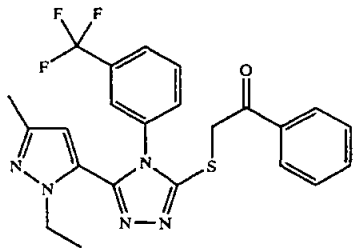
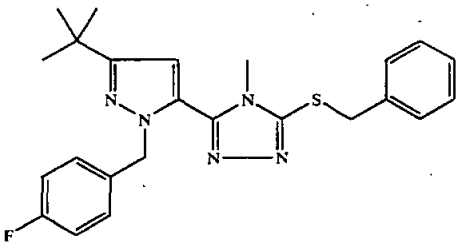
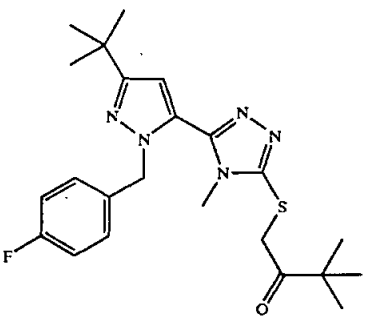
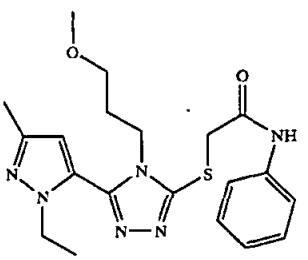
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ALL |  | * | *** | *** | * | * | * |
| ALM |  | ** | * | * | * | * | * |
| ALN |  | * | ** | * | * | ** | ** |
| ALO |  | * | *** | *** | * | * | * |
| ALP |  | ** | * | * | * | * | * |

TABLE 5-continued

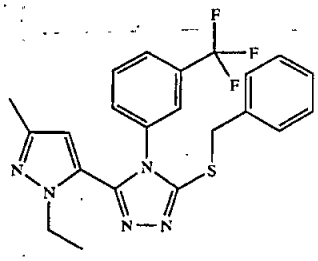
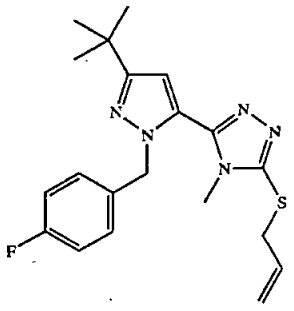
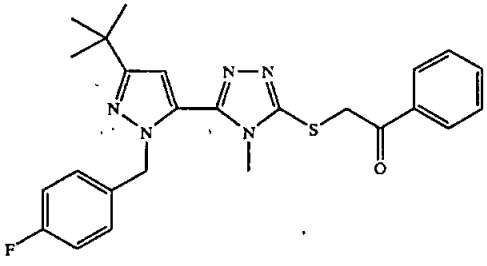
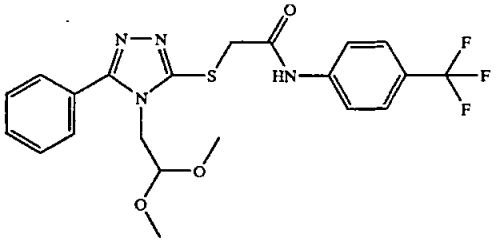
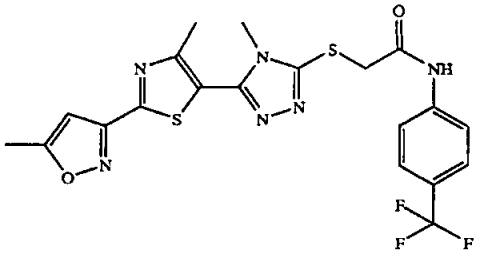
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ALQ |  | *** | *** | *** | * | * | * |
| ALR |  | * | ** | * | * | * | * |
| ALS |  | * | NT | NT | * | * | ** |
| ALT |  | *** | ** | NT | * | * | ** |
| ALU |  | * | ** | *** | * | ** | ND |

TABLE 5-continued

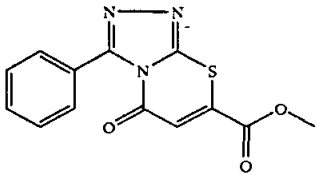
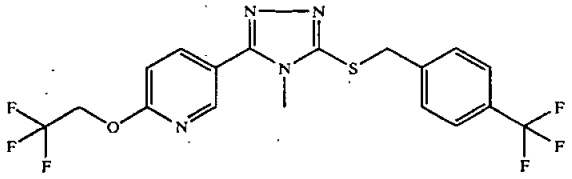
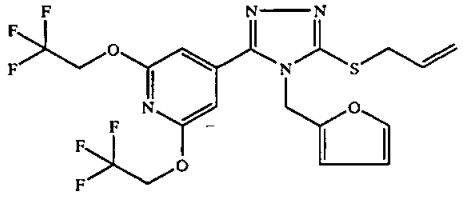
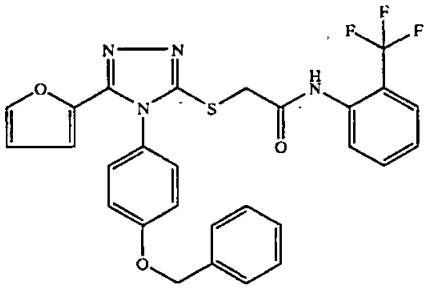
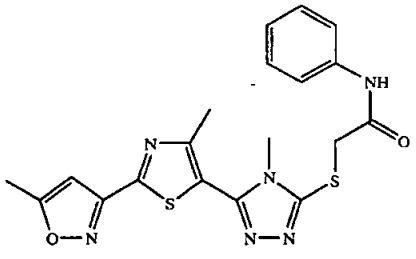
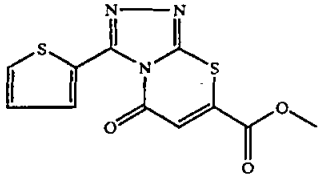
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ALV |  | ** | ** | *** | * | ** | ND |
| ALW |  | * | * | * | * | ** | ND |
| ALX |  | ** | *** | * | * | ** | * |
| ALY |  | ** | * | * | * | ** | ** |
| ALZ |  | * | * | * | * | ** | ** |
| AMA |  | * | NT | NT | * | ** | * |

TABLE 5-continued

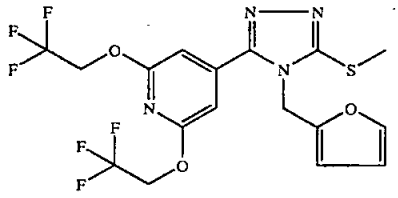
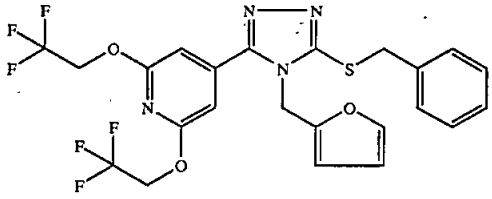
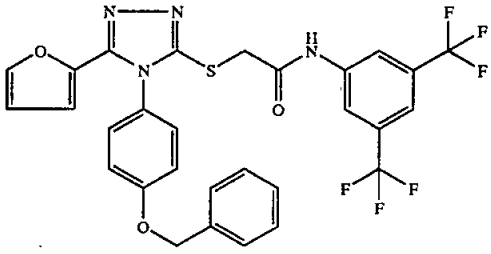
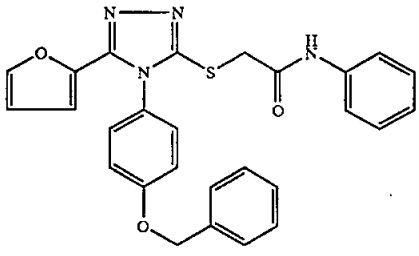
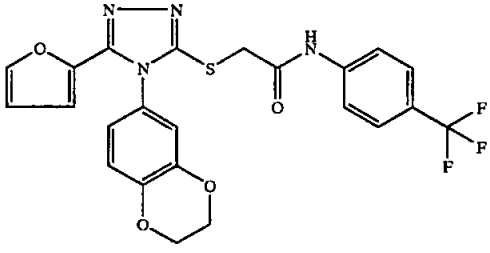
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AMB |  | *** | *** | ** | * | ** | ** |
| AMC |  | * | ** | ** | * | ** | ** |
| AMD |  | * | * | * | * | ** | * |
| AME |  | * | * | * | * | ** | * |
| AMF |  | * | * | * | * | ** | ** |

TABLE 5-continued

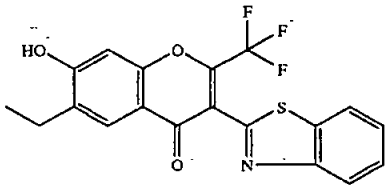
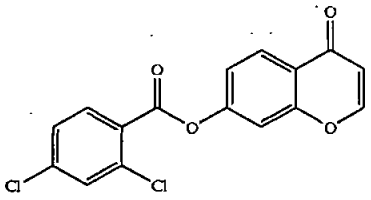
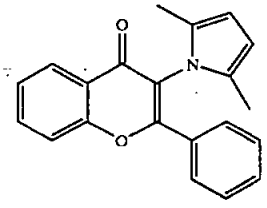
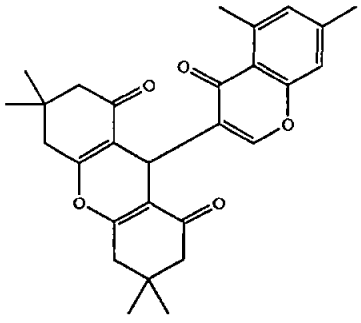
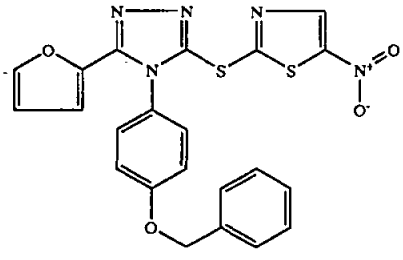
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AMG |  | *** | *** | *** | * | ** | ND |
| AMH |  | * | NT | NT | * | ** | ** |
| AMI |  | * | * | * | * | ** | ** |
| AMJ |  | * | * | *** | * | ** | ND |
| AMK |  | ** | *** | * | * | ** | ** |

TABLE 5-continued

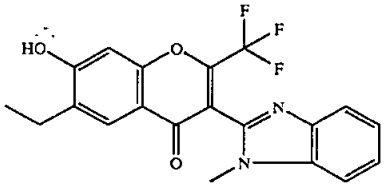
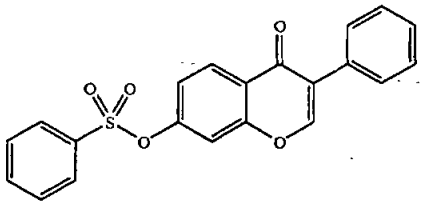
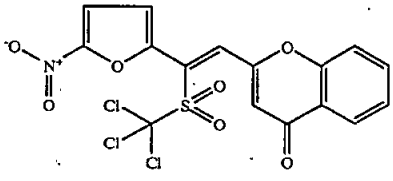
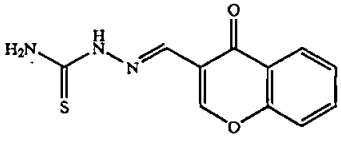
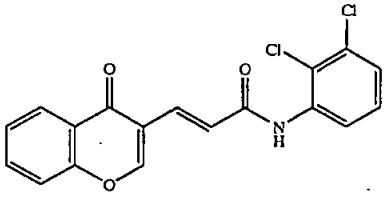
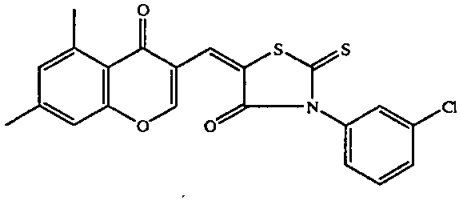
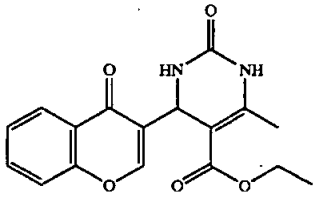
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD- | CV | Luc |
|---|---|---|---|---|---|---|---|
| AML |  | * | * | * | * | ** | ND |
| AMM |  | ** | * | ** | * | ** | ND |
| AMN |  | * | ** | * | * | * | * |
| AMO |  | * | * | ** | * | ** | ** |
| AMP |  | * | * | ** | * | ** | ND |
| AMQ |  | * | ** | * | * | ** | ND |
| AMR |  | * | NT | NT | * | * | * |

TABLE 5-continued

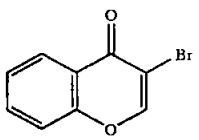
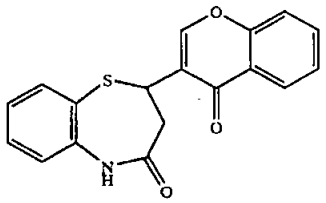
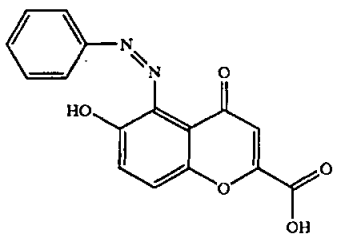
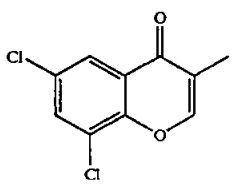
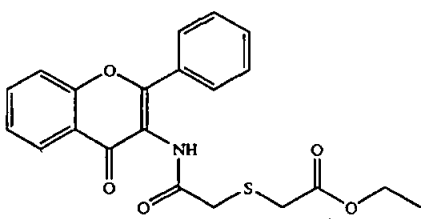
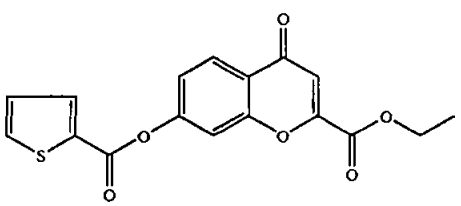
| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| AMS |  | ** | * | ** | * | ** | ND |
| AMT |  | * | ** | ** | * | * | ** |
| AMU |  | *** | *** | * | * | ** | ** |
| AMV |  | * | * | * | * | * | * |
| AMX |  | NT | *** | *** | * | ** | ** |
| AMY |  | *** | * | *** | * | ** | ** |

TABLE 5-continued

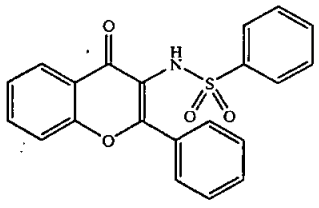
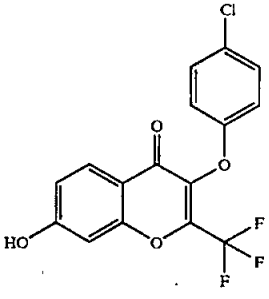
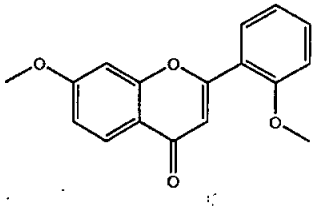
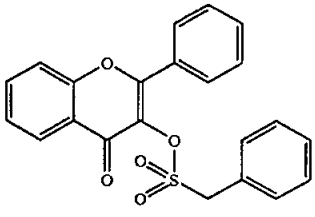
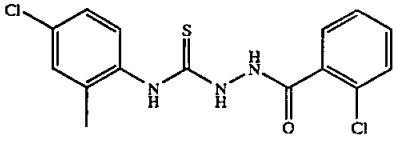
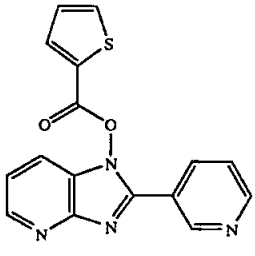
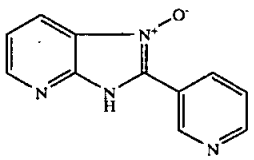
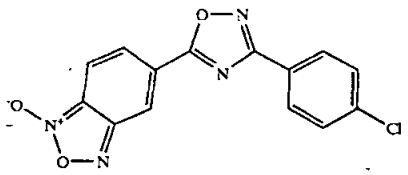
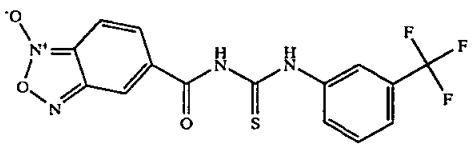
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|---|---|---|---|---|---|---|---|
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| ANA |  | * | * | * | * | ** | ** |
| ANB |  | * | * | ** | * | ** | ** |
| ANC |  | * | ** | ** | * | ** | ** |
| AND |  | NT | *** | *** | * | ** | ** |
| ANE |  | * | * | * | * | ** | ** |

TABLE 5-continued

| ID | STRUCTURE | MarA Lance | SoxS Lance | Rob Lance | OD | CV | Luc |
|---|---|---|---|---|---|---|---|
| ANF |  | *** | *** | *** | * | ** | ** |
| ANG |  | * | * | * | * | ** | ** |
| ANH |  | ** | *** | NT | * | ** | * |

[0685]

SEQUENCE LISTING

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tgcgggcatt attggcagcg tgattttcgg taaactgggt aatcagtatg cgtctgcgtt 180

ggtgagtacg gcgattgcgc tgttgctggt gtgcctggca ttgctgttac ctgcggcgaa 240

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gttaatttgg tcaatcatta tatttcgccg ctggccagtg acactcgaag aacagacgca 540

atagttgaaa ggcccattcg ggccttttt aatggtacgt tttaatgatt tccaggatgc 600

cgttaataat aaactgcaca cccatacata ccagcaggaa tcccatcaga cgggagatcg 660

cttcaatgcc acccttgccc accagccgca taattgcgcc ggagctgcgt aggcttcccc 720

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tgccatgcag tcgctccccg aaagtaaacg cgccaagcat gatagtaaaa aacgcctgtg   3180

cctgtaacac cagcgaagcc agtccagcag gcataccgaa gttaatggca caaaaaagaa   3240

aagcaaactg cgcaaaactg atggttaatc catacccccag cagcaaattc agtggtactt   3300

tcggtcgtgc gacaaaaaag atagccggaa aagcgaccag cataaagcgc aaaccggcca   3360

gcatcagcgt ggcatgttat gaagccccac tttgatgacc acaaaattta gccccccatac   3420

gaccactacc agtagcgcca acaccccatc ttttcgcgac attctaccgc ctctgaattt   3480

catcttttgt aagcaatcaa cttagctgaa tttacttttc tttaacagtt gattcgttag   3540

tcgccggtta cgacggcatt aatgcgcaaa taagtcgcta tacttcggat ttttgccatg   3600

ctatttcttt acatctctaa aacaaaacat aacgaaacgc actgccggac agacaaatga   3660

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acattggctg accatcggtc cgccgctcgg cacgctgttg gta atg cag agc atc      4135
                                              Met Gln Ser Ile
                                               1

aat ctg ccc ttc tgg ctg gca gct atc tgt tcc gcg ttt ccc atg ctt      4183
Asn Leu Pro Phe Trp Leu Ala Ala Ile Cys Ser Ala Phe Pro Met Leu
 5                  10                  15                  20

ttc att caa att tgg gta aag cgc agc gag aaa atc atc gcc acg gaa      4231
Phe Ile Gln Ile Trp Val Lys Arg Ser Glu Lys Ile Ile Ala Thr Glu
                25                  30                  35

aca ggc agt gtc tgg tcg ccg aaa gtt tta tta caa gat aaa gca ctg      4279
Thr Gly Ser Val Trp Ser Pro Lys Val Leu Leu Gln Asp Lys Ala Leu
            40                  45                  50

ttg tgg ttt acc tgc tct ggt ttt ctg gct tct ttt gta agc ggc gca      4327
Leu Trp Phe Thr Cys Ser Gly Phe Leu Ala Ser Phe Val Ser Gly Ala
        55                  60                  65

ttt gct tca tgc att tca caa tat gtg atg gtg att gct gat ggg gat      4375
Phe Ala Ser Cys Ile Ser Gln Tyr Val Met Val Ile Ala Asp Gly Asp
    70                  75                  80

ttt gcc gaa aag gtg gtc gcg gtt gtt ctt ccg gtg aat gct gcc atg      4423
Phe Ala Glu Lys Val Val Ala Val Val Leu Pro Val Asn Ala Ala Met
 85                  90                  95                 100

gtg gtt acg ttg caa tat tcc gtg ggc cgc cga ctt aac ccg gct aac      4471
Val Val Thr Leu Gln Tyr Ser Val Gly Arg Arg Leu Asn Pro Ala Asn
                105                 110                 115

atc cgc gcg ctg atg aca gca ggc acc ctc tgt ttc gtc atc ggt ctg      4519
Ile Arg Ala Leu Met Thr Ala Gly Thr Leu Cys Phe Val Ile Gly Leu
            120                 125                 130

gtc ggt ttt att ttt tcc ggc aac agc ctg cta ttg tgg ggt atg tca      4567
Val Gly Phe Ile Phe Ser Gly Asn Ser Leu Leu Leu Trp Gly Met Ser
        135                 140                 145
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atg ttg att gac cat att gcg ccg cca gaa atg aaa gcc agc tat ttt     4663
Met Leu Ile Asp His Ile Ala Pro Pro Glu Met Lys Ala Ser Tyr Phe
165                 170                 175                 180

tcc gcc cag tct tta ggc tgg ctt ggt gcc gcg att aac cca tta gtg     4711
Ser Ala Gln Ser Leu Gly Trp Leu Gly Ala Ala Ile Asn Pro Leu Val
                185                 190                 195

agt ggc gta gtg cta acc agc ctg ccg cct tcc tcg ctg ttt gtc atc     4759
Ser Gly Val Val Leu Thr Ser Leu Pro Pro Ser Ser Leu Phe Val Ile
            200                 205                 210

tta gcg ttg gtg atc att gct gcg tgg gtg ctg atg tta aaa ggg att     4807
Leu Ala Leu Val Ile Ile Ala Ala Trp Val Leu Met Leu Lys Gly Ile
        215                 220                 225

cga gca aga ccg tgg ggg cag ccc gcg ctt tgt tga tttaagtcga          4853
Arg Ala Arg Pro Trp Gly Gln Pro Ala Leu Cys  *
    230                 235

acacaataaa gatttaattc agccttcgtt taggttacct ctgctaatat ctttctcatt   4913

gagatgaaaa ttaaggtaag cgaggaaaca caccacacca taaacggagg caaataatgc   4973

tgggtaatat gaatgttttt atggccgtac tgggaataat tttatttttct ggttttctgg  5033

ccgcgtattt cagccacaaa tgggatgact aatgaacgga gataatccct cacctaaccg   5093

gccccttgtt acagttgtgt acaaggggcc tgatttttat gacggcgaaa aaaaaccgcc   5153

agtaaaccgg cggtgaatgc ttgcatggat agatttgtgt tttgctttta cgctaacagg   5213

cattttcctg cactgataac gaatcgttga cacagtagca tcagttttct caatgaatgt   5273

taaacggagc ttaaactcgg ttaatcacat tttgttcgtc aataaacatg cagcgatttc   5333

ttccggtttg cttaccctca tacattgccc ggtccgctct tccaatgacc acatccagag   5393

gctcttcagg aaatgcgcga ctcacacctg ctgtcacggt aatgttgata tgcccttcag   5453

aatgtgtgat ggcatggtta tcgactaact ggcaaattct gacacctgca cgacatgctt   5513

cttcatcatt agccgctttg acaataatga taaattcttc gcccccgtag cgataaaccg   5573

tttcgtaatc acgcgtccaa ctggctaagt aagttgccag ggtgcgtaat actacatcgc   5633

cgattaaatg cccgtagtat cattaaccaa tttaaatcgg tcaatatcca acaacattaa   5693

ataaagattc agaggctcag cgttgcgtaa ctgatgatca aaggattcat caagaacccg   5753

acgacccggc aatcccgtca aaacatccat attgctacgg atcgtcagca aataaatttt   5813

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gtcctgccag tgattttcaa caatagccag cattaattcc cgaccacagt tatgcatatg   5933

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cgtcaatatt ttcatagaaa tccgaagtta tgagtcatct ctgagataac attgtgattt   6293

aaaacaaaat cagcggataa aaaagtgttt aattctgtaa attacctctg cattatcgta   6353

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tctcctttgt gagaaatcaa attaccgcgt agtgaaaccg aagcgccatc gtgcatggtt   6653

tttgcgaaat caacggtcat ttttttgca tcatcggttc cgcgatagcc atcttctatt   6713

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ttgccaattg ttataaggca tttaaaatca gccaactagc tgtcaaatat acagagaatt   6953

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tccagatctt cgctgttacc tctggaaagg atcgcctcgc gaaaacgcag cccatttca    7793

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            20                  25                  30

Ile Ala Thr Glu Thr Gly Ser Val Trp Ser Pro Lys Val Leu Leu Gln
        35                  40                  45

Asp Lys Ala Leu Leu Trp Phe Thr Cys Ser Gly Phe Leu Ala Ser Phe
    50                  55                  60

Val Ser Gly Ala Phe Ala Ser Cys Ile Ser Gln Tyr Val Met Val Ile
65                  70                  75                  80

Ala Asp Gly Asp Phe Ala Glu Lys Val Val Ala Val Val Leu Pro Val
                85                  90                  95
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Asn Ala Ala Met Val Val Thr Leu Gln Tyr Ser Val Gly Arg Arg Leu
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Asn Pro Ala Asn Ile Arg Ala Leu Met Thr Ala Gly Thr Leu Cys Phe
        115                 120                 125

Val Ile Gly Leu Val Gly Phe Ile Phe Ser Gly Asn Ser Leu Leu Leu
    130                 135                 140

Trp Gly Met Ser Ala Ala Val Phe Thr Val Gly Glu Ile Ile Tyr Ala
145                 150                 155                 160

Pro Gly Glu Tyr Met Leu Ile Asp His Ile Ala Pro Pro Glu Met Lys
                165                 170                 175

Ala Ser Tyr Phe Ser Ala Gln Ser Leu Gly Trp Leu Gly Ala Ala Ile
            180                 185                 190

Asn Pro Leu Val Ser Gly Val Val Leu Thr Ser Leu Pro Pro Ser Ser
        195                 200                 205

Leu Phe Val Ile Leu Ala Leu Val Ile Ile Ala Ala Trp Val Leu Met
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Leu Lys Gly Ile Arg Ala Arg Pro Trp Gly Gln Pro Ala Leu Cys
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 1               5                  10                  15

cat ctg gat cag ccc ctg tcg ctc gac aat gta gcg gcg aaa gca ggt      96
His Leu Asp Gln Pro Leu Ser Leu Asp Asn Val Ala Ala Lys Ala Gly
            20                  25                  30

tat tcc aag tgg cac tta cag aga atg ttt aaa gat gtc act ggc cat     144
Tyr Ser Lys Trp His Leu Gln Arg Met Phe Lys Asp Val Thr Gly His
        35                  40                  45

gct att ggc gcg tat att cgt gct cgt cgt ttg tcg aaa tcg gcg gtc     192
Ala Ile Gly Ala Tyr Ile Arg Ala Arg Arg Leu Ser Lys Ser Ala Val
    50                  55                  60

gca cta cgc ctg act gcg cgt ccg att ctg gac atc gcg ctg caa tac     240
Ala Leu Arg Leu Thr Ala Arg Pro Ile Leu Asp Ile Ala Leu Gln Tyr
65                  70                  75                  80

cgc ttc gac tct caa cag aca ttt acc cgc gca ttc aag aag cag ttt     288
Arg Phe Asp Ser Gln Gln Thr Phe Thr Arg Ala Phe Lys Lys Gln Phe
                85                  90                  95

gcc cag act cct gca ctt tac cgc cgt tct cct gaa tgg agc gcc ttt     336
Ala Gln Thr Pro Ala Leu Tyr Arg Arg Ser Pro Glu Trp Ser Ala Phe
            100                 105                 110

ggt att cgc ccg ccg cta cgc ctg ggt gaa ttc act atg cca gag cac     384
Gly Ile Arg Pro Pro Leu Arg Leu Gly Glu Phe Thr Met Pro Glu His
        115                 120                 125

aaa ttt gtc acc ctg gaa gat acg ccg ctg att ggt gtt acc cag agc     432
Lys Phe Val Thr Leu Glu Asp Thr Pro Leu Ile Gly Val Thr Gln Ser
    130                 135                 140

tac tcc tgt tcg ctg gag caa atc tct gat ttc cgc cat gaa atg cgt     480
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145                 150                 155                 160

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Tyr Gln Phe Trp His Asp Phe Leu Gly Asn Ala Pro Thr Ile Pro Pro
                165                 170                 175

gtg ctc tac ggc ctg aat gaa acg cgt ccg agt cag gat aaa gac gac      576
Val Leu Tyr Gly Leu Asn Glu Thr Arg Pro Ser Gln Asp Lys Asp Asp
            180                 185                 190

gag caa gag gta ttc tat acc acc gcg tta gcc cag gat cag gca gat      624
Glu Gln Glu Val Phe Tyr Thr Thr Ala Leu Ala Gln Asp Gln Ala Asp
        195                 200                 205

ggc tat gta ctg acg ggg cat ccg gtg atg ctg cag ggc ggc gaa tat      672
Gly Tyr Val Leu Thr Gly His Pro Val Met Leu Gln Gly Gly Glu Tyr
    210                 215                 220

gtg atg ttt acc tat gaa ggt ctg gga acc ggc gtg cag gag ttt atc      720
Val Met Phe Thr Tyr Glu Gly Leu Gly Thr Gly Val Gln Glu Phe Ile
225                 230                 235                 240

ctg acg gta tac gga acg tgc atg cca atg ctc aac ctg acg cgc cgt      768
Leu Thr Val Tyr Gly Thr Cys Met Pro Met Leu Asn Leu Thr Arg Arg
                245                 250                 255

aaa ggt cag gat att gag cga tac tac ccg gca gaa gat gcc aaa gcg      816
Lys Gly Gln Asp Ile Glu Arg Tyr Tyr Pro Ala Glu Asp Ala Lys Ala
            260                 265                 270

gga gat cgc cca att aat cta cgc tgt gaa ctg ctg att ccg atc cgt      864
Gly Asp Arg Pro Ile Asn Leu Arg Cys Glu Leu Leu Ile Pro Ile Arg
        275                 280                 285

cgt taa                                                             870
Arg  *


<210> SEQ ID NO 4
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Echerichia coli

<400> SEQUENCE: 4

Met Asp Gln Ala Gly Ile Ile Arg Asp Leu Leu Ile Trp Leu Glu Gly
 1               5                  10                  15

His Leu Asp Gln Pro Leu Ser Leu Asp Asn Val Ala Ala Lys Ala Gly
            20                  25                  30

Tyr Ser Lys Trp His Leu Gln Arg Met Phe Lys Asp Val Thr Gly His
        35                  40                  45

Ala Ile Gly Ala Tyr Ile Arg Ala Arg Arg Leu Ser Lys Ser Ala Val
    50                  55                  60

Ala Leu Arg Leu Thr Ala Arg Pro Ile Leu Asp Ile Ala Leu Gln Tyr
65                  70                  75                  80

Arg Phe Asp Ser Gln Gln Thr Phe Thr Arg Ala Phe Lys Lys Gln Phe
                85                  90                  95

Ala Gln Thr Pro Ala Leu Tyr Arg Arg Ser Pro Glu Trp Ser Ala Phe
            100                 105                 110

Gly Ile Arg Pro Pro Leu Arg Leu Gly Glu Phe Thr Met Pro Glu His
        115                 120                 125

Lys Phe Val Thr Leu Glu Asp Thr Pro Leu Ile Gly Val Thr Gln Ser
    130                 135                 140

Tyr Ser Cys Ser Leu Glu Gln Ile Ser Asp Phe Arg His Glu Met Arg
145                 150                 155                 160
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-continued

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Tyr Gln Phe Trp His Asp Phe Leu Gly Asn Ala Pro Thr Ile Pro Pro
                165                 170                 175

Val Leu Tyr Gly Leu Asn Glu Thr Arg Pro Ser Gln Asp Lys Asp Asp
            180                 185                 190

Glu Gln Glu Val Phe Tyr Thr Thr Ala Leu Ala Gln Asp Gln Ala Asp
        195                 200                 205

Gly Tyr Val Leu Thr Gly His Pro Val Met Leu Gln Gly Gly Glu Tyr
    210                 215                 220

Val Met Phe Thr Tyr Glu Gly Leu Gly Thr Gly Val Gln Glu Phe Ile
225                 230                 235                 240

Leu Thr Val Tyr Gly Thr Cys Met Pro Met Leu Asn Leu Thr Arg Arg
                245                 250                 255

Lys Gly Gln Asp Ile Glu Arg Tyr Tyr Pro Ala Glu Asp Ala Lys Ala
            260                 265                 270

Gly Asp Arg Pro Ile Asn Leu Arg Cys Glu Leu Leu Ile Pro Ile Arg
        275                 280                 285

Arg
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1. A method for reducing antibiotic resistance of a microbial cell, comprising:

contacting said cell with a transcription factor modulating compound, such that the antibiotic resistance of said cell is reduced.

2. The method of claim 1, wherein said transcription factor modulating compound is of the formula (I):

A-E (I)

wherein

A is a polar moiety;

E is a hydrophobic moiety, and pharmaceutically acceptable salts thereof.

3. A method for modulating a transcription, comprising contacting a transcription factor with a transcription factor modulating compound, such that the transcription is modulated, wherein said transcription factor modulating compound is of the formula (I):

A-E (I)

wherein

A is a polar moiety; and

E is a hydrophobic moiety, and pharmaceutically acceptable salts thereof.

4. The method of claim 2 or 3, wherein said polar moiety comprises at least one heterocycle.

5. The method of claim 4, wherein said heterocycle is bicyclic.

6. The method of claim 4, wherein said heterocycle comprises at least one nitrogen atom.

7. The method of claim 6, wherein said heterocycle is selected from the group consisting of benzoimidazole, imidazopyridine, pyridine, pyrollidine, quinoline, triazole, pyrimidine, tetrazole, and porphyin.

8. The method of claim 4, wherein said heterocycle comprises at least one oxygen atom.

9. The method of claim 8, wherein said heterocycle is chromenone.

10. The method of claim 4, wherein said polar moiety is a fused ring moiety.

11. The method of any one of claims 4-10, wherein said heterocycle is substituted.

12. The method of claim 11, wherein the substituent is nitro, alkoxy, aryl, anidyl, ester, thioester, alkyl, araalkyl, halogen, hydroxy, or halogen.

13. The method of claim 12, wherein said substituent is hydroxyl.

14. The method of claim 12, wherein said substituent is halogen.

15. The method of any one of claims 2-3, wherein said hydrophobic moiety comprises at least one alkyl, alkenyl, alkynyl, or aryl moiety.

16. The method of claim 15, wherein said hydrophobic moiety is aryl.

17. The method of claim 16, wherein said hydrophobic moiety is substituted or unsubstituted phenyl.

18. The method of claim 17, wherein said phenyl is substituted with alkyl, alkoxy, halogen, amino, thiol, hydroxy, alkoxy, or nitro.

19. The method of claim 17, wherein said phenyl ispara-substituted.

20. The method of claim 19, wherein said para-substituent is alkyl.

21. The method of claim 20, wherein said alkyl substituent is selected from the group consisting of methyl, ethyl, propyl, butyl, or pentyl.

22. The method of claim 19, wherein said para-substituent is hydroxyl.

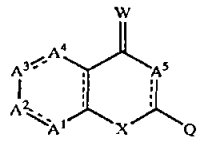
23. The method of claim 19, wherein said para-substituent is amino.

24. The method of claim 19, wherein said para-substituent is halogen.

25. The method of claim 16, wherein said aryl moiety is heterocyclic.

26. The method of claim 25, wherein said moiety is imidazopyridine, quinolinyl, or pyridinyl.

27. The method of anyone of claims 2-3, wherein said transcription factor modulating compound is of the formula (VII):



(VII)

wherein

W is NH, O or S;

X is O, S, or C, optionally linked to Q;

$A^1$ is C-$Z^1$, O, or S;

$A^2$ is C-$Z^2$, O, or S;

$A^3$ is C-$Z^3$, O, or S;

$A^4$ is C-$Z^4$, or S;

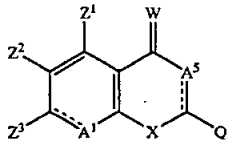
$A^5$ is C-$Z^5$, or N-$Z^5$;

$Z^1$, $Z^2$, $Z^3$, and $Z^4$ are each independently selected from the group consisting of hydrogen, alkoxy, hydroxy, halogen, and alkyl;

$Z^5$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, or carbonyl;

Q is hydrogen, alkyl, alkenyl, alkynyl, halogen, hydroxy, aryl, and pharmaceutically acceptable salts thereof.

28. The method of claim 27, wherein said transcription factor modulating compound is of the formula (II):



(II)

wherein

W is O or S;

X is O, S, or C, optionally linked to Q;

$A^1$ is C-$Z^4$, O, or S;

$A^2$ is C-$Z^5$, or N-$Z^5$;

$Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

$Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

29. The method of claim 28, wherein W and X are oxygen.

30. The method of claim 28, wherein $A^1$ is C-$Z^4$ and $Z^4$ is hydrogen.

31. The method of claim 28, wherein $A^2$ is C-$Z^5$, and $Z^5$ is hydrogen or hydroxy.

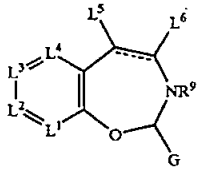
32. The method of claim 28, wherein $Z^1$ is hydrogen or hydroxy.

33. The method of claim 28, wherein $Z^2$ is hydrogen or halogen.

34. The method of claim 28, wherein $Z^3$ is hydrogen, alkoxy or hydroxy.

35. The method of claim 28, wherein Q is substituted phenyl.

36. The method of claim 2 or 3, wherein said transcription factor modulating compound is of the formula (VIII):



(VIII)

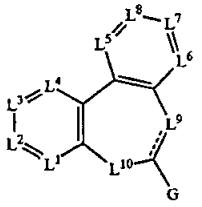
wherein

G is a substituted or unsubstituted aromatic moiety, alkyl, alkenyl, alkynyl, hydrogen;

$L^1$, $L^2$, $L^3$, and $L^4$ are each independently selected from oxygen, nitrogen, sulfur and or substituted or unsubstituted carbon; and

$R^9$, $L^5$ and $L^6$ are each independently hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, acyl, or aryl, and $L_5$ and $L_6$ may optionally be linked with a chain of one to six atoms to form a fused ring, and pharmaceutically acceptable salts thereof.

37. The method of claim 36, wherein said transcription factor modulating compound is of the formula (III):



(III)

wherein

G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

$L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$, $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

38. The method of claim 36 or 37, wherein $R^9$ is hydrogen.

39. The method of any one of claims 36-38, wherein G is substituted or unsubstituted phenyl or cyclohexenyl.

40. The method of any one of claims 36-38, wherein G is heteroaryl.

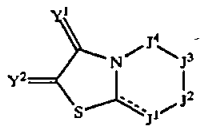
41. The method of claim 37, wherein $L^1$, $L^2$, $L^3$, and $L^4$ are each substituted or unsubstituted carbon and $L^5$, $L^6$, and $L^8$ are each nitrogen.

42. The method of claim 37 or 41, wherein $L^7$ is substituted carbon.

43. The method of claim 42, wherein said substituted carbon is substituted with a thioether moiety.

44. The method of claim 2 or 3, wherein said transcription factor modulating compound is of the formula (X):

(X)



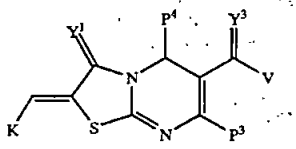
wherein

$Y^1$ and $Y^2$ are each oxygen, sulfur, or substituted or unsubstituted carbon;

$J^1$, $J^2$, $J^3$, and $J^4$ are each oxygen, nitrogen, or optionally substituted carbon, and pharmaceutically acceptable salts thereof.

45. The method of claim 44, wherein said transcription factor modulating compound is of the formula (IV):

(IV)



wherein

$Y^1$ and $Y^2$ are each oxygen or sulfur;

J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

46. The method of claim 45, wherein $Y^1$ and $Y^3$ are each oxygen.

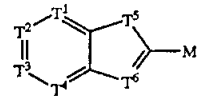
47. The method of claim 45 or 46, wherein V is alkoxy and J is lower alkyl.

48. The method of any one of claims 45-47, wherein P is substituted or unsubstituted phenyl.

49. The method of any one of claims 45-48, wherein K is substituted or unsubstituted heteroaryl.

50. The method of claim 2 or 3, wherein said transcription factor modulating compound is of the formula (V):

(V)



wherein

$T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ are each independently substituted or unsubstituted carbon, oxygen, substituted or unsubstituted nitrogen, or sulfur;

M is hydrogen, alkyl, alkenyl, heterocyclic, alkynyl, or aryl, or pharmaceutically acceptable salts thereof.

51. The method of claim 50, wherein M is substituted or unsubstituted aryl.

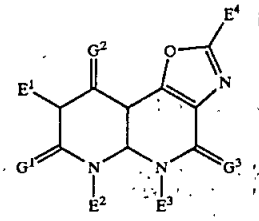
52. The method of claim 50 or 51, wherein $T^5$ is substituted nitrogen.

53. The method of anyone of claims 50-52, wherein $T^1$, $T^2$, $T^3$ and $T^4$ are each substituted or unsubstituted carbon.

54. The method of anyone of claims 50-52, wherein one of $T^1$, $T^2$, $T^3$, and $T^4$ is nitrogen.

55. The method of claims 2 or 3, wherein said transcription factor modulating compound is of the formula (VI):

(VI)



wherein

$G^1$, $G^2$, and $G^3$ are each independently O, S, substituted or unsubstituted nitrogen, or substituted or unsubstituted carbon;

$E^1$, $E^2$, and $E^3$ are each independently hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, or acyl; and

$E^4$ is alkyl, alkenyl, alkynyl, aryl, halogen, cyano, amino, nitro, or acyl, and pharmaceutically acceptable salts thereof.

56. The method of claim 55, wherein $G^1$, $G^2$ and $G^3$ are each oxygen.

57. The method of claim 55, wherein $E^1$, $E^2$, and $E^3$ are each alkyl.

58. The method of any one of claims 1-57, wherein said transcription factor is a helix-turn-helix protein.

59. The method of any one of claims 1-57, wherein said transcription factor is a transcriptional activation factor.

60. The method of claims 59, wherein said transcriptional activation factor is an AraC family polypeptide.

61. The method of claim 59, wherein said transcriptional activation factor is a MarA family polypeptide.

62. The method of any one of claims 1-61, wherein said transcription factor modulating compound is a transcription factor inhibiting compound.

63. The method of any one of claims 1-62, wherein said transcription factor is prokaryotic.

64. The method of claim 61, wherein said MarA family polypeptide is MarA, SoxS, or Rob.

65. The method of claim 1, wherein said microbial cell is selected from the group consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas putida, Stenotrophomonas maltophilia, Burkholderia cepacia, Aeromonas hydrophilia, Escherichia coli, Citrobacter freundii, Salmonella typhimurium, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia alcalifaciens, Providencia rettgeri, Providencia stuartii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia intermedia, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus haemolyticus, Haemophilus parahaemolyticus, Haemophilus ducreyi, Pasteurella multocida, Pasteurella haemolytica, Branhamella catarrhalis, Helicobacter pylori, Campylobacter fetus, Campylobacter jejuni, Campylobacter coli, Borrelia burgdorferi, Vibrio cholerae, Yibrio parahaemolyticus, Legionella pneumophila, Listeria monocytogenes, Neisseria gonorrhoeae, Neisseria meningitidis, Gardnerella vaginalis, Bacteroides fragilis, Bacteroides distasonis*, Bacteroides 3452A homology group, *Bacteroides vulgatus, Bacteroides ovalus, Bacteroides thetaiotaomicron, Bacteroides uniformis, Bacteroides eggerthii, Bacteroides splanchnicus, Clostridium difficile, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium leprae, Corynebacterium diphtheriae, Corynebacterium ulcerans, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus intermedius, Staphylococcus hyicus* subsp. *hyicus, Staphylococcus haemolyticus, Staphylococcus hominis, and Staphylococcus saccharolyticus.*

66. A method for identifying a transcription factor modulating compound, comprising:

contacting a microbial cell with a test compound, wherein said microbial cell comprises a selective marker under the direct control of a transcription factor responsive element and a transcription factor, under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of said test compound to affect said microbial cell, such that transcription factor modulating compounds are identified.

67. The method of claim 66, wherein the responsive element is activated by a transcription factor.

68. The method of claim 67, wherein the responsive element is selected from the group consisting of: marO, fum, inaA, galT, and micF.

69. The method of claim 66, wherein the responsive element is repressed by a transcription factor.

70. The method of claim 69, wherein the responsive element is selected from the group consisting offecA, purA, and guab.

71. The method of claim 66, wherein the selective marker is selected from the group consisting of ccdB, kan, cat, bla, purA, GuaB, and URA3.

72. The method of claim 66, wherein the ability of said test compound to modulate the activity of a transcription factor is demonstrated by the ability of the test compound to promote the in vitro or in vivo growth or survival of said microbial cell.

73. The method of claim 66, wherein the ability of said test compound to modulate the activity of a transcription factor is demonstrated by the ability of the compound to decrease the in vitro or in vivo growth or survival said cell in an animal model of infection.

74. The method of claim 66, wherein the method is used to identify a transcription factor agonist.

75. The method of claim 66, wherein the method is used to identify a transcription factor antagonist.

76. The method of claim 66, wherein said transcription factor is expressed from a plasmid.

77. The method of claim 76, wherein said transcription factor is expressed under the control of an inducible promoter.

78. The method of claim 77, wherein said inducible promoter is selected from the group consisting of: trp, tac, tet, and GAL1.

79. The method of claim 76, wherein said transcription factor is expressed under the control of a constitutive promoter.

80. The method of claim 66, wherein said transcription factor is a MarA family polypeptide.

81. The method of claim 66, wherein said microbial cell comprises a chromosomal deletion of at least one gene encoding a transcription factor.

82. The method of claim 66, wherein said transcription factor responsive element comprises a Marbox domain.

83. The method of claim 66, wherein said transcription factor comprises at least one HTH domain.

84. The method of claim 66, wherein said transcription factor is prokaryotic.

85. The method of claim 80, wherein said transcription factor is MarA.

86. A method for identifying a transcription factor modulating compound in vivo or in vitro, comprising:

contacting a microbial cell comprising: 1) a selective marker under the control of a transcription factor responsive element and 2) a transcription factor, with a test compound under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of the test compound to affect the growth or survival of the microbial cell as an indication of whether the compound modulates the activity of a transcription factor, wherein the inactivation of the transcription factor leads to a decrease in cell survival.

87. A method for identifying a transcription factor modulating compound in vivo or in vitro, comprising:

contacting a microbial cell comprising: 1) a selective marker under the control of a transcription factor responsive element and 2) a transcription factor, with a test compound under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of said test compound to affect the growth or survival of the microbial cell as an indication of whether the compound modulates the activity of a transcription factor, wherein the activation of the transcription factor leads to a decrease in cell survival.

**88**. A method for identifying a transcription factor modulating compound in vivo or in vitro, comprising:

contacting a microbial cell comprising: 1) a selective marker under the control of a transcription factor responsive element and 2) a transcription factor, with test compound under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of said test compound to affect the growth or survival of the microbial cell as an indication of whether said test compound modulates the activity of a transcription factor, wherein the inactivation of the transcription factor leads to an increase in cell survival.

**89**. A method for identifying a transcription factor modulating compound in vivo or in vitro, comprising:

contacting a microbial cell comprising: 1) a selective marker under the control of a trascription factor responsive element and 2) a transcription factor, with a compound under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of the compound to affect the growth or survival of the microbial cell as an indication of whether the compound modulates the activity of a transcription factor, wherein the activation of the transcription factor leads to an increase in cell survival.

**90**. A method for identifying a transcription factor modulating compound in vivo or in vitro, comprising:

contacting a microbial cell comprising: 1) a chromosomal deletion in a guaB or purA gene, 2) heterologous guaB or purA gene under the control of its natural promoter, and 3) a transcription factor, with a test compound under conditions which allow interaction of the compound with the microbial cell; and

measuring the ability of the compound to affect the growth or survival of the microbial cell as an indication of whether the compound modulates the activity of a transcription factor, wherein the ability of a compound to modulate the activity of a transcription factor leads to an increase in cell growth.

**91**. A transcription factor modulating compound identified by the method of any one of claims **66, 86, 87, 88, 89,** or **90**.

**92**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound affects the growth or development of gram negative bacteria.

**93**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound affects the growth or development of prokaryotic bacteria.

**94**. The method of any one of claims **86-90**, wherein said transcription factor is prokaryotic.

**95**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound affects the growth or development of gram positive bacteria.

**96**. The method of claim 95, wherein said gram positive bacteria are Enterococcus, Staphylococcus, Clostridium or Streptococcus.

**97**. The method of claim 95, wherein said transcription factor modulating compound affects the growth or development of bacteria from the family Enterobacteriaceae.

**98**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound affects the growth or development of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas putida, Stenotrophomonas maltophilia, Burkholderia cepacia, Aeromonas hydrophilia, Escherichia coli, Citrobacter freundii, Salmonella typhimurium, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia alcalifaciens, Providencia rettgeri, Providencia stuartii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia intermedia, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus haemolyticus, Haemophilus parahaemolyticus, Haemophilus ducreyi, Pasteurella multocida, Pasteurella haemolytica, Branhamella catarrhalis, Helicobacter pylori, Campylobacter fetus, Campylobacter jejuni, Campylobacter coli, Borrelia burgdorferi, Vibrio cholerae, Yibrio parahaemolyticus, Legionella pneumophila, Listeria monocytogenes, Neisseria gonorrhoeae, Neisseria meningitidis, Gardnerella vaginalis, Bacteroides fragilis, Bacteroides distasonis,* Bacteroides 3452A homology group, *Bacteroides vulgatus, Bacteroides ovalus, Bacteroides thetaiotaomicron, Bacteroides uniformis, Bacteroides eggerthii, Bacteroides splanchnicus, Clostridium difficile, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium leprae, Corynebacterium diphtheriae, Corynebacterium ulcerans, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus intermedius, Staphylococcus hyicus* subsp. *hyicus, Staphylococcus haemolyticus, Staphylococcus hominis,* and *Staphylococcus saccharolyticus.*

**99**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound is a nucleic acid molecule.

**100**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound is an antisense or sense oligonucleotide.

**101**. The method of any one of claims **86-90**, wherein said transcription factor modulating compound is a small molecule.

**102**. The method of any one of claims **86-101**, wherein said transcription factor is a MarA family polypeptide.

**103**. The method of any one of claims **86-101**, wherein said transcription factor is a AraC family polypeptide.

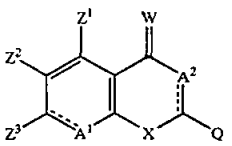
**104**. A kit for identifying a transcription factor modulating compound which modulates the activity of a transcription factor comprising a microbial cell comprising:

1) a selective marker under the control of a transcription factor responsive element and

2) a transcription factor.

105. A pharmaceutical composition comprising an effective amount of a transcription factor modulating compound, and a pharmaceutically acceptable carrier, wherein said transcription factor modulating compound is of the formula (II):

(II)



wherein

W is O or S;

X is O, S, or C, optionally linked to Q;

$A^1$ is C-$Z^4$, O, or S;

$A^2$ is C-$Z^5$, or N-$Z^5$;

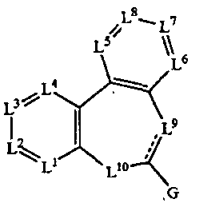
$Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

$Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

106. A pharmaceutical composition comprising an effective amount of an transcription factor modulating compound, and a pharmaceutically acceptable carrier, wherein said transcription factor modulating compound is of the formula (III):

(III)



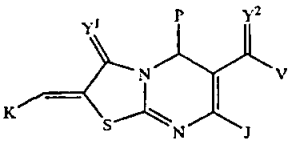
wherein

G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

$L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$, $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

107. A pharmaceutical composition comprising an effective amount of a transcription factor modulating compound, and a pharmaceutically acceptable carrier, wherein said transcription factor modulating compound is of the formula (IV):

(IV)



wherein

$Y^1$ and $Y^2$ are each oxygen or sulfur;

J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

108. The pharmaceutical composition of any one of claims 105-107, further comprising an antibiotic.

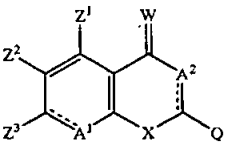
109. A pharmaceutical composition of any one of claims 105-107, wherein said effective amount is effective to treat a biofilm associated state in said subject.

110. The pharmaceutical composition of claim 109, wherein said biofilm associated state is selected from the group consisting of middle ear infections, cystic fibrosis, osteomyelitis, acne, dental cavities, endocarditis, and prostatitis.

111. A method of inhibiting a biofilm, comprising administering a composition comprising a transcription factor modulating compound, such that said biofilm is inhibited.

112. The method of claim 111, wherein said transcription factor modulating compound is of the formula (II):

(II)



wherein

W is O or S;

X is O, S, or C, optionally linked to Q;

$A^1$ is C-$Z^4$, O, or S;

$A^2$ is C-$Z^5$, or N-Z;

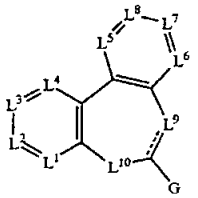
$Z^1$, $Z^2$, $Z^3$, $Z^4$ and $Z^5$ are each independently hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, or cyano;

$Z^3$ is hydrogen, alkoxy, hydroxy, halogen, alkyl, alkenyl, alkynyl, aryl, heterocyclic, amino, nitro, cyano, carbonyl, or thiocarbonyl;

Q is an aromatic or heterocyclic moiety, and pharmaceutically acceptable salts thereof.

113. The method of claim 111, wherein said transcription factor modulating compound is of the formula (III):

(III)



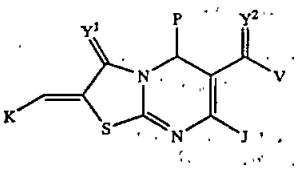
wherein

G is substituted or unsubstituted aromatic moiety, heterocyclic, alkyl, alkenyl, alkynyl, hydroxy, cyano, nitro, amino, carbonyl, or hydrogen; and

$L^1$, $L^2$, $L^3$, $L^4$, $L^5$, $L^6$, $L^7$, $L^8$, $L^9$, and $L^{10}$ are each independently oxygen, substituted or unsubstituted nitrogen, sulfur and or substituted or unsubstituted carbon, and pharmaceutically acceptable salts thereof.

**114**. The method of claim 111, wherein said transcription factor modulating compound is of the formula (IV):

(IV)



wherein

$Y^1$ and $Y^2$ are each oxygen or sulfur;

J is hydrogen, substituted or unsubstituted alkyl, alkenyl, alkynyl, cyano, nitro, amino, or halogen;

V is substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkylamino, or alkylthio;

P and K are each independently substituted or unsubstituted aryl, and pharmaceutically acceptable salts thereof.

**115**. The method of claim 111, wherein said transcription factor modulating compound is a compound of Table 4 or Table 5.

**116**. The method of any one of claims **111-115**, wherein said composition further comprises a surfactant.

**117**. The method of claim 116, wherein said surfactant is Sodium Dodecyl Sulfate; Quaternary Ammonium Compounds; alkyl pyridinium iodides; Tween 80, Tween 85, Triton X-100; Brij 56; biological surfactants; Rhamnolipid, Surfactin, Visconsin, or sulfonates.

**118**. The method of claim 117 wherein said biofilm development is diminished by the administration of said composition.

**119**. A method of inhibiting the formation of a biofilm, comprising administering a transcription factor modulating compound, such that the formation of said biofilm is inhibited.

**120**. A method for cleaning and disinfecting contact lenses comprising administering a composition comprising an acceptable carrier and a transcription factor modulating compound, such that said contact lenses are cleaned and disinfected.

**121**. A method of treating medical indwelling devices comprising administering a composition comprising a transcription factor modulating compound, such that said medical indwelling devices are treated.

**122**. The method of claim 121, wherein said device is selected from the group consisting of catheters, orthopedic devices and implants.

**123**. A method for treating or preventing a biofilm associated state in a subject, comprising administering to said subject an effective amount of a transcription factor modulating compound, such that said biofilm associated state in said subject is treated.

**124**. The method of claim 123, wherein said biofilm associated state is selected from the group consisting of middle ear infections, cystic fibrosis, osteomyelitis, acne, dental cavities, endocarditis, and prostatitis.

**125**. The method of claim 123, further comprising administering a pharmaceutically acceptable carrier.

**126**. The method of claim 123, wherein said subject is a mammal.

**127**. The method of claim 126, wherein said mammal is a human.

**128**. The method of claim 123, wherein said subject is immunocompromised.

**129**. A method of identifying a transcription factor modulating compounds, comprising:

obtaining the structure of said transcription factor;

using an appropriate program to identify a scaffold which have an interaction energy score of −20 or less with a portion of said transcription factor, such that transcription factor modulating compounds are identified.

**130**. The method of claim 129, wherein said transcription factor is a MarA family polypeptide.

**131**. The method of claim 129, wherein said scaffold has an interaction energy score of −40 or less.

**132**. The method of claim 131, wherein said scaffold has an interaction energy score of −60 or less.

**133**. The method of claim 129, wherein said transcription factor is MarA.

**134**. The method of claim 133, wherein said portion of MarA is selected from the group consisting of about residue 42 to about residue 50, about residue 54 to about residue 62, about residue 55 to about residue 65, about residue 15 to about residue 25, about residue 14 to about residue 25, about residue 24 to about residue 35, about residue 76 to about residue 83, and about residue 106 to about residue 112, of SEQ ID NO. 2.

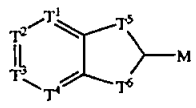
**135**. The method of claim **129-132**, wherein said transcription factor is Rob.

**136**. The method of claim 135, wherein said portion of Rob is selected from the group consisting of from about residue 37 to about residue 45, about residue 43 to about residue 54, about residue 51 to about residue 60, about residue 10 to about residue 20, about residue 9 to about residue 20, about residue 21 to about residue 29, about residue 66 to about residue 77, and about residue 101 to about residue 107, of SEQ ID NO. 4.

137. The method of any one of claims 129-136, wherein said method further comprises chemically modifying said scaffold.

138. A transcription factor modulating compound identified by any one of the methods of claims 129-137.

139. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a transcription factor modulating compound, wherein said compound is of the formula (V):



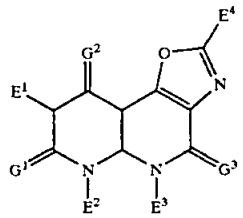
(V)

wherein

$T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ are each independently substituted or unsubstituted carbon, oxygen, substituted or unsubstituted nitrogen, or sulfur;

M is hydrogen, alkyl, alkenyl, heterocyclic, alkynyl, or aryl, or pharmaceutically acceptable salts thereof.

140. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a transcription factor modulating compound, wherein said compound is of the formula (VI):



(VI)

wherein

$G^1$, $G^2$, and $G^3$ are each independently O, S, substituted or unsubstituted nitrogen, or substituted or unsubstituted carbon;

$E^1$, $E^2$, and $E^3$ are each independently hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, or acyl; and

$E^4$ is alkyl, alkenyl, alkynyl, aryl, halogen, cyano, amino, nitro, or acyl, and pharmaceutically acceptable salts thereof.

141. A method for preventing a bacterial associated state in a subject, comprising administering to said subject an effective amount of a transcription factor modulating compound, such that the bacterial associated state in said subject is prevented.

142. The method of claim 141, wherein said subject is a human.

143. The method of claim 141, wherein said transcription factor modulating compound is a MarA family polypeptide inhibitor.

144. The method of claim 141, wherein said transcription factor modulating compound is a AraC family polypeptide inhibitor.

* * * * *

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(54) Title: COMPOUNDS FOR IMMUNOPOTENTIATION

(57) Abstract: Methods of stimulating an immune response and treating patients responsive thereto with 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones, staurosporine analogs, derivatized pyridazines, chromen-4-ones, indolinones, quinazolines, nucleoside analogs, and other small molecules are disclosed.

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# COMPOUNDS FOR IMMUNOPOTENTIATION

## FIELD OF THE INVENTION

[0001] This invention pertains generally to compounds capable of stimulating or modulating an immune response in a subject. In some embodiments, the compounds are 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones, staurosporine analogs, derivatized pyridazines, cliromen-4-ones, indolinones, quinazolines, nucleoside analogs, or other small molecules as described herein. In some further embodiments, the invention provides novel combinations of antigens with immune potentiators that may be used in vaccine therapies. The compounds in one embodiment can be used as immunotherapeutics for proliferative diseases, infectious diseases, autoimmune diseases and/or allergies/asthma.

## BACKGROUND OF THE INVENTION

[0002] With the number and diversity of diseases burgeoning and respective therapeutic treatments receding, a new therapeutic approach is needed. Such an approach should be focused less on targeting specific substrates in the disease state and more on bolstering the immune response to the disease. Since the discovery of penicillin, which targets bacteria-specific cell walls conveniently absent in man, the model of modem medicine has been to eliminate substrates in the disease state, while leaving the host system unaffected. Unfortunately, few therapies have ever reached that pinnacle and fewer still remain effective in the face of resistance mutations. Applied to cancer, upregulated kinases have been the targets of therapeutic development. Unfortunately, the only recent therapeutic agent to hit the bull's-eye is Gleevec®, and likely not solely because of its kinase inhibitory activity. Borg *et al.* J. Clin. Invest. 114:379-388 (2004).

[0003] There are numerous benefits to (or detriments to not) potentiating an immune response instead of, or in addition to, disease substrate inhibition. One advantage is that substrates in the disease and host are commonly shared, although possibly

upregulated in the disease state. For example, cancer drugs targeting kinases may be cytotoxic and may destroy cellular machinery in the host in addition to the cancer cells. Subsequently, the maximum tolerated doses (MTDs) necessary for treatment efficacy may result in undesirable side effects and even weaken the immune response in the patient. Such side effects may require cessation of treatment. Conversely, as seen with Gleevec®, the dual action of inhibiting bcr-abl, while stimulating an immune response, likely contributes to its efficacy and tolerability, particularly because NK cells, which are stimulated by administration of Gleevec®, independently play a role in tumor recession. This synergistic approach to cancer regression is extremely effective. Alternatively, cytotoxics that suppress the immune system may independently contribute to the disease state since they may inhibit separate pathways that are involved in recovery.

[0004] Another advantage to immune potentiation is that it provides a platform less easily bypassed by resistance mutations. Where therapeutic targets are so polarized and specific (which may be necessary in order to avoid targeting host cells), such as a particular substrate in a viral replicon or a kinase in a cancer cell line, a single point mutation in the disease state may render it unaffected by a drug resulting in even harsher strains of the disease in future generations.

[0005] Novel methods and mechanisms for treating patients having diseases resistant to, or inadequately treated by, conventional approaches utilizing agents targeting specific immune response mechanisms in the body is needed.

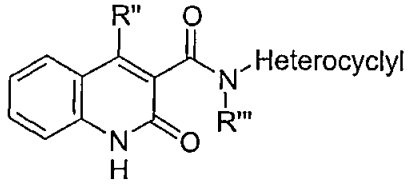
[0006] Substances that stimulate immune cells *in vitro* exhibit similar immuno-stimulatory effects *in vivo*. These compounds, such as recombinant cytokines, pathogen products (e.g. toxins, lipids, proteins/peptides, carbohydrates and nucleic acids) and other mammalian-derived immunostimulatory molecules (e.g. heat shock proteins, immune complexes and proteoglycans) all induce a measurable pro-inflammatory response both *in vitro* and *in vivo*.

[0007] Immune response to certain agents that are otherwise less potent can be enhanced through the use of adjuvants. Such adjuvants potentiate the immune response to the specific agents or antigens and are therefore the subject of considerable interest and study within the medical community. Historically, the classic adjuvants have been Freund's complete or incomplete (i.e., without mycobacteria) adjuvants. Edmund Coley described the potential of Coley's toxin for cancer immuno-therapy. Other materials, such as mineral oil and aluminum

hydroxide, have also been used as adjuvants, but they invariably suffer from disadvantages. For example, mineral oil is known to produce tissue irritation and to be potentially oncogenic. Alum, the only approved adjuvant in the United States, also induces granulomas at the inoculation site and furthermore it does not effectively induce cell-mediated immunity. Moreover, many of the adjuvants currently available have limited utility because they contain components that are not metabolizable in humans. Additionally, most adjuvants are difficult to prepare in that they may require time consuming procedures and the use, in some cases, of elaborate and expensive equipment to formulate a vaccine and adjuvant system.

[0008] Immunological adjuvants are described in "Current Status of Immunological Adjuvants", Ann. Rev. Immunol., 1986, 4, pp. 369-388, and "Recent Advances in Vaccine Adjuvants and Delivery Systems" by Derek T OH agan and Nicholas M. Valiante. See also U.S. Pat. Nos. 4,806,352; 5,026,543; and 5,026,546 for disclosures of various vaccine adjuvants appearing in the patent literature.

[0009] Compounds are described in issued U.S. Patent Nos. 4,547,51 1 and 4,738,971 with the general structure (a):

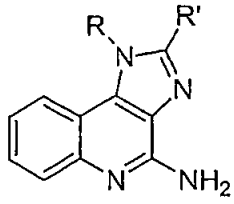



a

for the treatment of disorders responsive to agents that enhance cell-mediated immunity.

[0010] Immunostimulatory oligonucleotides and polynucleotides are described in PCT WO 98/55495 and PCT WO 98/16247. U.S. Patent Application No. 2002/0164341 describes adjuvants including an unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant. U.S. Patent Application No. 2002/0197269 describes compositions comprising an antigen, an antigenic CpG-ODN and a polycationic polymer.

[0011] Additionally, issued U.S. Patent Nos. 4,689,338, 5,389,640, 5,268,376, 4,929,624, 5,266,575, 5,352,784, 5,494,916, 5,482,936, 5,346,905, 5,395,937, 5,238,944, 5,525,612, WO99/29693 and U.S. Ser. No. 09/361,544 disclose compounds of the general structure (b):



b

wherein R' is focused around H and $C_{1-8}$ alkyl for the use as "immune response modifiers." Currently at the forefront of that genus is resiquimod. Although a potent cytokine stimulator, resiquimod has not been shown to directly inhibit mechanisms in the virus. As a result, it's efficacy in treating viral infections may be greatly reduced. Nevertheless, the fact that without direct antiviral activity resiquimod is still capable of ameliorating HSV infections indicates the effectiveness of treating by immune potentiation.

[0012] Many of existing cancer therapeutics focus on targeted cytotoxicity, ideally eradicating cancer cell lines, while leaving normal cells intact. Unfortunately, the cancer cell targets for a particular drug, although upregulated in cancer cells, typically exist in normal non-carcinogenic cells as well. Subsequently, with high drug concentrations necessary for treatment, damage to healthy cells results, thereby effecting general health and immune response in the patient as well as causing toxicities such as mucositis, stomatitis, anorexia, vomiting, hypersensitivity, neurotoxicity, oncogenesis and/or mutagenesis. Furthermore, cytotoxic drugs are typically only effective against cancers with rapid growth rates, limiting their applicability and effective patient populations.

[0013] Compounding the problem of toxicity and weakened immune response is the potential for an increased fraction of treatment resistant carcinogenic cells as therapy progresses. In order for treatment to be successful (beyond palliative therapy), it is necessary that all of the cancer cells be eliminated from the patient. Where the drug is so selective in its targeted mechanism of action, the likelihood of drug resistance increases since cancer cell survival depends solely on modifying a single targeted point in the cellular machinery.

[0014] Drug regimens have been designed to kill as many tumor cells as possible by treating with "maximum tolerated doses" (MTDs) of these cytotoxic agents. In an effort to balance toxicity with efficacy, a conventional dosing schedule calls for episodic application of a cytotoxic drug at or near the MTD, followed by periods of

rest to allow normal tissues to recover. Many such chemotherapy regimens are initially efficacious, resulting in tumor regression, but eventually lead relapses often marked by aggressive cancers that are resistant to the cytotoxic drug.

[0015] Subsequently, an altered regimen with multiple mechanisms of action, functioning at doses low enough to avoid toxicity, while stimulating an immune response, would be beneficial. Such a regimen could be used in patients with both rapidly growing tumor cell lines as well as those with low growth rates and/or those in remission to prevent a relapse, since toxicities would be low and immune system functionality would be enhanced as opposed to being suppressed.

[0016] It is therefore an object of the invention to provide a new treatment regimen involving a small molecule immune potentiator, either alone or in combination with another agent, for treatment against a disease characterized by decreased immune capacity, particularly cancer, whereby the regimen targets both substrates integral in cancer cell line survival as well as host immune potentiation, allowing the patient to benefit from both palliative and curative therapies at decreased compound doses.

[0017] The current invention also seeks to provide individual therapeutic and prophylactic agents for treatment of disease states characterized by other immune deficiencies or abnormalities, including autoimmune diseases responsive to compounds with the capacity to modulate cytokines and/or TNF-a, such as multiple sclerosis and Crohn's disease. This need could be met by small molecule immune potentiators (SMIPs) because the small molecule platform provides diverse compounds for the selective manipulation of the immune response, necessary for increasing the therapeutic index immune modulators.

[0018] Furthermore, it would be desirable to provide novel compounds with a varied capacity to alter levels and/or profiles of cytokine production in human immune cells. Compounds with structural disparities will often times elicit a desired response through a different mechanism of action, or with greater specificity to a target, such as a dendritic cell, modulating potency and lowering side effects when administered to a patient.

[0019] Furthermore, it would be desirable to provide novel sole acting agents with a varied capacity to alter levels and/or profiles of cytokine production in human immune cells. Compounds with structural disparities will often times elicit a desired response through a different mechanism of action, or with greater specificity to a

target, such as a dendritic cell, modulating potency and lowering side effects when administered to a patient.

[0020] The immunosuppressive effect of cytostatic substances has rendered them useful in the therapy of autoimmune diseases such as multiple sclerosis, psoriasis and certain rheumatic diseases. Unfortunately, their beneficial effect has to be weighed against the serious side effects that necessitate too low dosages and/or interruption of the treatment.

[0021] It is one object of the present invention to provide an agent or combination of active substances that results in a significantly improved cytostatic or cytotoxic effect as compared to conventional cytostatics e.g. vincristin, methotrexate, cisplatin etc. Thereby, chemotherapies may be offered that combine increasing efficiency with a large reduction of side effects and therapeutic doses. Thus, the therapeutic efficiency of known cytostatic drugs is increased. Also, certain cell lines that are insensitive to chemotherapeutic treatment may become susceptible to chemotherapy by applying the combination of active substances.

[0022] The current invention also seeks to provide individual therapeutic and prophylactic agents for treatment of disease states characterized by other immune deficiencies, abnormalities, or infections including autoimmune diseases and viral and bacterial infections responsive to compounds with the capacity to modulate cytokines and/or TNF-a, such as multiple sclerosis, Crohn's disease, HIV, HSV, and HCV, among others.

[0023] Therapeutics that could serve to augment natural host defenses against viral infections with reduced toxicity would be very beneficial. The present invention provides such therapeutic agents, and further provides other related advantages.

## SUMMARY OF THE INVENTION

[0024] In one aspect, the present invention provides immunogenic compositions and novel methods of administering a vaccine by administering 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones in combination with antigens or other agents. In accordance with the first aspect, the invention further provides 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones as pharmaceutical compositions, for use in the treatment of cancer, infectious diseases, allergies, and asthma. More particularly the invention provides 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones for treatment of diseases in patients with



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suppressed immune systems. For the treatment of cancer at lowered doses, 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-diones surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0025] As adjuvants, the 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds are combined with numerous antigens and delivery systems to form a final vaccine product.

[0026] As immuno-therapeutics, the 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0027] As immunotherapeutics, the 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, mAbs or other immune potentiators). In addition, certain 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-diones with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN's) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds may be used for example for the treatment of BCG, cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds may be used in an anti cell proliferative effective amount for the treatment of cancer. The 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0028] In preferred embodiments of the first aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione and a mAb or antigen, wherein said

combination is more effective to inhibit such cell growth than when said mAb or antigen is administered individually.

[0029] Additional embodiments, methods and compositions contemplated to be useful in the first aspect of the instant invention are disclosed in USSN 10/814,480, 10/762,873, 60/582,654 and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0030] In a second aspect, the present invention provides immunogenic compositions and novel methods of administering a vaccine by administering indolinones in combination with antigens or other agents. In accordance with the second aspect of the invention, the invention further provides indolinones as pharmaceutical compositions, for use in the treatment of cancer, infectious diseases, allergies, and asthma. More particularly the invention provides indolinones for treatment of diseases in patients with suppressed immune systems. For the treatment of cancer at lowered doses, indolinones surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0031] As adjuvants, the indolinone compounds are combined with numerous antigens and delivery systems to form a final vaccine product.

[0032] As immuno-therapeutics, the indolinone compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0033] As immunotherapeutics, the indolinone compounds also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, mAbs or other immune potentiators). In addition, certain indolinones with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN's) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The indolinone compounds may be used for example for the treatment of BCG, cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The indolinone compounds may be used in an anti cell proliferative effective amount for the treatment of cancer.

The indolinone compounds may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0034] In preferred embodiments of the second aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with indolinone compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one indolinone and a mAb or antigen, wherein said combination is more effective to inhibit such cell growth than when said mAb or antigen is administered individually.

[0035] Preferred SMIPs of the second aspect of the present invention include indolinone compositions, specifically N-(2-(dimethylamino)ethyl)-5-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2,4-dimethyl-lH-pyrrole-3-carboxamide; 3-((3,5-dimethyl-lH-pyrrol-2-yl)methylene)indolin-2-one; 3-(2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl)-lH-pyrrol-3-yl)propanoic acid; and 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodoindolin-2-one.

[0036] Additional embodiments, methods and compositions contemplated to be useful in the second aspect of the invention are disclosed in USSN 10/814,480, 10/762,873, and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0037] In a third aspect, the present invention provides immunogenic compositions and novel methods of administering a vaccine by administering chromen-4-ones in combination with antigens or other agents. The third aspect of the invention further provides chromen-4-ones as pharmaceutical compositions, for use in the treatment of cancer, infectious diseases, allergies, and asthma. More particularly the invention provides chromen-4-ones for treatment of diseases in patients with suppressed immune systems. For the treatment of cancer at lowered doses, chromen-4-ones surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0038] As adjuvants, the chromen-4-one compounds are combined with numerous antigens and delivery systems to form a final vaccine product.

[0039] As immuno-therapeutics, the chromen-4-one compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such

as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0040] As immunotherapeutics, the chromen-4-one compounds also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, mAbs or other immune potentiators). In addition, certain chromen-4-ones with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN' s) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The chromen-4-one compounds may be used for example for the treatment of BCG, cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The chromen-4-one compounds may be used in an anti cell proliferative effective amount for the treatment of cancer. The chromen-4-one compounds may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0041] In preferred embodiments of the third aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with chromen-4-one compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one chromen-4-one and a mAb or antigen, wherein said combination is more effective to inhibit such cell growth than when said mAb or antigen is administered individually.

[0042] Preferred SMIPs of the third aspect of the present invention include chromen-4-one compositions, specifically 2-(2-chlorophenyl)-2,3-dihydro-5,7-dihydroxy-8-(3-hydroxy-l-methylpiperidin-4-yl)chromen-4-one; 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one; and 2,3-dihydro-5,7-dihydroxy-6-methoxy-2-(3 ,4-dimethoxyphenyl)chromen-4-one.

[0043] Additional embodiments, methods and compositions contemplated to be useful in the third aspect instant invention are disclosed in USSN 10/814,480, 10/762,873, 60/582,654 and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0044] In a fourth aspect, the present invention provides immunogenic compositions and novel methods of administering a vaccine by administering derivatized pyridazines in combination with antigens or other agents. In accordance with the

fourth aspect, the invention further provides derivatized pyridazines as pharmaceutical compositions, for use in the treatment of cancer, infectious diseases, allergies, and asthma. More particularly the invention provides derivatized pyridazines for treatment of diseases in patients with suppressed immune systems. For the treatment of cancer at lowered doses, derivatized pyridazines surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0045] As adjuvants, the derivatized pyridazine compounds are combined with numerous antigens and delivery systems to form a final vaccine product.

[0046] As immuno-therapeutics, the derivatized pyridazine compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0047] As immunotherapeutics, the derivatized pyridazine compounds also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, niAbs or other immune potentiators). In addition, certain derivatized pyridazines with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN' s) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The derivatized pyridazine compounds may be used for example for the treatment of BCG, cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The derivatized pyridazine compounds may be used in an anti cell proliferative effective amount for the treatment of cancer. The derivatized pyridazine compounds may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0048] In preferred embodiments of the fourth aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with derivatized pyridazine compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one derivatized pyridazine and a

mAb or antigen, wherein said combination is more effective to inhibit such cell growth than when said mAb or antigen is administered individually.

[0049] Preferred SMIPs of the fourth aspect of the present invention include derivatized pyridazine compositions, specifically 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine.

[0050] Additional embodiments, methods and compositions contemplated to be useful in the fourth aspect of the instant invention are disclosed in USSN 10/814,480, 10/762,873, 60/582,654, US 6,258,812 and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0051] In a fifth aspect, the present invention provides immunogenic compositions and novel methods of administering a vaccine by administering staurosporine analogs in combination with antigens or other agents. The fifth aspect of the invention further provides staurosporine analogs as pharmaceutical compositions, for use in the treatment of cancer, infectious diseases, allergies, and asthma. More particularly the invention provides staurosporine analogs for treatment of diseases in patients with suppressed immune systems. For the treatment of cancer at lowered doses, staurosporine analogs surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0052] As adjuvants, the staurosporine analog compounds are combined with numerous antigens and delivery systems to form a final vaccine product.

[0053] As immuno-therapeutics, the staurosporine analog compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0054] As immunotherapeutics, the staurosporine analogs also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, niAbs or other immune potentiators). In addition, certain staurosporine analogs with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN's) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The staurosporine analog compounds may be used for example for the treatment of BCG,

cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The staurosporine analog compounds may be used in an anti cell proliferative effective amount for the treatment of cancer. The staurosporine analog compounds may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0055] In preferred embodiments of the fifth aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with staurosporine analog compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one staurosporine analog and a mAb or antigen, wherein said combination is more effective to inhibit such cell growth than when said mAb or antigen is administered individually.

[0056] Preferred SMIPs of the fifth aspect of the present invention include staurosporine analog compositions, specifically 9,13-Epoxy-lH,9H-diindolo[l,2,3-gh^'^U'-Mpyrrolop^^fl^benzodiazonin-l-one, 2,3,10,1 1,12,13-hexahydro-11,12-dihydroxy- 10-methoxy-9-methyl; 9,13-Epoxy- 1H,9H-diindolo[ 1,2,3-gh:3\2\ Y-lm]pyrrolo[3 ,4-j][1,7]benzodiazonin-l -one, 2,3,10,11,12,13-hexahydro- 10-methoxy-9-methyl-ll-(methylamino); 9,13-Epoxy-lH,9H-diindolo[l,2,3-gh:3',2',r-lm]pyrrolo[3,4-j][l,7]benzodiazonin-l-one, 2,3,10,1 1,12,13-hexahydro-3-hydroxy-10-methoxy-9-methyl-ll-(methylammo); 9,12-Epoxy-lH-diindolo[l,2,3-fg:3',2',r-kl]pyrrolo[3 ,4-i][1,6]benzodiazocin-l -one, 2,3,9,10,11,12-hexahydro- 10-hydroxy- 10-(hydroxymethyl)-9-methyl; and benzamide, N-[(9S,10R,llR,13R)-2,3,10,ll, 12,13-hexahydro- 10-methoxy-9-methyl- 1-oxo-9,13-epoxy- lH,9H-diindolo[ 1,2,3-gh:3',2',1'-lm]pyrrolo [3,4-j][1,7]benzodiazonin- 11-yl]-N-methyl.

[0057] Additional embodiments, methods and compositions contemplated to be useful in the fifth aspect of the instant invention are disclosed in USSN 10/814,480, 10/762,873, 60/582,654 and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0058] In a sixth aspect, the instant invention provides novel immune potentiators, immunogenic compositions, and novel methods of administering a vaccine, by administering nucleoside analogs alone or in combination with antigens/other agents.

The sixth aspect of the invention further provides compositions, novel compounds and pharmaceutical compositions, for use in the treatment of infectious diseases.

[0059] The nucleoside analogs used in the methods and compositions of the sixth aspect of the invention are inexpensive to produce and easy to administer. They have potential for finer specificity compared to existing immunostimulants, thus providing improved efficacy and safety profiles.

[0060] As adjuvants, the nucleoside analogs are combined with numerous antigens and delivery systems to form a final vaccine product.

[0061] As immuno-therapeutics, the nucleoside analogs are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as Kaposi's sarcoma, anogenital warts, HIV, HCV, SARS, HBV, HPV, HSV, and H. pylori.

[0062] The nucleoside analogs of the present invention target substrates in the virus or disease state, such as, for example proteases, replicases, DNA polymerase, and/or RNA polymerase.

[0063] In addition, certain nucleoside analogs with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN's) could be useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae. The nucleoside analogs may be used for example for the treatment of BCG, cholera, plague, typhoid, hepatitis B infection, influenza, inactivated polio, rabies, measles, mumps, rubella, oral polio, yellow fever, tetanus, diphtheria, hemophilus influenzae b, meningococcus infection, and pneumococcus infection. The nucleoside analogs may be used in an anti cell proliferative effective amount for the treatment of cancer. The nucleoside analogs may also be used in anti-Th2/Type2 cytokine amount for the deviation of allergic/asthmatic immune responses.

[0064] Additional embodiments, methods and compositions contemplated to be useful in the sixth aspect of instant invention are disclosed in USSN 10/814,480, 10/762,873, 60/582,654 and 10/748,071 which are incorporated by reference as if set forth fully herein.

[0065] Methods of manufacturing compounds and compositions described herein are provided and contemplated to fall within the scope of the invention. Further provided are pharmaceutical compositions comprising any one of the compounds of Table 1, or

compounds encompassed by Formulas I, II, or III, of Section VI of the detailed description.

[0066] In a seventh aspect, the present invention immunogenic compositions and novel methods of administering a vaccine, by administering fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-3 17615, squalamine, UCN-Ol, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, or tetrathiomolybdate in combination with antigens or other agents. The seventh aspect of the invention further provides one of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, or tetrathiomolybdate as a pharmaceutical composition, for use in the treatment of diseases associated or complicated with immune suppression, such as cancer, infectious diseases, allergies, and asthma. For the treatment of cancer at attenuated doses, fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-3 176 15, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate surpass existing therapies in that they function by independently targeting substrates involved in cancer pathways, while separately inducing an immune response in the subject thereby attacking different modes of the disease state.

[0067] As adjuvants, the SMIP compounds (namely fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-3 17615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, or tetrathiomolybdate) are combined with numerous antigens and delivery systems to form a final vaccine product.

[0068] As immuno-therapeutics, the SMIP compounds are used alone or in combination with other therapies (e.g. anti-virals, anti-bacterials, other immune modulators or in therapeutic vaccine antigens) for treatment of autoimmune diseases, chronic infections such as HIV, HCV, HBV, HSV, and H. pylori, as well as medicaments for the reduction of tumor growth.

[0069] More particularly, one embodiment of the seventh aspect of the invention provides administering a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-Ol, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate to treat one of the following diseases: bacterial diseases autoimmune-, allergic- and viral-diseases, disturbances caused by the incidence of mental diseases, central nervous system depressants, habitual alcohols, and disorders of the respiratory system, CNS, urinary system, lymphatic system, reproductive system, or digestive system.

[0070] As immunotherapeutics, the SMIP compounds also may be used for the treatment of cancer either alone or in combination with other anti-cancer therapies (e.g. chemotherapeutic agents, mAbs or other immune potentiators). In addition, fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, or tetrathiomolybdate with the capacity to induce Type 1 cytokines (e.g. IL-12, TNF or IFN's) are useful for the treatment of allergies or asthma due to their capacity to steer the immune response towards more benign sequelae.

[0071] In preferred embodiments of the seventh aspect of the invention, methods of treating cancer are provided wherein known anticancer agents are combined with SMIP compounds to reduce tumor growth in a subject. A method of inhibiting tumor cell growth is provided, comprising administering to a subject an effective dose of a combination containing at least one SMIP and a Mab or antigen, wherein said combination is more effective to inhibit such cell growth than when said Mab or antigen is administered individually.

[0072] Preferable SMIPs of the seventh aspect of the present invention include fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, or tetrathiomolybdate compositions as well as analogs disclosed in the following patents and patent applications: US 4,323,581, US 6,258,812, WO 98/35958, WO 01/60814, US

5,883,1 13, WO 99/61422, US 5,883,113, WO 99/61422, WO 03/24978, WO 03/04505, US 5,780,454, US 2003134846, WO 97/21701, US 5,621,100, WO 01/32651, WO 02/16351, US 6,727,256, WO 02/02552, US 5,457,105, US 5,616,582, US 5,770,599, US 5,747,498, WO, 96/30347, US 2003171303, WO 97/20842, WO 99/02162, WO 95/17182, WO 01/79255, WO 89/07105, US 5,439,936, WO 94/18151, WO 97/42949, WO 98/13344, US 5,554,519, WO 98/04541, US 6,025,387, US 2004073044, WO 02/62826, WO 04/06834, US 6,331,555, and WO 01/60814, wherein they are administered in reduced dosing amounts from existing regimens. More particularly, preferred compounds of the seventh aspect include those encompassed by Formula I in the aforementioned references.

[0073] Additional embodiments, methods and compositions contemplated to be useful in the seventh aspect of the instant invention are disclosed in USSN 10/814,480, 10/762,873, and 10/748,071.

**[0074]** Further embodiments of the several aspects of the invention include those described in the detailed description.

## DETAILED DESCRIPTION OF THE INVENTION

**[0075]** In each of the aspects of the invention, the "method of modulating an immune response in a patient comprising administering a compound" of a particular formula, such as formula (I), can be replaced with: "use of a compound of formula (I) in the manufacture of a medicament for modulating an immune response in a patient." In other embodiments the compounds are used in the manufacture of a medicament for treating an infectious disease, autoimmune disease, allergies, or cancer. In other embodiments the compounds are used in the manufacture of a medicament for use as an adjuvant. Preferred compounds of the present invention include those having a particular formula as described in the aspects of the invention.

**[0076]** Other embodiments provide the use of a compound of formula (I) and another agent for simultaneous separate or sequential administration. In a more particular embodiment the other agent is an antigen. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

**[0077]** Other embodiments provide a pharmaceutical preparation or system, comprising **(a)** a **first** pharmaceutical agent, **which** comprises a compound of formula

(I); and (b) a second pharmaceutical agent, wherein said first and second agents are either in admixture or are separate compositions. In a more particular embodiment the second agent is an antigen. More specifically, the agents are for simultaneous separate or sequential administration. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

[0078] A kit comprising (a) a first pharmaceutical agent, which comprises a SMIP of formula I-L; and (b) a second pharmaceutical agent. In a more particular embodiment the second agent is an antigen. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

[0079] Another embodiment provides the use of a compound of formula (I) and another agent in the manufacture of a combination medicament. In a more particular embodiment the other agent is an antigen. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

[0080] Another embodiment provides the use of a compound of formula (I) in the manufacture of a medicament, wherein the medicament is co-administered with another agent. In a more particular embodiment the second agent is an antigen. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

[0081] Another embodiment provides the use of an antigen in the manufacture of a medicament, wherein the medicament is co-administered with a compound of formula (I).

[0082] The two agents are preferably administered within 4 hours of each other.

[0083] Another embodiment provides the use of a compound of formula (I) in the manufacture of a medicament, wherein the medicament is for administration to a patient who has been pre-treated with another agent. In a more particular embodiment the second agent is an antigen. In another more particular embodiment the use is for

modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

**[0084]** Another embodiment provides the use of an antigen in the manufacture of a medicament, wherein the medicament is for administration to a patient who has been pre-treated with a compound of formula (I). The pre-treatment may be recent (e.g. within the 24 hours preceding administration of said medicament), intermediate (e.g. more than 24 hours previous, but no longer than 4 weeks), more distant (e.g. at least 4 weeks previous), or very distant (e.g. at least 6 months previous), with these time periods referring to the most recent pre treatment dose. The patient may be refractory to treatment by the pharmaceutical agent that was administered in the pre-treatment. In another more particular embodiment the use is for modulating an immune response in a patient. In another embodiment the use is for treating an infectious disease, autoimmune disease, allergies, or cancer. In another embodiment the use is as an adjuvant.

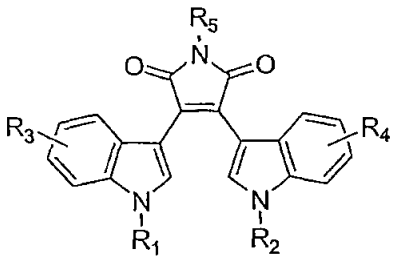
**[0085]** Another embodiment provides, the use of a compound of formula (I) in the manufacture of a medicament, wherein the medicament is for administration to a patient who has a tumor or infection that is resistant to treatment with another agent.

**[0086] Section I - First Aspect of the Invention -** Quinazolines **for** Immuiiopotentiation

**[0087]** All definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

**[0088]** In accordance with the first aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

**[0089]** One embodiment of the first aspect invention includes a method of modulating an immune response in a subject comprising administering a compound of fonnula I:

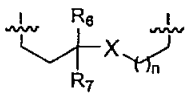


I

wherein,

Ri and $R_2$ are each independently H, alkyl, aryl, alkoxy, $-C(O)R_c$, or heterocyclyl; or

Ri and $R_2$ are taken together to form a bridge in subformula Ia:



Ia

the binding being achieved at the bonds crossed with a ~~~;

$R_3$ and $R_4$ each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-C(O)R_c$, $-S(O)_pR_d$, or heterocyclyl;

$R_5$ is H or alkyl;

$R_6$ is H, -OH, $-NH_2$, halogen, or alkyl;

$R_7$ is H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-C(O)R_0$, $-S(O)_pR_d$, or heterocyclyl;

X is $-CH(R_e)-$, -O-, -S-, or $-N(R_f)-$;

each $R_a$ and $R_b$ is independently H, alkyl, $-C(O)R_0$, aryl, heterocyclyl, or alkoxy;

each $R_0$ is independently H, alkyl, alkoxy, $-NH_2$, -NH(alkyl), $-N(alkyl)_2$, aryl, or heterocyclyl;

each Rd is independently H, alkyl, alkenyl, aryl, or $-NR_aR_b$;

Re is H, -OH, halogen, $-C(O)R_0$, aryl, heterocyclyl, or $-NR_aR_b$;

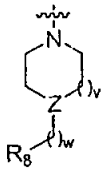
n is 0, 1, or 2;

each p is independently 0, 1, or 2;

each q is independently 0, 1, or 2.

[0090] Another embodiment is provided, wherein $R_5$ is H. In still another more particular embodiment $R_3$ and $R_4$ are both H. In still another more particular embodiment Ri is methyl, ethyl, propyl, or isopropyl, and $R_2$ is heterocyclyl.

[0091] In another more particular embodiment, $R_2$ is defined as in subformula $R_{2a}$:



$R_{2a}$

the binding being achieved at the bond crossed with a ~~~;

Z is N, O, S, or CH, provided that when Z is O or S, w is 0 and $R_8$ is absent;

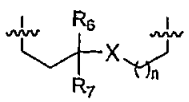
$R_8$ is absent, aryl or heterocyclyl;

v is 0, 1, or 2; and

w is 0, 1, or 2.

[0092] In another embodiment thereof, Z is N. In still another more particular embodiment, w and v are both 1. In still another more particular embodiment $R_8$ is pyridyl.

[0093] In a separate embodiment thereof, $R_1$ and $R_2$ are taken together to form a bridge in subformula Ia:



Ia

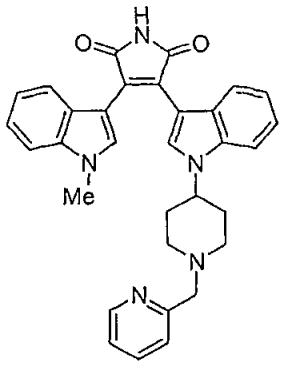
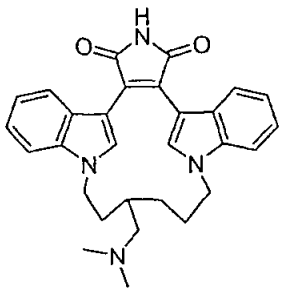
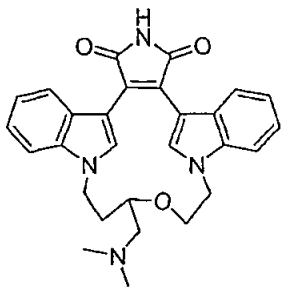
the binding being achieved at the bonds crossed with a ~~~.

[0094] In another more particular embodiment of substructure Ia, $R_6$ is H. In another more particular embodiment of substructure Ia, n is 1.

[0095] In another more particular embodiment of substructure Ia, X is O. In still another more particular embodiment of substructure Ia, $R_7$ is $-CH_2-NH(alkyl)$, $-CH_2-NH_2$, $-CH_2-CH_2-NH_2$, $-CH_2-CH_2-NH(alkyl)$, $-CH_2-CH_2-N(alkyl)_2$ or $-CH_2-N(alkyl)_2$. In a preferred embodiment thereof said alkyl with $R_7$ is methyl, ethyl, propyl, or isopropyl.

[0096] In another more particular embodiment of substructure Ia, X is $-CH_2-$. In still another more particular embodiment of substructure Ia, $R_7$ is $-CH_2-NH(alkyl)$, $-CH_2-NH_2$, $-CH_2-CH_2-NH_2$, $-CH_2-CH_2-NH(alkyl)$, $-CH_2-CH_2-N(alkyl)_2$ or $-CH_2-N(alkyl)_2$. In a preferred embodiment thereof said alkyl with $R_7$ is methyl, ethyl, propyl, or isopropyl.

[0097] Another embodiment of the invention provides a method of modulating an immune response in a subject comprising administering a compound selected from the group consisting of:


,

, and

.

[0098] In a more particular embodiment of any of the above methods, said modulating is inducing. In another embodiment said inducing stimulates production of cytokines, chemokines, and/or growth factors.

[0099] In another embodiment said compound is administered in a subcytotoxic amount to said subject.

[00100] In another embodiment said subject is in remission from cancer.

[00101] In another embodiment said compound is administered for the treatment of refractory cancer cells.

[00102] In another embodiment said compound is administered metronomically.

[00103] In another embodiment the subject is not suffering from cancer.

[00104] In another embodiment said compound is co-administered with another agent.

[00105] In another embodiment said compound is administered in a dose capable of increasing TNF-ce levels.

[00106] In another embodiment said compound has an average steady state drug concentration in the blood of less than 20 $\mu$M.

[00107] In another embodiment subject is suffering from an autoimmune disease. Further still, said autoimmune disease is multiple sclerosis or Crohn's disease.

[00108] In another embodiment said subject is suffering from a viral infection.

[00109] In another embodiment said viral infection is HCV, HIV, or HSV.

[00110] In another embodiment said subject is suffering from allergies.

[00111] In another embodiment said subject is suffering from asthma.

[00112] In another embodiment the subject is suffering from precancerous lesions, such as actinic keratosis, atypical or dysplastic nevi, or premalignant lentigos. In another embodiment the subject is suffering from a disease associated with abnormal cellular proliferation, such as, neuro-fibromatosis, atherosclerosis, pulmonary fibrosis, arthritis, psoriasis, glomerulonephritis, restenosis, proliferative diabetic retinopathy (PDR), hypertrophic scar formation, inflammatory bowel disease, transplantation rejection, angiogenesis, or endotoxic shock.

[00113] Another embodiment provides a pharmaceutical composition containing any of the aforementioned compounds or embodiments of formula I.

[00114] In another embodiment the subject is suffering from precancerous lesions, such as actinic keratosis, atypical or dysplastic nevi, or premalignant lentigos.

[00115] Furthermore, it is contemplated that the invention encompasses all possible combinations of the preceding embodiments.

[00116] The 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compounds can be used with or without an antigen in therapeutic applications, for example to treat cancer or infectious diseases. The 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compounds also may be used in combination with other therapeutic agents, such as anti-virals and monoclonal antibodies in different therapeutic applications.

[00117] One preferred embodiment of the method of inducing an immunostimulatory effect in a patient is directed to administering an immunogenic composition comprising a vaccine in an amount effective to stimulate an immune response such as a cell-mediated immune response and, as a vaccine adjuvant, an 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound, in an amount effective to potentiate the immune response such as the cell-mediated immune response to the vaccine.

[00118] Agents combined with the 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compounds, contemplated to be useful in treating the aforementioned diseases include those well known in the art, such as, anesthetics, hypnotic sedatives, anti-anxieties, antiepileptics, antipyretic antiphlogistics, stimulants, wake amines, anti-parkinson drugs, agents for psychoneuroses, agents for central nervous system, skeletal muscle relaxants, agents for autonomic nervous system, antispastic agents, cytotoxic agents, monoclonal antibodies, drugs for eye, drugs for nose and ear, anti-vertiginous drugs,

cardiotonics, antiarrhythmic drugs, diuretics, pressure reduction drugs, vasoconstrictors, coronary vaso-dilators, peripheral vasodilating drugs, hyper-lipemia drugs, breath stimulants, antitussive and expectorant drugs, bronchodilators, drugs for allergy, antidiarrheal drugs, drugs for intestinal disorders, peptic ulcer drugs, stomachic digestants, antacids, cholagogouses, pituitary hormone drugs, salivary gland hormones, thyroid hormone drugs, antithyroid drugs, anabolic steroids, corticosteroids, androgen drugs, estrogen drugs, corpus luteum hormone drugs, mixed hormones, urinary/genital organ drugs, anus drugs, surgical sterilizations/antiseptics, wound protectives, externals for purulent diseases, analgesics, antipruritics, astringents, antiphlogistics, externals for parasite skin diseases, skin-softening drugs, caustics, dental/oral drugs, vitamins, inorganic preparations, supplemental liquids, hemostatics, anticoagulation drugs, drugs for liver diseases, antidotes, habitual intoxication drugs, drugs for treatment of gout, enzyme preparations, diabetic drugs, antioncotics, antihistaminics, antibiotics (such as ketolides, aminoglycosides, sulphonamides, and/or beta lactams), chemotherapeutics, biological preparations, anthelmintics, anti-Protozoas, drugs for preparations, X-ray contrast media, and diagnostic drugs.

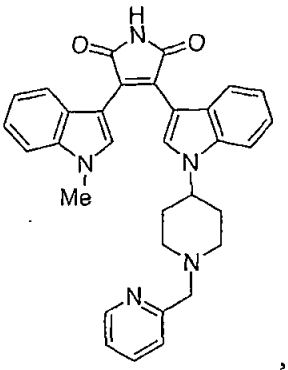
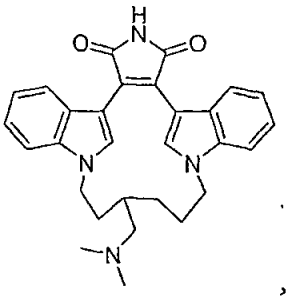
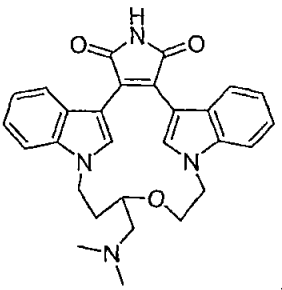
[00119] Further in accordance with the first aspect of the invention, methods of the invention are provided wherein compositions described herein are used for the treatment of cancer and reduction of tumor growth. In one embodiment an 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound of the invention is combined with a known mAb for the treatment of cancer. In a presently preferred embodiment of this embodiment of the present invention, an antibody and a 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound are administered. It may be particularly preferred that said antibody, individually, has an inhibiting effect upon tumor cell growth and that the 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound induces the production of cytokines.

[00120] In accordance with another embodiment of the first aspect of the present invention, there is provided a therapeutic composition for inhibiting tumor cell growth in a subject, which composition comprises an effective amount of a combination of at least an 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound and a mAb and a pharmaceutically acceptable carrier, wherein said combination is more effective to inhibit growth of certain mammalian tumor cells than are either of the agents when administered individually.

[00121] In another embodiment of the first aspect of the invention, methods of treating allergies are provided comprising administering an 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound alone or in combination with another agent known to be effective against allergies, wherein said combination is more effective in treating an allergic condition than the known agent(s) are without the addition of said 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compound. In a more preferred embodiment the known agent is an antihistamine and/or a leukotriene inhibitor. In another preferred embodiment, the allergic condition is asthma. In another preferred embodiment, the allergic condition is selected from the group consisting of allergic rhinitis, dermatosis, and urticaria. In an even more preferred embodiment the combination is administered to a subject enterally, parenterally, intranasally, subcutaneously, or intraarterially.

[00122] Preferred SMIPs in accordance with the first aspect of the invention are 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones, as well as analogs disclosed in the following patents and patent applications: US 5,552,396, US 5,057,614, and WO 95/17182. More particularly, preferred SMIPs of the first aspect of the invention include flavones, isoflavones and those encompassed by Formula I as described in this section, or contained within the aforementioned references.

[00123] Reference to "3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones" indicates compounds having the general structure of Formula I as described herein. Preferred 3,4-di( lH-indol-3-yl)-l H-pyrrole-2,5-diones are

[00124]  ,  , or 

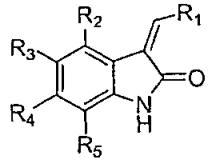
[00125] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially available, or are easily synthesized by one skilled in the art from procedures described in patents/applications, such as US 5,552,396, US 5,057,614, WO 95/17182 and other patents/applications listed herein.

[00126] **Section K - Second Aspect of the Invention -** Indolinones **for** Immunopotentiation

[00127] AU definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

In accordance with the second aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

[00128] In one embodiment of the second aspect of the invention, a method of modulating an immune response in a subject is provided, comprising administering a compound of formula I:



I

wherein,

Ri is alkyl, aryl or heterocyclyl;

$R_2$, $R_3$, $R_4$, and $R_5$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aRb$, -C(O)Rc, $-S(O)_nRd$, or heterocyclyl;

each $R_a$ and $R_b$ is independently H, alkyl, $-C(O)R_c$, aryl, heterocyclyl, or alkoxy;

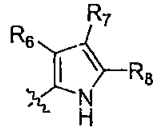
each $R_c$ is independently H, alkyl, alkoxy, $-NH_2$, -NH(alkyl), $-N(alkyl)_2$, aryl, or heterocyclyl;

each $R_d$ is independently H, alkyl, alkenyl, aryl, or -NRaRb!

each n is independently o, 1, or 2; and

each q is independently 0, 1, or 2.

[00129] In a more particular embodiment, Ri is heterocyclyl. In a more particular embodiment still, Ri is R]$_a$:



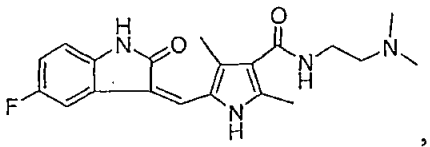
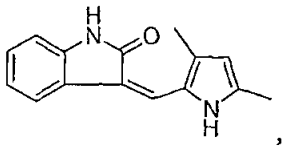
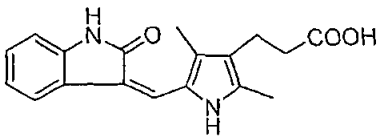
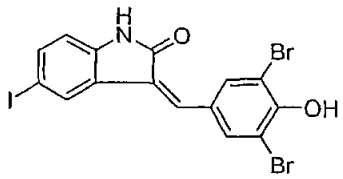
Rla

wherein,

$R_6$, $R_7$ and $R_8$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aRb$, $-C(O)R_0$, $-S(O)_nRd$, or heterocyclyl. In yet another more particular embodiment, wherein Ri is R]$_a$, $R_7$ is $-C(O)-(CH_2)_p-N(H)_{(2-r)}(alkyl)_r$, wherein p is 0, 1, 2, 3, 4, or 5 and r is 0, 1, or 2. In yet another more particular embodiment, wherein Ri is Ria, $R_3$ is F. In yet another more particular embodiment, wherein Ri is $Ri_a$, $R_7$ is $-(CH_2)_tCOOH$, wherein t is 1, 2, 3, or 4. In yet another more particular embodiment, wherein Ri is $Ri_a$, $R_6$ and $R_8$ are both methyl. In yet another more particular embodiment, wherein $R_1$ is R]$_a$, $R_7$ is H.

[00130] In another more particular embodiment of the second aspect of the invention, Ri is aryl. More particular still, Ri is substituted or unsubstituted phenyl. In another embodiment $R_3$ is iodide.

[00131] In another embodiment of the second aspect of the invention, a method of modulating an immune response in a subject is provided, comprising administering a compound selected from the group consisting of:


,

,

, and

.

[00132] In a more particular embodiment of any of the above methods, said modulating is inducing. In another embodiment said inducing stimulates production of cytokines, chemokines, and/or growth factors.

[00133] In another embodiment said compound is administered in a subcytotoxic amount to said subject.

[00134] In another embodiment said subject is in remission from cancer.

[00135] In another embodiment said compound is administered for the treatment of refractory cancer cells.

[00136] In another embodiment said compound is administered metronomically.

[00137] In another embodiment the subject is not suffering from cancer.

[00138] In another embodiment said compound is co-administered with another agent.

[00139] In another embodiment said compound is administered in a dose capable of increasing TNF-$\alpha$ levels.

[00140] In another embodiment said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

[00141] In another embodiment subject is suffering from an autoimmune disease. Further still, said autoimmune disease is multiple sclerosis.

[00142] In another embodiment said subject is suffering from a viral infection.

[00143] In another embodiment said viral infection is HCV, HIV, or HSV.

[00144] In another embodiment said subject is suffering from allergies.

[00145] In another embodiment said subject is suffering from asthma.

[00146] It is contemplated that the invention encompasses all possible combinations of the embodiments described herein.

[00147] Preferred SMIPs in accordance with the second aspect of the invention are indolinones, such as N-(2-(dimethylamino)ethyl)-5-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2,4-dimethyl-lH-pyrrole-3-carboxamide; 3-((3,5-dimethyl-lH-pyrrol-2-yl)methylene)mdolin-2-one; 3-(2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl)-lH-pyrrol-3-yl)propanoic acid; and 3-(3,5-dibromo-4-hydroxybcnzylidene)-5-iodoindolin-2-one, as well as analogs disclosed in the following patents and patent applications: WO 01/60814, US 5,883,1 13, WO 99/61422, and WO 99/61422. More particularly, preferred SMIPs include those encompassed by Formula I as described in this section, or contained within the aforementioned references.

[00148] Reference to "indolinones" indicates compounds having the general structure of Foπnula I as described in this section. Preferred indolinones are N-(2-(dimethylamino)ethyl)-5-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2,4-dimethyl-lH-pyrrole-3-carboxamide; 3-((3,5-dimethyl-lH-pyrrol-2-yl)methylene)indolin-2-one; 3-(2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl)-lH-pyrrol-3-yl)propanoic acid; and 3-(3,5-dibromo-4-hydiOxybenzylidene)-5-iodoindolin-2-one.

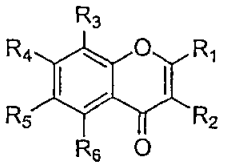
[00149] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially available, or are easily synthesized by one skilled in the art from procedures described in patents or patent applications listed herein.

[00150] **Section III - Third Aspect of the Invention** - Chromen-4-ones for Immiinopotentiation

[00151] AU definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

[00152] In accordance with the third aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

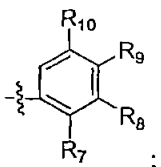
[00153] In one embodiment of the invention, a method of modulating an immune response in a subject is provided, comprising administering a compound of formula I:



I

wherein

one of Ri and $R_2$ is H, -CN, alkyl, -OH, $-NR_aR_b$, alkoxy, -C(O)Rc, $-S(O)_nRd$, heterocyclyl, or aryl, and the other is:



;

$R_3$, $R_4$, $R_5$, and $R_6$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, -NRaRb, -C(O)Rc, $-S(O)_nRd$, or heterocyclyl;

$R_7$ and $Ri_0$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-C(O)R_0$, $-S(O)_nRd$, or heterocyclyl;

$R_8$ and $R_9$ are each independently H, -CN, -OH, halogen, alkyl, aryl, $-O-(CH_2)_q-R_e$, -C(O)Rc, $-0-(CH_2)_q-0-R_e$, $-NR_aR_b$, $-S(O)_nR_d$, or heterocyclyl;

each $R_a$ and Rb is independently H, alkyl, $-C(O)R_C$ aryl, heterocyclyl, or alkoxy;

each $R_c$ is independently H, alkyl, alkoxy, $-NH_2$, -NH(alkyl), $-N(alkyl)_2$, aryl, or heterocyclyl;

each Rd is independently H, alkyl, alkenyl, aryl, or $-NRJR_b$;

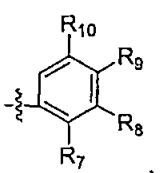
each Re is independently H, halogen, $-C(O)R_C$ aryl, heterocyclyl, or $-NR_aR_b$;

each n is independently 0, 1, or 2; and

each q is independently 0, 1, or 2.

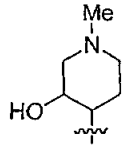
[00154] In a more particular embodiment $R_4$ and $R_6$ are both -OH.

[00155] In another more particular embodiment Ri is:



[00156] In another more particular embodiment $R_3$ is heterocyclyl.

[00157] In another more particular embodiment thereof, $R_3$ is:




[00158] In another more particular embodiment $R_7$ is Cl.

[00159] In another more particular embodiment $R_4$ and $R_6$ are both -OH.

[00160] In another more particular embodiment $R_5$ is alkoxy.

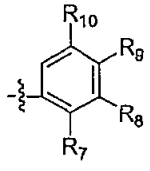
[00161] In another more particular embodiment $R_5$ is methoxy.

[00162] In another more particular embodiment $R_8$ and $R_9$ are both $-O-(CH_2)_q-Rc$

[00163] In another more particular embodiment $R_8$ and Rg are both $-OCH_3$.

[00164] In another more particular embodiment $R_2$ is H.

[00165] In another embodiment $R_2$ is:

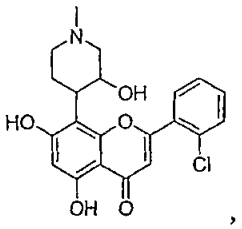
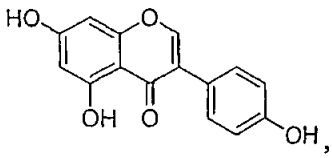
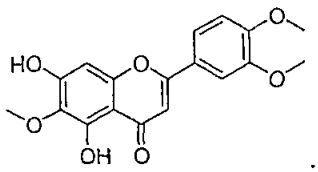



[00166] In another more particular embodiment Ri is H.

[00167] In another more particular embodiment $R_4$ and $R_6$ are both -OH.

[00168] In another more particular embodiment $R_9$ is -OH.

[00169] Another embodiment of the invention provides a method of modulating an immune response in a subject comprising administering a compound selected from the group consisting of:



, and


[00170] In a more particular embodiment of any of the above methods, said modulating is inducing. In another embodiment said inducing stimulates production of cytokines, chemokines, and/or growth factors.

[00171] In another embodiment said compound is administered in a subcytotoxic amount to said subject.

[00172] In another embodiment said subject is in remission from cancer.

[00173] In another embodiment said compound is administered for the treatment of refractory cancer cells.

[00174] In another embodiment said compound is administered metronomically.

[00175] In another embodiment the subject is not suffering from cancer.

[00176] In another embodiment said compound is co-administered with another agent.

[00177] In another embodiment said compound is administered in a dose capable of increasing TNF-$\alpha$ levels.

[00178] In another embodiment said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

[00179] In another embodiment subject is suffering from an autoimmune disease. Further still, said autoimmune disease is multiple sclerosis or Crohn's disease.

[00180] In another embodiment said subject is suffering from a viral infection.

[00181] In another embodiment said viral infection is HCV, HIV, or HSV.

[00182] In another embodiment said subject is suffering from allergies.

[00183] In another embodiment said subject is suffering from asthma.

[00184] In another embodiment the subject is suffering from a disease associated with abnormal cellular proliferation, such as, neuro-fibromatosis, atherosclerosis, pulmonary fibrosis, arthritis, psoriasis, glomerulonephritis, restenosis, proliferative diabetic retinopathy (PDR), hypertrophic scar formation, inflammatory bowel disease, transplantation rejection, angiogenesis, or endotoxic shock.

[00185] It is contemplated that the invention encompasses all possible combinations of the embodiments described herein.

[00186] Preferred SMIPs in accordance with the third aspect of the invention are chromen-4-ones, such as 2-(2-chlorophenyl)-2,3-dihydro-5,7-dihydroxy-8-(3-hydroxy- l-methylpiperidin-4-yl)chromen-4-one; 5,7-dihydroxy-3 -(4-hydroxyphenyl)-4H-chromen-4-one; and 2,3-dihydro-5,7-dihydroxy-6-methoxy-2-(3,4-dimethoxyphenyl)chromen-4-one, as well as analogs disclosed in the following patents and patent applications: WO 97/42949, and WO 98/13344, US 5,554,519, WO 98/04541, and US 6,025,387. More particularly, preferred SMIPs include flavones, isoflavones and those encompassed by Formula I as described in this section, or contained within the aforementioned references.

[00187] Reference to "chromen-4-ones" indicates compounds having the general structure of Formula I as described in this section. Preferred chromen-4-ones are flavones and isoflavones selected from the group consisting of 2-(2-chlorophenyl)-2,3-dihydro-5,7-dihydroxy-8-(3-hydroxy-l-methylpiperidin-4-yl)chromen-4-one; 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one; and 2,3-dihydro-5,7-dihydroxy-6-methoxy-2-(3,4-dimethoxyphenyl)chromen-4-one.

[00188] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially available, or are easily synthesized by one skilled in the art from procedures described in patents or patent applications listed herein.

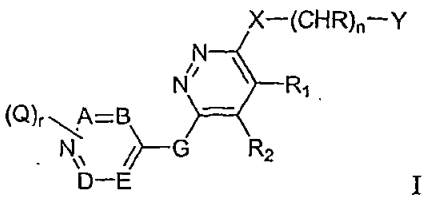
[00189] **Section IV - Fourth Aspect of the Invention - Derivatized** Pyridazines **for** Immunopotentiation

[00190] All definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

[00191] In accordance with the fourth aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

[00192] One embodiment of the invention provides a method of modulating an immune response comprising administering a derivatized pyridazine or a pharmaceutically acceptable salt thereof to a subject in need thereof.

[00193] In one embodiment of the invention, a method of modulating an immune response in a subject is provided, comprising administering a compound of formula I:




I

wherein

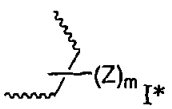
r is 0 to 2,

n is 0 to 2,

m is 0 to 4,

$R_1$ and $R_2$ (i) are lower alkyl, or

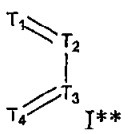
(ii) together form a bridge in subformula I*




I*

the binding being achieved via the two terminal carbon atoms, or

(iii) together form a bridge in subformula I**




I**

wherein one or two of the ring members $T_1$, $T_2$, $T_3$ and $T_4$ are nitrogen, and the others are in each case CH, and the binding is achieved via Ti and $T_4$;

A, B, D, and E are, independently of one another, N or CH, with the stipulation that not more than 2 of these radicals are N;

G is lower alkylene, lower alkylene substituted by acyloxy or hydroxy, -$CH_2$-O-, -$CH_2$-S-, -$CH_2$-NH-, oxa (-O-), thia (-S-), or imino (-NH-);

Q is lower alkyl, especially methyl;

R is H or lower alkyl;

X is imino, oxa, or thia;

Y is aryl, pyridyl, or unsubstituted or substituted cycloalkyl; and

Z is mono- or disubstituted amino, halogen, alkyl, substituted alkyl, hydroxy, etherified or esterified hydroxy, nitro, cyano, carboxy, esterified carboxy, alkanoyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amidino, guanidino, mercapto, sulfo, phenylthio, phenyl lower alkylthio, alkylphenylthio, phenylsulfinyl, phenyl-lower alkylsulfinyl, alkylphenylsulfinyl, phenylsulfonyl, phenyl-lower alkylsulfonyl, or alkylphenylsulfonyl, wherein---if more than 1 radical Z (m=2) is present---the substituents Z are the same or different from one another;

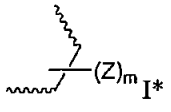
and wherein the bonds characterized, if present, by a wavy line are either single or double bonds;

or an N-oxide of the defined compound, wherein one or more N atoms carry an oxygen atom;

with the stipulation that, if Y is pyridyl or unsubstituted cycloalkyl, X is imino, and the remaining radicals are as defined, G is selected from the group comprising lower alkylene, $-CH_2-O-$, $-CH_2-S-$, oxa and thia;

or a pharmaceutically acceptable salt thereof.

[00194] In a more particular embodiment, $R_1$ and $R_2$ together form a bridge in subformula I*



I*

the binding being achieved via the two terminal carbon atoms, wherein both wavy lines represent double bonds.

[00195] In another more particular embodiment, G is methylene.

[00196] In another more particular embodiment, r is 0.

[00197] In another more particular embodiment, X in -NH-.

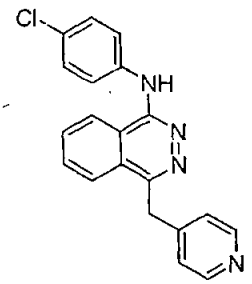
[00198] In another more particular embodiment, n is 0.

[00199] In another more particular embodiment, Y is substituted phenyl.

[00200] In another more particular embodiment, said phenyl is substituted with chloro.

[00201] In another more particular embodiment, said chloro is bound para to the phenyl group's point of attachment.

[00202] Another embodiment of the invention provides a method of modulating an immune response in a subject comprising administering a compound having the structure:




or a pharmaceutically acceptable salt thereof .

[00203] In a more particular embodiment of any of the above methods, said modulating is inducing. In another embodiment said inducing stimulates production of cytokines, chemokines, and/or growth factors.

[00204] In another embodiment said compound is administered in a subcytotoxic amount to said subject.

[00205] In another embodiment said subject is in remission from cancer.

[00206] In another embodiment said compound is administered for the treatment of refractory cancer cells.

[00207] In another embodiment said compound is administered metronomically.

[00208] In another embodiment the subject is not suffering from cancer.

[00209] In another embodiment said compound is co-administered with another agent.

[00210] In another embodiment said compound is administered in a dose capable of increasing TNF-$\alpha$ levels.

[00211] In another embodiment said compound has an average steady state drug concentration in the blood of less than 20 /xM.

[00212] In another embodiment subject is suffering from an autoimmune disease. Further still, said autoimmune disease is multiple sclerosis or Crohn's disease.

[00213] In another embodiment said subject is suffering from a viral infection.

[00214] In another embodiment said viral infection is HCV, HIV, or HSV.

[00215] In another embodiment said subject is suffering from allergies.

[00216] In another embodiment said subject is suffering from asthma.

[00217] In another embodiment the subject is suffering from precancerous lesions, such as actinic keratosis, atypical or dysplastic nevi, or premalignant lentigos.

[00218] In another embodiment the subject is suffering from a disease associated with abnormal cellular proliferation, such as, neuro-fibromatosis, atherosclerosis, pulmonary fibrosis, arthritis, psoriasis, glomerulonephritis, restenosis, proliferative diabetic retinopathy (PDR), hypertrophic scar formation, inflammatory bowel disease, transplantation rejection, angiogenesis, or endotoxic shock.

[00219] The following compositions may be used in any of the methods or indications described herein.

[00220] The invention also relates to a compound of formula I, or a pharmaceutically acceptable salt thereof, or an N-oxide thereof, for use in modulating an immune response of a subject, wherein said compound n is 0 and any of r, m, $R_1$, $R_2$, A, B, D, E, G, Q, R, X, Y and Z is as defined above or below.

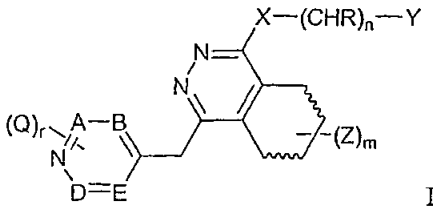
[00221] The invention also relates to a compound of the formula I, a salt thereof or an N-oxide thereof, wherein n is 0 and X is imino or thia, and any of r, m, $R_1$, $R_2$, A, B, D, E, G, Q, R, Y and Z is as defined above or below.

[00222] The prefix "lower" denotes a radical having up to and including a maximum of 7, especially up to and including a maximum of 4 carbon atoms, the radicals in question being either linear or branched with single or multiple branching.

[00223] Where the plural form is used for compounds, salts, and the like, this is taken to mean also a single compound, salt, or the like.

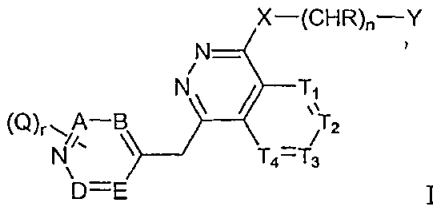
[00224] Any asymmetric carbon atoms (for example in compounds of formula I [or an N-oxide thereof], wherein n=1 and R is lower alkyl) may be present in the (R)-, (S)- or (R,S)-configuration, preferably in the (R)- or (S)- configuration. Substituents at a double bond or a ring may be present in cis (-Z-) or trans (-E-) form. The compounds may thus be present as mixtures of isomers or as pure isomers, preferably as enantiomer-pure diastereomers.

[00225] IfRj and $R_2$ together form a bridge in subformula I*, the pertinent compound of formula I has formula IA (compounds of this formula are hereinbefore and hereinafter especially preferred when compounds of formula I are mentioned),



IA

wherein the radicals are as defined above for compounds of formula I. IfRi and $R_2$ together form a bridge in subformula I**, the pertinent compound of formula I has formula IB,



IB

wherein the radicals are as defined above for compounds of formula I.

[00226] Of the ring members $T_1$, $T_2$, $T_3$, and $T_4$, preferably only one is nitrogen, the remaining three being CH; preferably only $T_3$, especially $T_4$, is nitrogen, whereas the other ring members $T_1$, $T_2$, and $T_4$ or $T_1$, $T_2$, and $T_3$ are CH.

[00227] The index r is preferably 0 or 1.

[00228] The index n is preferably 0 or 1, especially 0.

[00229] The index m is preferably 0, 1, or 2, especially 0 or also 1.

[00230] Of ring members A, B, D, and E in formula I, not more than 2 are N, and the remaining ones are CH. Preferably, each of the ring members A, B, D and E are CH.

[00231] If G is a bivalent group $-CH_2-O-$, $-CH_2-S-$, or $-CH_2-NH-$, the methylene group in each case is bound to the ring with ring members A, B, D r and E, whereas the heteroatom (O, S, or NH) is bound to the phthalazine ring in formula I.

[00232] Lower alkylene G may be branched or preferably linear and is especially branched or preferably linear Ci -$C_4$ alkylene, especially methylene ($-CH_2-$), ethylene ($-CH_2-CH_2-$), trimethylene ($-CH_2-CH_2-CH_2-$) or tetramethylene ($-CH_2-CH_2-CH_2-CH_2-$). G is preferably methylene.

[00233] Acyl in lower alkylene substituted by acyloxy is preferably arylcarbonyloxy, wherein aryl is defined as below, especially benzoyloxy or lower alkanoyloxy, especially benzoyloxy; lower alkylene substituted by acyloxy is especially methylene substituted by benzoyloxy.

[00234] Lower alkylene substituted by hydroxy is preferably hydroxymethylene (-CH(OH)-).

[00235] G as lower alkylene substituted by acyloxy or hydroxy is preferred, or G as otherwise defined hereinbefore and hereinafter is in each case especially preferred.

[00236] Q is preferably bound to A or D (r=l) or to both (r=2), where in the event of binding of Q, A and/or D are/is C(-Q).

[00237] Lower alkyl is especially Ci -$C_4$ alkyl, e.g. n-butyl, sec-butyl, tert-butyl, n-propyl, isopropyl, or especially methyl or also ethyl.

[00238] In the preferred embodiment, aryl is an aromatic radical having 6 to 14 carbon atoms, especially phenyl, naphthyl, fluorenyl or phenanthrenyl, the radicals defined above being unsubstituted or substituted by one or more, preferably up to three, especially one or two substituents, especially selected from amino, mono- or disubstituted amino, halogen, alkyl, substituted alkyl, hydroxy, etherified or esterified hydroxy, nitro, cyano, carboxy, esterified carboxy, alkanoyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amidino, guanidino, mercapto, sulfo, phenylthio, phenyl-lower alkylthio, alkylphenylthio, phenylsulfmyl, phenyl-lower alkylsulfmyl, alkylphenylsulfmyl, phenylsulfonyl, phenyl- lower alkylsulfonyl, and alkylphenylsulfonyl, or (as an alternative or in addition to the above group of substituents) selected from lower alkenyl, such as ethenyl, phenyl, lower alkylthio, such as methylthio, lower alkanoyl, such as acetyl, lower alkylmercapto, such as methylmercapto (-S-$CH_3$), halogen-lower alkylmercapto, such as trifluoromethylmercapto (-S-$CF_3$), lower alkylsulfonyl, halogen-lower alkylsulfonyl, such as especially trifluoromethane sulfonyl, dihydroxybora (-$B(OH)_2$), heterocyclyl, and lower alkylene dioxy bound at adjacent C-atoms of the ring, such as methylene dioxy; aryl is preferably phenyl which is either unsubstituted or independently substituted by one or two substituents selected from the group comprising amino; lower alkanoylamino, especially acetylamino; halogen, especially fluorine, chlorine, or bromine; lower alkyl, especially methyl or also ethyl or propyl; halogen-lower alkyl, especially trifluoromethyl; hydroxy; lower alkoxy, especially methoxy or also ethoxy; phenyl-lower alkoxy, especially benzyloxy; and cyano, or (as an alternative or in addition to the previous group of substituents) $C_8$ -$Ci_2$ alkoxy, especially n-decyloxy, carbamoyl, lower alkylcarbamoyl, such as n-methyl- or n-tert-butylcarbamoyl, lower alkanoyl, such as acetyl, phenyloxy, halogen-lower alkyloxy,

such as trifluoromethoxy or 1,1,2,2-tetrafluoroethyloxy, lower alkoxycarbonyl, such as ethoxycarbonyl, lower alkylmercapto, such as methylmercapto, halogen-lower alkylniercapto, such as txifluoromethylmercapto, hydroxy-lower alkyl, such as hydroxymethyl or 1-hydroxymethyl, lower alkylsulfonyl, such as methane sulfonyl, halogen-lower alkylsulfonyl, such as trifluoromethane sulfonyl, phenylsulfonyl, dihydroxybora ($-B(OH)_2$), 2-methylpyrimidin-4-yl, oxazol-5-yl, 2-methyl-l,3-dioxolan-2-yl, lH-pyrazol-3-yl, l-methyl-pyrazol-3-yl and lower alkylene dioxy bound to two adjacent C-atoms, such as methylene dioxy.

[00239] Mono- or disubstituted amino is especially amino substituted by one or two radicals selected independently of one another from lower alkyl, such as methyl; hydroxy-lower alkyl, such as 2-hydroxyethyl; phenyl-lower alkyl; lower alkanoyl, such as acetyl; benzoyl; substituted benzoyl, wherein the phenyl radical is unsubstituted or especially substituted by one or more, preferably one or two, substituents selected from nitro or amino, or also from halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and phenyl-lower alkoxycarbonyl, wherein the phenyl radical is unsubstituted or especially substituted by one or more, preferably one or two, substituents selected from nitro or amino, or also from halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and is preferably N-lower alkylamino, such as N-methylamino, hydroxy-tower alkylamino, such as 2-hydroxyethylamino, phenyl-lower alkylamino, such as benzylamino, N,N-di-lower alkylamino, N-phenyl-lower alkyl-N-lower alkylamino, N,N-di-lower alkylphenylamino, lower alkanoylamino, such as acetylamino, or a substituent

selected from the group comprising benzoylamino and phenyl-lower alkoxycarbonylamino, wherein the phenyl radical in each case is unsubstituted or especially substituted by nitro or amino, or also by halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl or carbamoyl, or as an alternative or in addition to the previous group of radicals by aminocarbonylamino.

[00240] Halogen is especially fluorine, chlorine, bromine, or iodine, especially fluorine, chlorine, or bromine and halo is especially fluoro, chloro, bromo, or iodo, especially fluoro, chloro, or bromo.

[00241] In the preferred embodiment, alkyl has up to a maximum of 12 carbon atoms and is especially lower alkyl, especially methyl, or also ethyl, n- propyl, isopropyl, or tert-butyl.

[00242] Substituted alkyl is alkyl as last defined, especially lower alkyl, preferably methyl, where one or more, especially up to three, substituents may be present, primarily from the group selected from halogen, especially fluorine, and also from amino, N-lower alkylamino, N,N-di-lower alkylamino, N-lower alkanoylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, and phenyl-lower alkoxycarbonyl. Trifluoromethyl is especially preferred.

[00243] Etherified hydroxy is especially $C_8$ -$C_{20}$ alkyloxy, such as n-decyloxy, lower alkoxy (preferred), such as methoxy, ethoxy, isopropyloxy, or n-pentyloxy, phenyl-lower alkoxy, such as benzyloxy, or also phenyloxy, or as an alternative or in addition to the previous group $C_8$ -$C_{20}$ alkyloxy, such as n-decyloxy, halogen-lower alkoxy, such as trifluoromethyloxy or 1,1,2,2-tetrafluoroethoxy.

[00244] Esterified hydroxy is especially lower alkanoyloxy, benzoyloxy, lower alkoxycarbonyloxy, such as tert-butoxycarbonyloxy, or phenyl-lower alkoxycarbonyloxy, such as benzyloxcarbonyloxy.

[00245] Esterified carboxy is especially lower alkoxycarbonyl, such as tert-butoxycarbonyl or ethoxycarbonyl, phenyl-lower alkoxycarbonyl, or phenyloxycarbonyl .

[00246] Alkanoyl is primarily alkylcarbonyl, especially lower alkanoyl, e.g. acetyl.

[00247] N-mono- or N,N-disubstituted carbamoyl is especially substituted by one or two substituents, lower alkyl, phenyl-lower alkyl, or hydroxy- lower alkyl, at the terminal nitrogen atom.

[00248] Alkylphenylthio is especially lower alkylphenylthio.

[00249] Alkylphenylsulfmyl is especially lower alkylphenylsulfmyl.

[00250] Alkylphenylsulfonyl is especially lower alkylphenylsulfonyl.

[00251] Pyridyl Y is preferably 3- or 4-pyridyl.

[00252] Z is preferably amino, hydroxy-lower alkylamino, such as 2-hydroxyethylamino, lower alkanoylamino, such as acetylamino, nitrobenzoylamino, such as 3-nitrobenzoylamino, aminobenzoylamino, such as 4-aminobenzoylamino, phenyl-lower alkoxycarbonylamino, such as benzyloxycarbonylamino, or halogen, such as bromine; preferably only one substituent is present (m=l), especially one of the last mentioned, especially halogen. A compound of formula I (or an N-oxide thereof), wherein Z is absent (m=0), is especially preferred.

[00253] Unsubstituted or substituted cycloalkyl is preferably $C_3$ -$C_8$ cycloalkyl, which is unsubstituted or substituted in the same way as aryl, especially as defined for phenyl. Cyclohexyl or also cyclopentyl or cyclopropyl are preferred.

[00254] Heterocyclyl is especially a five or six-membered heterocyclic system with 1 or 2 heteroatoms selected from the group comprising nitrogen, oxygen, and sulfur, which may be unsaturated or wholly or partly saturated, and is unsubstituted or substituted especially by lower alkyl, such as methyl; a radical selected from 2-methylpyrimidm-4-yl, oxazol-5- yl, 2-methyl-l,3-dioxolan-2-yl, lH-pyrazol-3-yl, and l-methyl-pyrazol-3- yl is preferred.

[00255] Aryl in the form of phenyl which is substituted by lower alkylene dioxy bound to two adjacent C-atoms, such as methylenedioxy, is preferably 3,4-methylenedioxyphenyl .

[00256] The bonds in formula I characterized by wavy lines are present either as single or as double bonds. Preferably both are at the same time either single or double bonds.

[00257] An N-oxide of a compound of formula I is preferably an N-oxide in which a phthalazine-ring nitrogen or a nitrogen in the ring with ring members A, B, D, and E carries an oxygen atom, or several of the said nitrogen atoms carry an oxygen atom.

[00258] Salts are especially the pharmaceutically acceptable salts of compounds of formula I (or an N-oxide thereof).

[00259] Such salts are formed, for example, as acid addition salts, preferably with organic or inorganic acids, from compounds of formula I (or an N- oxide thereof with a basic nitrogen atom, especially the pharmaceutically acceptable salts. Suitable

inorganic acids are, for example, halogen acids, such as hydrochloric acid, sulfuric acid, or phosphoric acid. Suitable organic acids are, for example, carboxylic, phosphonic, sulfonic or sulfamic acids, for example acetic acid, propionic acid, octanoic acid, decanoic acid, dodecanoic acid, glycolic acid, lactic acid, 2-hydroxybutyric acid, gluconic acid, glucosemonocarboxylic acid, fumaric acid, succinic acid, adipic acid, pimelic acid, suberic acid, azelaic acid, malic acid, tartaric acid, citric acid, glucaric acid, galactaric acid, amino acids, such as glutmic acid, asparic acid, N-methylglycine, acetylaminoacetic acid, N-acetylasparagine or N-acetylcysteine, pyruvic acid, acetoacetic acid, phosphoserine, 2- or 3-glycerophosphoric acid, glucose-6-pliosphonc acid, glucose- -phosphoric acid, fructose- 1,6-bis- phosphoric acid, maleic acid, hydroxymaleic acid, methylmaleic acid, cyclohexanecarboxylic acid, adamantanecarboxylic acid, benzoic acid, salicylic acid, 1- or 3-hydroxynaphthyl-2-carboxylic acid, 3,4,5- trimethoxybenzoic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, 4- aminosalicylic acid, phthalic acid, phenylacetic acid, mandelic acid, cinnamic acid, glucuronic acid, galacturonic acid, methane- or ethane- sulfonic acid, 2-hydroxyethanesulfonic acid, ethane- 1,2-disutfonic acid, benzenesulfonic acid, 2-naphthalenesultonic acid, 1,5-naphthalene-disulfonic acid, 2-, 3- or 4-methylbenzenesulfonic acid, methylsulfuric acid, ethylsulfuric acid, dodecylsulfuric acid, N-cyclohexylsulfamic acid, N-methyl-, N-ethyl-or N-propyl-sulfamic acid, or other organic protonic acids, such as ascorbic acid.

[00260] In the presence of negatively charged radicals, such as carboxy or sulfo, salts may also be formed with bases, e.g. metal or ammonium salts, such as alkali metal or alkaline earth metal salts, for example sodium, potassium, magnesium or calcium salts, or ammonium salts with ammonia or suitable organic amines, such as tertiary monoamines, for example triethylamine or tri(2-hydroxyethyl)amine, or heterocyclic bases, for example N-ethylpipperidine or N, N'-dimethylpiperazine.

[00261] When a basic group and an acid group are present in the same molecule, a compound of formula I (or an N-oxide thereof) may also form internal salts.

[00262] For isolation or purification purposes it is also possible to use pharmaceutically unacceptable salts, for example picrates or perchlorates. For therapeutic use, only pharmaceutically acceptable salts or free compounds are employed (where applicable in the form of pharmaceutical preparations), and these are therefore preferred.

[00263] In view of the close relationship between the novel compounds in free form and those in the form of their salts, including those salts that can be used as intermediates, for example in the purification or identification of the novel compounds, any reference to the free compounds hereinbefore and hereinafter is to be understood as referring also to the corresponding salts, as appropriate and expedient.

[00264] The compounds of formula I (or an N-oxide thereof have valuable pharmacological properties, as described hereinbefore and hereinafter.

[00265] With the groups of preferred compounds of formula I mentioned hereinafter, definitions of substitoents from the general definitions mentioned hereinbefore may reasonably be used, for example, to replace more general definitions with more specific definitions or especially with definitions characterized as being preferred;

[00266] (A) Preference is given to a compound of formula I comprised in a pharmaceutical preparation or to be used according to the invention wherein

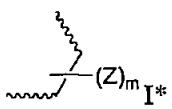
r is 0 to 2, preferably 0,

n is 0 or 1,

mis 0 or also 1,

Ri and $R_2$ (i) are lower alkyl, especially methyl, or

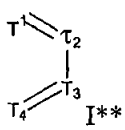
(ii) together form a bridge in subfonnula I*



I*

the binding being achieved via the two terminal carbon atoms, or

(iii) together form a bridge in subformula I**



I**

wherein one of the ring members Ti, $T_2$, $T_3$ and $T_4$ is nitrogen, and the others are in each case CH, and the binding is achieved via Ti and $T_4$;

A, B, D, and E are in each case CH, or also A, D, and E are each CH and B is N;

G is lower alkylene, especially methylene or ethylene (-$CH_2$-$CH_2$-), -$CH_2$-NH-, -$CH_2$-O-, hydroxymethylene, or benzoyloxymethylene,

Q is methyl, which is bound to A, D, or to A and D;

R is H or lower alkyl, especially H or methyl,

X is imino, oxa, or thia,

Y is phenyl, which is unsubstituted or is substituted by one or two substituents independently of one another from the group comprising amino; lower alkanoylamino, especially acetylamino; halogen, especially fluorine, chlorine, or bromine; lower alkyl, especially methyl or also ethyl or propyl; halogen-lower alkyl, especially trifluoromethyl; hydroxy; lower alkoxy, especially methoxy or also ethoxy; phenyl-lower alkoxy, especially benzyloxy; and cyano, or (as an alternative or in addition to the previous group of substituents) lower alkenyl, such as ethenyl, $C_8$-$Ci_2$ alkoxy, especially n-decyloxy, lower alkoxycarbonyl, such as tert-butoxycarbonyl, carbamoyl, lower alkylcarbamoyl, such as N-methyl- or N-tert-butylcarbamoyl, lower alkanoyl, such as acetyl, phenyloxy, halogen- lower alkyloxy, such as trifluoromethoxy or 1,1,2,2-tetrafluoroethyloxy, lower alkoxycarbonyl, such as ethoxycarbonyl, lower alkylmercapto, such as methylmercapto, halogen-lower alkylmercapto, such as trifluoromethylmercapto, hydroxy-lower alkyl, such as hydroxymethyl or 1-hydroxymethyl, lower alkylsulfonyl, such as methanesulfonyl, halogen- lower alkylsulfonyl, such as trifluoromethanesulfonyl, phenylsulfonyl, dihydroxybora (-$B(OH)_2$), 2-methylpyrimidin-4-yl, oxazol-5-yl, 2-methyl- 1,3-dioxolan-2-yl, lH-pyrazol-3-yl, l-methylpyrazol-3-yl and lower alkylenedioxy bound to two adjacent C-atoms, such as methylenedioxy, or is also pyridyl, especially 3-pyridyl; especially phenyl, 2-, 3- or 4- aminophenyl, 2-, 3- or 4-acetylaminophenyl, 2-, 3- or 4-fluorophenyl, 2-, 3- or 4-chlorophenyl, 2-, 3- or 4-bromophenyl, 2,3-, 2,4-, 2,5- or 3,4- dichlorophenyl, chlorofluorophenyl, such as 3-chloro-4-fluorophenyl or also 4-chloro-2-fluoroanilino, 2-, 3- or 4-methylphenyl, 2-, 3- or 4-ethylphenyl, 2-, 3- or 4-propylphenyl, methylfluorophenyl, such as 3-fluoro-4-methylphenyl, 2-, 3- or 4-trifluoromethylphenyl, 2-, 3- or 4-hydroxyphenyl, 2-, 3- or 4-methoxyphenyl, 2-, 3- or 4-ethoxyphenyl, methoxychlorophenyl, such as 3-chloro-4-methoxycarbonyl, 2-, 3- or 4- benzyloxyphenyl, 2-, 3- or 4-cyanophenyl, or also 2-, 3- or 4-pyridyl;

Z is amino; N-lower alkylamino, such as N-methylamino; hydroxy-lower alkylamino, such as 2-hydroxyethylamino; phenyl-lower alkylamino, such as benzylamino; N,N-di-lower alkylamino; n-phenyl-lower alkyl-N-lower alkylamino; N,N-di-lower alkylphenylamino; lower alkanoylamino, such as acetylamino; or a substituent from the group comprising benzoylamino or phenyl-lower alkoxycarbonylamino, wherein the phenyl radical in each case is unsubstituted or especially substituted by nitro or

amino, or also by halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl or carbamoyl; or is halogen, especially bromine; especially amino, acetylamino, nitrobenzoylamino, aminobenzoylamino, 2-hydroxyethylamino, benzyloxycarbonylamino or bromine; and,

if present (in formula IA), the bonds characterized by a wavy line are in each case a double bond or in each case a single bond;

or of a pharmaceutically acceptable salt thereof; or to such a compound or pharmaceutically acceptable salt thereof wherein n=0 and the other moieties are as defined under (A) for use in the treatment of a disease mentioned hereinbefore or hereinafter; or to such a compound wherein n=0 and X is thia or is imino, and the other moieties are as defined under (A), or a pharmaceutically acceptable salt thereof.

[00267] (B) Special preference is given to a compound of formula I, especially formula IA, comprised in a pharmaceutical preparation or to be used according to the invention wherein

r is 0;

n is 0 or 1,

m is 0;

A, B, 0, and E are in each case CH,

G is lower alkylene, especially methylene,

R is H,

X is imino,

Y is phenyl, which is unsubstituted or is substituted by one or two substituents independently of one another selected from the group comprising amino; lower alkanoylamino, especially acetylamino; halogen, especially fluorine, chlorine, or bromine; lower alkyl, especially methyl; halogen-lower alkyl, especially trifluoromethyl; hydroxy; lower alkoxy, especially methoxy; phenyl-lower alkoxy, especially ben2yloxy; and cyano; especially phenyl, 2-, 3- or 4-aminophenyl, 2-, 3- or 4-acetylaminophenyl, 2-, 3- or 4-fluorophenyl, 2-, 3- or 4-chlorophenyl, 2-, 3- or 4-bromophenyl, 2,3-, 2,4-, 2,5- or 3,4-dichlorophenyl, chlorofluorophenyl, such as 3-chloro-4-fluorophenyl, 2,-3- or 4-methylphenyl, 2-, 3- or 4- trifluoromethylphenyl, 2-, 3- or 4-hydroxyphenyl, 2-, 3- or 4- methoxycarbonyl, methoxychlorophenyl, such as 3-chloro-4-methoxycarbonyl, 2-, 3- or 4-benzyloxyphenyl, or 2-, 3- or 4-cyanophenyl; and

the bonds characterized by a wavy line are double bonds;

or a pharmaceutically acceptable salt thereof; or to such a compound of formula I, especially IA, wherein n=0 and the other moieties are as defined under (B), or a salt thereof.

[00268] Special preference is given to a compound of formula I, especially formula IA, such as is mentioned in the Examples below, or a pharmaceutically acceptable salt thereof, especially a compound specifically mentioned in the Examples or a salt thereof.

[00269] High preference is given to a compound selected from

1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Chloroanilino)-4-(4-pyridylmethyl)phthalazine;

1-Anilino-4-(4-pyridylmethyl)phthalazine;

1-Benzylamino-4-(4-pyridylmethyl)phthalazine;

1-(4-Methoxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Benzyloxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Methoxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(2-Methoxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(4-Trifluoromethylanilino)-4-(4-pyridy=methyl)phthalazine;

1-(4-Fluoroanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Hydroxyanilino)-4-(4-pyridyl methyi)phthalazine;

1-(4-Hydroxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Aminoanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3,4-Dichloroanilino)-4-(4-pyridylmethyl)phthalazine;

1-(4-Bromoanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Chloro-4-methoxyanilino)-4-(4-pyridylmethyl)phthalazine;

1-(4-Cyanoanilino)-4-(4-pyridylmethyl)phthalazine;

1-(4-Methylanilino)-4-(4-pyridylmethyl)phthalazine; and also

1-(3-Chloro-4-fluoroanilino)-4-(4-pyridylmethyl)phthalazine;

1-(3-Methylanilino)-4-(4-pyridylmethyl)phthalazine;

or a pharmaceutically acceptable salt thereof.

[00270] It is contemplated that the invention encompasses all possible combinations of the embodiments described herein.

[00271] Preferred SMIPs in accordance with the fourth aspect of the invention are derivatized pyridazines, such as 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine,

as well as analogs disclosed in US 6,258,812. More particularly, preferred SMIPs include derivatized pyridazines encompassed by Formula I as described in this section, or contained within the aforementioned references.

[00272] Reference to "derivatized pyridazines" indicates compounds having the general structure of Formula I as described in this section. A preferred derivatized pyridazine is a phthalazine such as I-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine.

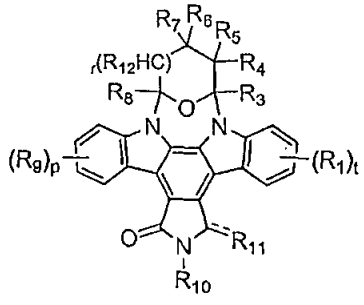
[00273] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially available, or are easily synthesized by one skilled in the art from procedures described in patents or patent applications listed herein.

[00274] **Section V - Fifth Aspect of the Invention** - Staurosporine Analogs **for** Immimopotentiation

[00275] All definitions, descriptors of constituent variables for chemical fonnulas, and descriptions appearing in the section shall be understood to apply to this section only.

[00276] In accordance with the fifth aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

[00277] In one embodiment of the invention, a method of modulating an immune response in a subject is provided, comprising administering a compound of formula I:




wherein,

Ri and $R_9$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, -$NR_aRb$, -C(O)Rc, -$S(O)_nRd$, orheterocyclyl;

$R_3$ and $R_8$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, -$NR_aRb$, -C(O)Rc, -$S(O)_nR_d$, or heterocyclyl;

$R_4$ and $R_7$ are each independently H, -CN, -OH, halogen, alkyl, -0-$(CH_2)_q$-$R_e$, -C(O)Rc, -0-$(CH_2)_q$-0-Re, -$NR_aR_b$, and -$S(O)_nRa$;

$R_5$ and $R_6$ are each independently H, -CN, -OH, halogen, alkyl, aryl, alkoxy, -$NR_aRb$, -$C(O)R_c$, or heterocyclyl;

Rio is H, alkyl, aryl, -$C(O)R_0$, or heterocyclyl;

the dotted line is absent and Rn is H, -CN, -OH, halogen, alkyl, aryl, alkoxy, -$NR_aR_b$, -C(O)Rc, -$S(O)_nR_d$, orheterocyclyl; or

the dotted line is present and Rn is O or S;

$Ri_2$ is H, alkyl, or alkoxy;

each $R_a$ and $R_b$ is independently H, alkyl, -$C(O)R_O$ aryl, heterocyclyl, or alkoxy;

each $R_c$ is independently H, alkyl, alkoxy, -$NH_2$, -NH(alkyl), -$N(alkyl)_2$, aryl, or heterocyclyl;

each $R_d$ is independently H, alkyl, alkenyl, aryl, or -NRaRbJ

each $R_s$ is independently H, halogen, -$C(O)R_0$, aryl, heterocyclyl, or -$NR_aR_b$;

each n is independently o, 1, or 2;

each p is independently 0, 1, 2, or 3;

each q is independently o, 1, or 2;

each r is o, thereby forming a covalent bond, or 1; and

each t is independently o, 1, 2 or 3.

[00278] In another embodiment, Ri and $R_9$ are both H.

[00279] In another more particular embodiment, Rio is H.

[00280] In another more particular embodiment, $R_3$ is methyl.

[00281] In another more particular embodiment, r is 1 and $R_{12}$ is H.

[00282] In another more particular embodiment, $R_4$ is -$OCH_3$ and $R_5$ is H.

[00283] In another more particular embodiment, $R_6$ is H and $R_7$ is -$NR_aR_b$.

[00284] In another more particular embodiment, $R_4$ is -$OCH_3$, $R_5$ is H, $R_6$ is H and $R_7$ is -$NR_aRb$-

[00285] In another more particular embodiment, $R_a$ within $R_7$ is methyl and $R_b$ within $R_7$ is H.

[00286] In another more particular embodiment, $Ri_1$ is -H.

[00287] In another more particular embodiment, R]i is -OH.

[00288] In another more particular embodiment, Rii is =0.

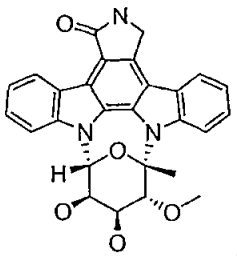
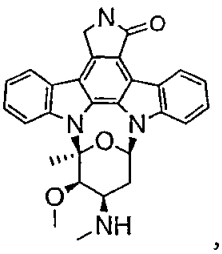
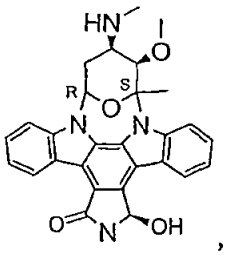
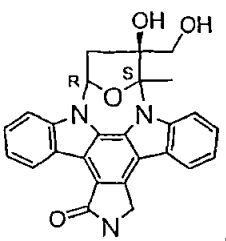
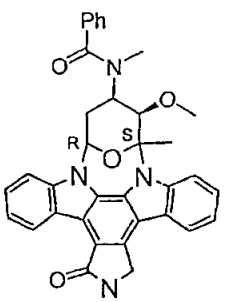
[00289] In another more particular embodiment, r is 0.

[00290] In another more particular embodiment, $R_5$ is -OH.

[00291] In another more particular embodiment, $R_4$ is $-CH_2OH$.

[00292] In another more particular embodiment, $R_4$ is $-C(O)R_0$.

[00293] Another embodiment of the invention provides a method of modulating an immune response in a subject comprising administering a compound selected from the group consisting of:


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,

,

, and

[00294] In a more particular embodiment of any of the above methods, said modulating is inducing. In another embodiment said inducing stimulates production of cytokines, chemokines, and/or growth factors.

[00295] In another embodiment said compound is administered in a subcytotoxic amount to said subject.

[00296] In another embodiment said subject is in remission from cancer.

[00297] In another embodiment said compound is administered for the treatment of refractory cancer cells.

[00298] In another embodiment said compound is administered metronomically.

[00299] In another embodiment the subject is not suffering from cancer.

[00300] In another embodiment said compound is co-administered with another agent.

[00301] In another embodiment said compound is administered in a dose capable of increasing TNF-$\alpha$ levels.

[00302] In another embodiment said compound has an average steady state drug concentration in the blood of less than 20 $\mu$M.

[00303] In another embodiment subject is suffering from an autoimmune disease. Further still, said autoimmune disease is multiple sclerosis or Crohn's disease.

[00304] In another embodiment said subject is suffering from a viral infection.

[00305] In another embodiment said viral infection is HCV, HIV, or HSV.

[00306] In another embodiment said subject is suffering from allergies.

[00307] In another embodiment said subject is suffering from asthma.

[00308] In another embodiment the subject is suffering from a disease associated with abnormal cellular proliferation, such as, neuro-fibromatosis, atherosclerosis, pulmonary fibrosis, arthritis, psoriasis, glomerulonephritis, restenosis, proliferative diabetic retinopathy (PDR), hypertrophic scar formation, inflammatory bowel disease, transplantation rejection, angiogenesis, or endotoxic shock.

[00309] Any asymmetric carbon atoms (for example in compounds of formula I [or an N-oxide thereof], wherein n=l and R is lower alkyl) may be present in the (R)-, (S)- or (R,S)-configuration, preferably in the (R)- or (S)- configuration. Substituents at a double bond or a ring may be present in cis (-Z-) or trans (-E-) form. The compounds may thus be present as mixtures of isomers or as pure isomers, preferably as enantiomer-pure diastereomers.

[00310] It is contemplated that the invention encompasses all possible combinations of the embodiments described herein.

[00311] Preferred SMIPs in accordance with the fifth aspect of the invention are staurosporine analogs, such as 9,13-Epoxy-lH,9H-diindolo[l,2,3-gh:3',2',r-lm]pyrrolo[3,4-j][1,7]benzodiazonin- 1-one, 2,3,10,11,12,13-hexahydro- 11,12-dihydroxy- 10-methoxy-9-methyl; 9,13-Epoxy- lH,9H-diindolo[ 1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-l-one, 2,3,10,1 1,12,13-hexahydro-10-methoxy-9-methyl-ll-^ethylam^^ ,1S-Epoxy-lH^H-diindolotl^ ,3-gh:3¦2¦,!'-lm]pyrrolo[3,4-j][1,7]benzodiazonin- 1-one, 2,3,10,11,12, 13-hexahydro-3-hydroxy-10-methoxy-9-methyl-ll-(methylammo); 9,12-Epoxy-lH-diindolo[l,2,3-fg:3',2',l'-

kl]pyrrolo[3 ,4-i][1,6]benzodiazocin- 1-one, 2,3,9, 10,11,12-hexahydro-l O-hydroxy- 10-(hydroxymethyl)-9-methyl; andbenzamide, N-[(9S,1OR,11R,13R)-2,3,1O,1 1,12,13-hexahydro-1 0-methoxy-9-methyl-l-oxo-9,l 3-epoxy-lH,9H-diindolo[l ,2,3-gh:3',2', 1'-lm]pyiτolo[3,4-j][l,7]benzodiazonin-ll-yl]-N-methyl, as well as analogs disclosed in the following patents and patent applications: WO 02/30941, WO 97/07081, WO 89/07105, US 5,621,100, WO 93/07153, WO 89/07105, WO 01/04125, WO 02/30941, WO 93/08809, WO 94/06799 WO 00/27422, WO 96/13506, and WO 88/07045. More particularly, preferred SMIPs include flavones, isoflavones and those encompassed by Formula I as described in this section, or contained within the aforementioned references.

[00312] Reference to "staurosporine analogs" indicates compounds having the general structure of Formula I as described in this section. Preferred staurosporine analogs are selected from the group consisting of 9,13-Epoxy-lH,9H-diindolo[l,2,3-gh:3',2', 1'-lm]pyrrolo[3,4-j] [1,7]benzodiazonin- 1-one, 2,3, 10,11,12,13-hexahydro-11,12-dihydroxy- 10-methoxy-9-methyl; *9,*13-Epoxy- lH,9H-diindolo[ 1,2,3-gh:3\2\ 1'-lm]pyrrolo[3,4-j] [1,7]benzodiazonin- 1-one, 2,3,10,11,12,13-hexahydro- 10-methoxy-9-methyl-ll-(methylamino); 9,13-Epoxy-lH,9H-diindolo[1,2,3-gh:3',2',l'-lm]pyrrolo[3 ,4-j][1,7]benzodiazonin- 1-one, 2,3,10,11, 12,13-hexahydro-3-hydroxy-10-methoxy-9-methyl-ll-(methylamino); 9,12-Epoxy-lH-diindolo[l,2,3-fg:3',2',l'-kl]pyrrolo[3 ,4-i][1,6]benzodiazocin- 1-one, 2,3,9, 10,11,12-hexahydro- lO-hydroxy- 10-(hydroxymethyl)-9-methyl; andbenzamide, N-[(9S,10R,l 1R,13R)-2,3,1O,1 1,12,13-hexahydro- 10-methoxy-9-methyl- 1-oxo-9, 13-epoxy- lH,9H-diindolo[ 1,2,3-gh:3',2', l'-lm]pyrrolo[3,4-j][l,7]benzodiazonin-ll-yl]-N-methyl, as well as analogs disclosed in the following patents and patent applications: WO 02/30941, WO 97/07081, WO 89/07105, US 5,621,100, WO 93/07153, WO 89/07105, WO 01/04125, WO 02/30941, WO 93/08809, WO *94/06799* WO 00/27422, WO 96/13506, and WO 88/07045. Subsequently, it will become apparent to the skilled artisan that not only 9,13-epoxy analogs are encompassed by the term "staurosporine analogs", but additionally homologs, such as 9,12-epoxy compounds, for example K-252, are encompassed as well.

[00313] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially

available, or are easily synthesized by one skilled in the art from procedures described in patents or patent applications listed herein.

[00314]

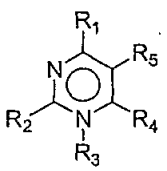
[00315] **Section VI - Sixth Aspect of the Invention - Nucleoside Analogs for Immunopotentiation**

[00316] All definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

[00317] In accordance with the sixth aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

[00318] One embodiment of the invention provides a method of treating a subject suffering from a viral infection comprising administering a small molecule in an amount such that said small molecule concomitantly inhibits viral replication while stimulating an immune response in said subject. In another embodiment thereof said small molecule is a nucleoside analog. In another embodiment thereof said small molecule inhibits viral DNA polymerase. In another embodiment thereof said small molecule is a nucleoside analog. In another embodiment said small molecule is a substituted purine or pyrimidine.

[00319] Another embodiment of the invention provides a method of stimulating an immune response in a subject comprising administering a nucleoside analog of formula I:



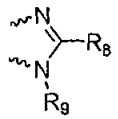
I

wherein,

Ri and $R_2$ are each independently H, halo, $-NR_3Rb$, =O, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, heterocyclyl, substituted heterocyclyl, $C_{6-10}$ aryl, substituted $C_{6-10}$ aryl, $Ci_{-6}$ alkyl, or substituted $Ci_{-6}$ alkyl;

$R_3$ is absent, H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, $C_{6-10}$ aryl, substituted $C_{6-10}$ aryl, heterocyclyl, or substituted heterocyclyl;

$R_4$ and $R_5$ are each independently H, halo, heterocyclyl, substituted heterocyclyl, -C(O)-$R_d$, Ci-$_6$ alkyl, substituted Ci-$_6$ alkyl, or bound together to form a 5 membered ring as in $R_{4-5}$:



R4-5

the binding being achieved at the bonds indicated by a ~~~ ;

$R_8$ is H, halo, Ci-$_6$ alkyl, $C_{2-6}$ alkenyl, $C_{2-6}$ alkynyl, -OH, -$NR_aR_b$, -$(CH_2)_n$-O-$R_c$, -O-(Ci-$_6$ alkyl), -$S(O)_pR_e$, or -C(O)-$R_d$;

$R_9$ is H, Ci-6 alkyl, substituted Ci-$_6$ alkyl, heterocyclyl, or substituted heterocyclyl;

each $R_a$ and Rb is independently H, Ci-$_6$ alkyl, substituted Ci-$_6$ alkyl, -C(O)$R_d$, or $C_{6-10}$ aryl;

each $R_c$ is independently H, phosphate, diphosphate, triphosphate, Ci-$_6$ alkyl, or substituted Ci-$_6$ alkyl;

each $R_d$ is independently H, halo, Ci-$_6$ alkyl, substituted Ci-$_6$ alkyl, Ci-$_6$ alkoxy, substituted Ci-$_6$ alkoxy, -$NH_2$, -NH(Ci-$_6$ alkyl), -NH(substituted Ci-$_6$ alkyl), -N(Ci-$_6$ alkyl)$_2$, -N(substituted Ci-$_6$ alkyl)$_2$, $C_{6-10}$ aryl, or heterocyclyl;

each $R_e$ is independently H, Ci-$_6$ alkyl, substituted Ci-$_6$ alkyl, $C_{6-10}$ aryl, substituted $C_{6-10}$ aryl, heterocyclyl, or substituted heterocyclyl;

each $R_f$ is independently H, Ci-$_6$ alkyl, substituted Ci-$_6$ alkyl, -C(O)$R_d$, phosphate, diphosphate, or triphosphate;

each n is independently 0, 1, 2, or 3;

each p is independently 0, 1, or 2; or

a pharmaceutically acceptable salt thereof, a tautomer thereof, or a pharmaceutically acceptable salt of the tautomer;

with the stipulation that if $R_{4-5}$ is present, $R_3$ is H or absent.

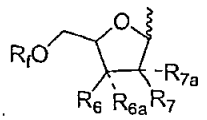
[00320] In another embodiment of formula I, Ri is -$NH_2$.

[00321] In another embodiment of formula I, each $R_f$ is H.

[00322] In another embodiment of formula I, $R_2$ is =0.

[00323] In another embodiment of formula I, $R_3$ is -$(CH_2)_n$-O-$R_c$. In still another embodiment thereof, $R_3$ is -$CH_2$-O-($C_2$ alkyl or substituted $C_2$ alkyl)-O-(H, phosphate, diphosphate, triphosphate, Ci-$_6$ alkyl or substituted Ci-$_6$ alkyl).

[00324] In another embodiment of formula I, $R_3$ is $R_{3a}$:



$R_3a$

the binding being achieved at the bond indicated by a ~~~;

$R_6$ and $R_7$ are each independently H, halo, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, $C_{2-6}$ alkenyl, substituted $C_{2-6}$ alkenyl, $-NR_aRb$, $-N_3$, or -OH;

$R_{6a}$ and $R_{7a}$ are each independently H, halo, or $Ci_{-6}$ alkyl;

each $R_a$ and $R_b$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -C(O)Rd, or $C_{6-10}$ aryl;

each $R_d$ is independently H, halo, $C_{1-6}$ alkyl, substituted $Ci_{-6}$ alkyl, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, $-NH_2$, -NH($Ci_{-6}$ alkyl), -NH(substituted $Ci_{-6}$ alkyl), -N($Ci_{-6}$ alkyl)$_2$, -N(substituted $Ci_{-6}$ alkyl)$_2$, $C_{6-10}$ aryl, or heterocyclyl; and

$R_f$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -C(O)Rd, phosphate, diphosphate, or triphosphate.

[00325] In another embodiment wherein $R_3$ is $R_{3a}$, $R_6$ is -OH and $R_7$ is -CH=CHF.

[00326] In another embodiment wherein $R_3$ is $R_{3a}$, $R_2$ is =0. In still another embodiment wherein $R_3$ is $R_{3a}$ and $R_2$ is =0, $R_4$ and $R_5$ are both H. In still another embodiment wherein $R_3$ is $R_{3a}$ and $R_2$ is =0, Ri is $-NH_2$. In still another embodiment wherein $R_3$ is $R_{3a}$ and $R_2$ is =0, Rf is H.

[00327] In another embodiment wherein $R_3$ is $R_{3a}$, $R_2$ is =0, and $R_f$ is H, $R_6$ and $R_7$ are both H.

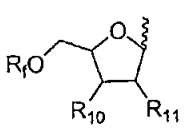
[00328] In another embodiment wherein $R_3$ is $R_{3a}$, $R_2$ is =0, and $R_f$ is H, $R_6$ is -OH. In still another embodiment thereof $R_7$ is H, F, or -OH.

[00329] In another embodiment wherein $R_3$ is $R_{3a}$, $R_2$ is =0, and $R_f$ is H, $R_6$ is $-N_3$.

[00330] In another embodiment wherein $R_3$ is $R_{3a}$, $R_{6a}$ and $R_{7a}$ are both H.

[00331] In another embodiment wherein $R_3$ is $R_{3a}$, both $R_7$ and $R_{7a}$ are methyl.

[00332] In another embodiment of formula I, said substituted heterocyclyl within $R_9$ is $R_{9a}$:



$R_{9a}$

the binding being achieved at the bond indicated by a ~~~ ;

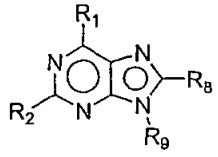
Rio and Rn are each independently H, halo, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, -$NR_aRb$, or -OH;

$R_a$ and $R_b$ are independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -$C(O)R_d$, or $C_{6-10}$ aryl;

$R_d$ is H, halo, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, -$NH_2$, -NH($Ci_{-6}$ alkyl), -NH(substituted $Ci_{-6}$ alkyl), -N($Ci_{-6}$ alkyl)$_2$, -N(substituted $Ci_{-6}$ alkyl)$_2$, $C_{6-10}$ aryl, or heterocyclyl; and

Rfis independently H, $C_{1-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -$C(O)R_d$, phosphate, diphosphate, or triphosphate.

[00333] Another embodiment of the invention provides a method of stimulating an immune response in a subject comprising administering a nucleoside analog of formula II:



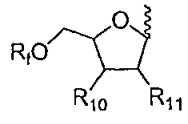
II

wherein,

Ri and $R_2$ are each independently H, halo, -$NR_aR_b$, =0, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, heterocyclyl, substituted heterocyclyl, $C_{6-10}$ aryl, substituted $C_{6-10}$ aryl, $Ci_{-6}$ alkyl, or substituted $Ci_{-6}$ alkyl;

$R_8$ is H, halo, $Ci_{-6}$ alkyl, $C_{2-6}$ alkenyl, $C_{2-6}$ alkynyl, heterocyclyl, substituted heterocyclyl, -OH, -$NR_aR_b$, -$(CH_2)_n$-O-$R_c$, -0-($C_{1-6}$ alkyl), -$S(O)_pR_e$, or -C(O)-$R_d$;

R9 is H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, heterocyclyl, or $R_{9a}$:



$R_{9a}$

the binding being achieved at the bond indicated by a ~~~*;

Rio and Rn are each independently H, halo, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, -$NR_aR_b$, or -OH;

each $R_a$ and $R_b$ is independently H, $C_{1-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -C(O)Rd, or $C_{6-10}$ aryl;

each $R_c$ is independently H, phosphate, diphosphate, triphosphate, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl;

each $R_d$ is independently H, halo, $Ci_{-6}$ alkyl, substituted $C]_{-6}$ alkyl, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, $-NH_2$, -NH($Ci_{-6}$ alkyl), -NH(substituted $Ci_{-6}$ alkyl), -N($Ci_{-6}$ alkyl)$_2$, -N(substituted $Ci_{-6}$ alkyl)$_2$, $C_{6-10}$ aryl, or heterocyclyl;

each $R_s$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, $C_{6-10}$ aryl, substituted $C_{6-10}$ aryl, heterocyclyl, or substituted heterocyclyl;

each $R_f$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -C(O)$R_d$, phosphate, diphosphate, or triphosphate;

each n is independently 0, 1, 2, or 3; and

each p is independently 0, 1, or 2.

[00334] In another embodiment of formula II, $R_9$ is -$(CH_2)_n$-O-$R_c$. In still another embodiment thereof, $R_9$ is -$CH_2$-O-($C_2$ alkyl or substituted $C_2$ alkyl)-O-(H, phosphate, diphosphate, triphosphate, $Ci_{-6}$ alkyl or substituted $Ci_{-6}$ alkyl).

[00335] In another embodiment of formula II, $R_2$ is $-NH_2$.

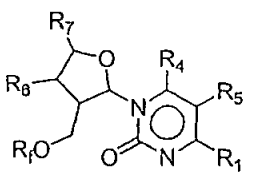
[00336] In another embodiment of formula II, Ri is =0.

[00337] In another embodiment of formula II, Ri is $-NH_2$.

[00338] In another embodiment of formula II, $R_8$ is H.

[00339] In another embodiment of formula II, $R_9$ is $R_{9a}$ and $R_f$ is H. In still another embodiment wherein $R_9$ is $R_{9a}$ and $R_f$ is H, $Ri_0$ and Rn are both H. In still another embodiment wherein $R_9$ is $R_{9a}$ and $R_f$ is H, $Ri_0$ and Rn are both -OH.

[00340] Another embodiment of the invention provides a method of stimulating an immune response in a subject comprising administering a compound of formula III:




III

wherein,

Ri and $R_2$ are each independently H, halo, -$NR_aRb$, =0, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, heterocyclyl, substituted heterocyclyl, $C_{6-1}$o aryl, substituted $C_{6-}$io aryl, $Ci_{-6}$ alkyl, or substituted $Ci_{-6}$ alkyl;

$R_4$ and $R_5$ are each independently H, halo, heterocyclyl, substituted heterocyclyl, -C(O)-Rd, $Ci_{-6}$ alkyl, or substituted $Ci_{-6}$ alkyl;

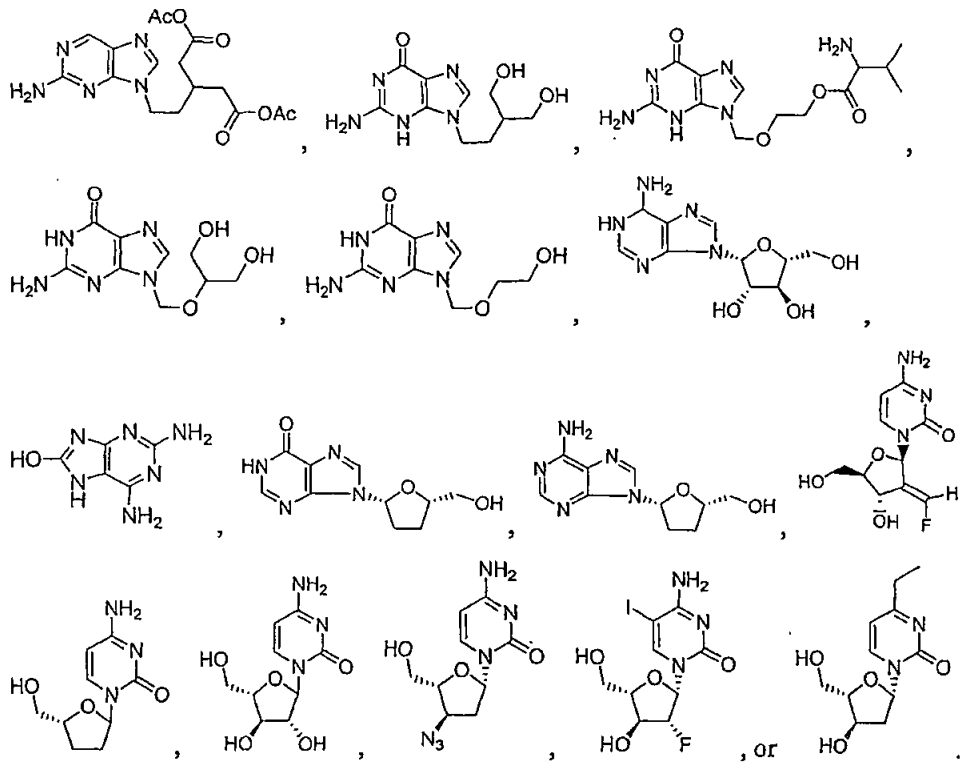
$R_6$ and $R_7$ are each independently H, halo, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, $C_{2-6}$ alkenyl, substituted $C_{2-6}$ alkenyl, -$NR_aR_b$, -N3, or -OH;

each Ra and $R_b$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -$C(O)R_d$, or $C_{6-}$io aryl;

each $R_d$ is independently H, halo, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, $Ci_{-6}$ alkoxy, substituted $Ci_{-6}$ alkoxy, -$NH_2$, -NH($Ci_{-6}$ alkyl), -NH(substituted $Ci_{-6}$ alkyl), -N($Ci_{-6}$ alkyl)$_2$, -N(substituted $Ci_{-6}$ alkyl)$_2$, $C_{6-}$io aryl, or heterocyclyl; and

$R_f$ is independently H, $Ci_{-6}$ alkyl, substituted $Ci_{-6}$ alkyl, -$C(O)R_d$, phosphate, diphosphate, or triphosphate.

[00341] In another embodiment of the invention the small molecule or nucleoside analog is:



[00342] Further provided is a composition comprising a cytokine or chemokine in combination with any compound(s) disclosed herein, such as a nucleoside, a compound encompassed by formula I, II, or III, or those listed in Table 1. In still a more particular embodiment thereof, the compound is present in the blood at less then 20 uM.

[00343] In another embodiment said nucleoside analog induces interferon biosynthesis.

[00344] In another embodiment said nucleoside analog is co-administered with another agent to the subject. In still another embodiment the agent is an antigen. In another embodiment the agent is a vaccine.

[00345] In another embodiment said nucleoside analog induces the production of TNF-cein the subject. In another embodiment thereof the nucleoside analog has an average steady-state drug concentration in the blood of less than 20 $\mu M$.

[00346] In another embodiment the subject is suffering from a microbial infection.

[00347] In another embodiment the subject is suffering from a viral infection. In another embodiment the viral infection is a viral infection caused by the hepatitis C virus (HCV). In yet another embodiment the viral infection is caused by the human immunodeficiency virus (HIV). In still another embodiment the viral infection is caused by the herpes simplex virus (HSV). In yet another embodiment the viral infection is caused by the SARS virus.

[00348] Further provided are any asymmetric carbon atoms (for example in compounds of formula I [or an N-oxide thereof]) in the (R)-, (S)- or (R,S)-configuration, preferably in the (R)- or (S)- configuration. Substituents at a double bond or a ring may be present in cis (-Z-) or trans (-E-) form. The compounds may thus be present as mixtures of isomers or as pure isomers, preferably as enantiomerically-pure.

[00349] Furthermore, it is contemplated that the invention encompasses all possible combinations of the preceding embodiments.

[00350] The nucleoside analog compounds can be used with or without an antigen in therapeutic applications, for example to treat cancer or infectious diseases. The nucleoside analog compounds also may be used in combination with other therapeutic agents, such as anti-virals and monoclonal antibodies in different therapeutic applications.

[00351] One preferred embodiment of the method of inducing an immunostimulatory effect in a patient is directed to administering an immunogenic composition comprising a vaccine in an amount effective to stimulate an immune response such as a cell-mediated immune response and, as a vaccine adjuvant, an nucleoside analog compound, in an amount effective to potentiate the immune response such as the cell-mediated immune response to the vaccine.

[00352] Agents combined with the nucleoside analog compounds, contemplated to be useful in treating the aforementioned diseases include those well known in the art, such as, anesthetics, hypnotic sedatives, anti-anxieties, antiepileptics, antipyretic antiphlogistics, stimulants, wake amines, anti-parkinson drugs, agents for psychoneuroses, agents for central nervous system, skeletal muscle relaxants, agents for autonomic nervous system, antispastic agents, cytotoxic agents, monoclonal antibodies, drugs for eye, drugs for nose and ear, anti-vertiginous drugs, cardiotonics, antiarrhythmic drugs, diuretics, pressure reduction drugs, vasoconstrictors, coronary vaso-dilators, peripheral vasodilating drugs, hyper-lipemia drugs, breath stimulants, antitussive and expectorant drugs, bronchodilators, drugs for allergy, antidiarrheal drugs, drugs for intestinal disorders, peptic ulcer drugs, stomachic digestants, antacids, cholagogouses, pituitary hormone drugs, salivary gland hormones, thyroid hormone drugs, antithyroid drugs, anabolic steroids, corticosteroids, androgen drugs, estrogen drugs, corpus luteum hormone drugs, mixed hormones, urinary/genital organ drugs, anus drugs, surgical sterilizations/antiseptics, wound protectives, externals for purulent diseases, analgesics, antipruritics, astringents, antiphlogistics, externals for parasite skin diseases, skin-softening drugs, caustics, dental/oral drugs, vitamins, inorganic preparations, supplemental liquids, hemostatics, anticoagulation drugs, drugs for liver diseases, antidotes, habitual intoxication drugs, drugs for treatment of gout, enzyme preparations, diabetic drugs, antioncotics, antihistaminics, antibiotics (such as ketolides, aminoglycosides, sulphonamides, and/or beta lactams), chemotherapeutics, biological preparations, anthelmintics, anti-Protozoas, drugs for preparations, X-ray contrast media, and diagnostic drugs.

[00353] In another embodiment methods of treating allergies are provided comprising administering an nucleoside analog compound alone or in combination with another agent known to be effective against allergies, wherein said combination is more effective in treating an allergic condition than the known agent(s) are without the addition of said nucleoside analog compound. In a more preferred embodiment the

known agent is an antihistamine and/or a leukotriene inhibitor. In another preferred embodiment, the allergic condition is asthma. In another preferred embodiment, the allergic condition is selected from the group consisting of allergic rhinitis, dermatosis, and urticaria. In an even more preferred embodiment the combination is administered to a subject enterally, parenterally, intranasally, subcutaneously, or intraarterially.

**[00354]** Preferred SMIPs in accordance with the sixth aspect of the invention include nucleoside analogs and those compounds encompassed by Formula I as described in this section, or contained within any reference cited herein.

**[00355]** A "substituted pyrimidine" refers to an aromatic ring containing two nitrogen atoms as shown:  , wherein any of the substituents defined herein or well known in the art may be appended via a single or double bond (excluding double bonds to the nitrogen atoms) to any atom of the ring.

**[00356]** A "substituted purine" refers to two condensed aromatic rings containing two nitrogen atoms each as shown:  , wherein any of the substituents defined herein or well known in the art may be appended via a single or double bond (excluding double bonds to the nitrogen atoms) to any atom of the ring.

**[00357]** Reference to "halo," "halide," or "halogen" refers to F, Cl, Br, or I atoms, especially F, Cl, and Br.

**[00358]** The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are easily synthesized by one skilled in the art from procedures described herein, as well as in patents or patent applications listed herein.

**[00359] Section VII - Seventh Aspect of the Invention - Small Molecule Compounds for Immunopotentiation**

**[00360]** AU definitions, descriptors of constituent variables for chemical formulas, and descriptions appearing in the section shall be understood to apply to this section only.

**[00361]** In accordance with the seventh aspect of the invention, Applicants have discovered methods of stimulating cytokine activity in cells and

immunotherapeutics and/or vaccine adjuvants, that will provide effective treatments for disorders described herein and those apparent to one skilled in the art.

[00362] One embodiment provides a method of modulating an immune response in a subject comprising administering a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. Another embodiment is provided wherein said compound is co-administered with another agent.

[00363] In another more particular embodiment said subject is in remission from cancer. In another more particular embodiment said compound is administered for the treatment of refractory cancer cells. In another more particular embodiment said compound is administered metronomically. In a more particular embodiment the subject is not suffering from cancer. Further still, said subject is suffering from a cancer selected from the group consisting of prostate, breast, ovarian, colon, epidermal, ductal, non-small-cell lung, colorectal, neuroendocrine, spinal, esophageal, pancreas, renal, stomach, lymphoidal, intestinal, bladder, uterine cervix, head and neck, brain, nasopharynx, leukemia, kaposis sarcoma, and mesothelioma. Another embodiment is provided wherein said compound is co-administered with another agent.

[00364] One embodiment provides a method of stimulating an immune response in a subject comprising administering a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-Ol, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-cn levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

[00365] In a more particular embodiment of said method of stimulating an immune response in a subject, said compound is administered in a dose capable of

inducing cytokines. In another more particular embodiment said subject is in remission from cancer. In another more particular embodiment said compound is administered for the treatment of refractory cancer cells. In another more particular embodiment said compound is administered metronomically. In a more particular embodiment the subject is not suffering from cancer. Further still, said subject is suffering from a cancer selected from the group consisting of prostate, breast, ovarian, colon, epidermal, ductal, non-small-cell lung, colorectal, neuroendocrine, spinal, esophageal, pancreas, renal, stomach, lymphoidal, intestinal, bladder, uterine cervix, head and neck, brain, nasopharynx, leukemia, kaposis sarcoma, and mesothelioma. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

[00366] Another embodiment of the invention provides a method of treating a subject in need of immune stimulation, comprising administering to a subject in need thereof, a subcytotoxic amount of a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu$M.

[00367] In a more particular embodiment of said method of treating a subject in need of immune stimulation, said amount is sufficient to stimulate cytokine production in the subject. In another more particular embodiment said subject is in remission from cancer. In another more particular embodiment said compound is administered for the treatment of refractory cancer cells. In another more particular embodiment said compound is administered metronomically. In another more particular embodiment the subject is not suffering from cancer. Further still, said subject is suffering from a cancer selected from the group consisting of prostate, breast, ovarian, colon, epidermal, ductal, non-small-cell lung, colorectal, neuroendocrine, spinal, esophageal, pancreas, renal, stomach, lymphoidal, intestinal,

bladder, uterine cervix, head and neck, brain, nasopharynx, leukemia, kaposis sarcoma, and mesothelioma. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu$M.

[00368] Another embodiment of the invention provides a method of identifying a subject in need of a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-Ol, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate, comprising:

a) taking a blood sample from said subject;

b) monitoring for cytokine levels in said sample; and

c) identifying said subject by decreased cytokine levels in said sample.

[00369] In a more particular embodiment of said method of identifying a subject, a further step of administering said compound to the subject, wherein upon administration of said compound, cytokine levels increase is provided. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu$M.

[00370] Another embodiment of the invention provides a method of identifying a subject in need of a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate, comprising:

a) taking a blood sample from said subject;

b) monitoring for leukocyte levels in said sample; and

c) identifying said subject by decreased leukocyte levels in said sample.

[00371] In a more particular embodiment of said method of identifying a subject, a further step of administering said compound to the subject, wherein upon administration of said compound, leukocyte levels increase is provided. Another

embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

[00372] Another embodiment of the invention provides a method of determining efficacy of a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotmib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate, comprising:

a) taking a first blood sample from a subject;

b) identifying a first cytokine level in said sample;

c) administering said compound to said subject;

d) taking a second blood sample from said subject;

e) identifying a second cytokine level in said second blood sample, wherein the compound is efficacious if said second cytokine level is greater than said first cytokine level.

[00373] Another embodiment of the invention provides a method of treating a subject suffering from an autoimmune disease, comprising administering to a subject in need thereof, a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. In a more particular embodiment said autoimmune disease is Addison's Disease, or Alopecia Areata, or Ankylosing Spondylitis, or Antiphospholipid Syndrome (APS), or Behcet's Disease, or Chronic Fatigue Syndrome, or Crohn's Disease, or Ulcerative Colitis, or Diabetes, or Fibromyalgia, or Goodpasture Syndrome, or Graft Versus Host Disease, or Graves' Disease, or Guillain-Barre Syndrome, or Lupus, or Meniere's, or Multiple Sclerosis, or Myasthenia Gravis, or Myositis, or Pemphigus Vulgaris, or Primary Biliary Cirrhosis, or Psoriasis, or Rheumatic Fever, or Sarcoidosis, or Scleroderma, or Vasculitis, or Vitiligo, or Wegener's Granulomatosis. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of

increasing TNF-a levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

**[00374]** Another embodiment of the invention provides a method of treating a subject suffering from a viral infection, comprising administering to a subject in need thereof, a compound selected from the group consisting of fenretinide, vatalanib, SU-11248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. In a more particular embodiment said viral infection is HTV, or HCV, or HBV, or HSV. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-ce levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 μM.

[00375] Another embodiment of the invention provides a method of treating a subject suffering from allergies or asthma, comprising administering to a subject in need thereof, a compound selected from the group consisting of fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate. Another embodiment is provided wherein said compound is co-administered with another agent. In a more particular embodiment still, said compound is administered in a dose capable of increasing TNF-$\alpha$ levels, more specifically, said compound has an average steady state drug concentration in the blood of less than 20 $\mu M$.

**[00376]** Preferred SMIPs in accordance with the seventh aspect of the invention include fenretinide, vatalanib, SU-1 1248, SU 5416, SU 6668, oxaliplatin, bortezomib, R 115777, CEP-701, ZD-6474, MLN-518, lapatinib, gefitinib, erlotinib, perifosine, CYC-202, LY-317615, squalamine, UCN-01, midostaurin, irofulven, alvocidib, genistein, DA-9601, avicine, docetaxel, IM 862, SU 101, and tetrathiomolybdate compositions as well as analogs disclosed in the following patents and patent applications: US 4,323,581, US 6,258,812, WO 98/35958, WO 01/60814, US 5,883,1 13, WO 99/61422, US 5,883,1 13, WO 99/61422, WO 03/24978, WO 03/04505, US 5,780,454, US 2003134846, WO 97/21701, US 5,621,100, WO

01/32651, WO 02/16351, US 6,727,256, WO 02/02552, US 5,457,105, US 5,616,582, US 5,770,599, US 5,747,498, WO, 96/30347, US 2003171303, WO 97/20842, WO 99/02162, WO 95/17182, WO 01/79255, WO 89/07105, US 5,439,936, WO 94/18151, WO 97/42949, WO 98/13344, US 5,554,519, WO 98/04541, US 6,025,387, US 2004073044, WO 02/62826, WO 04/06834, US 6,331,555, and WO 01/60814. More particularly, preferred SMIPs include those encompassed by Formula I in the aforementioned patents and patent applications.

[00377] The invention also includes compounds encompassed by Formula I, disclosed in the following patents and patent applications: US 4,323,581, US 6,258,812, WO 98/35958, WO 01/60814, US 5,883,1 13, WO 99/61422, US 5,883,113, WO 99/61422, WO 03/24978, WO 03/04505, US 5,780,454, US 2003134846, WO 97/21701, US 5,621,100, WO 01/32651, WO 02/16351, US 6,727,256, WO 02/02552, US 5,457,105, US 5,616,582, US 5,770,599, US 5,747,498, WO, 96/30347, US 2003171303, WO 97/20842, WO 99/02162, WO 95/17182, WO 01/79255, WO 89/07105, US 5,439,936, WO 94/18151, WO 97/42949, WO 98/13344, US 5,554,519, WO 98/04541, US 6,025,387, US 2004073044, WO 02/62826, WO 04/06834, US 6,331,555, and WO 01/60814.

[00378] The foregoing may be better understood by reference to the Examples, *infra,* that are presented for illustration and not to limit the scope of the inventive concepts. The Example compounds and their analogs are either commercially available, or are easily synthesized by one skilled in the art from procedures described in patents or patent applications listed herein.

Each aspect of the present invention also comprises compounds identified by the assay methods described herein.

**Definitions:**

The following Definitions apply throughout this document, in the absence of a countervailing definition in a given section.

| | |
|---|---|
| ATP: | Adenosine triphosphate |
| BCG | Mycobacterium bovis bacillus Calmette-Guerin |
| BSA: | Bovine Serum Albumin |
| FHA | Filamentous haemaglutinin |
| GCMS | Gas Chromatography / Mass Spectroscopy |
| H. Pylori | Helicobacter Pylori |
| HAV | Hepatitis A Virus |
| HBV | Hepatitis B Virus |
| HCV | Hepatitis C Virus |
| HIV | Human Immunodeficiency Virus |

| | |
|---|---|
| HPLC | High Performance Liquid Chromatography |
| HSV | Herpes Simplex Virus |
| IC50 value: | The concentration of an inhibitor that causes a 50 % reduction in a measured activity. |
| IFN | Interferon |
| IL | Interleukin |
| IMS | Immunomagnetic separation |
| IPV | Inactivated polio virus |
| LCMS | Liquid Chromatography / Mass Spectroscopy |
| LPS | Lip polysaccharide |
| MAb or mAb | Monoclonal Antibody |
| Men A | Neisseria Meningitidis Type A |
| Men C | Neisseria Meningitidis Type C |
| Men B | Neisseria Meningitidis Type B |
| Men W | Neisseria Meningitidis Type W |
| Men Y | Neisseria Meningitidis Type Y |
| MeOH: | Methanol |
| NANB | Non-A, non-B hepatitis |
| NMR | Nuclear magnetic resonance |
| OMV | Outer membrane vesicle |
| PBMC | Peripheral blood mononuclear cells |
| PT | Petussis holotoxin |
| Rt | Room temperature (25°C) |
| SMIP | Small Molecule Immune Potentiator |
| TLC | Thin-layer chromatography |
| TNF-$\alpha$ | Tumour necrosis factor-a |

The term "SMIP" refers to small molecule immunopotentiating compounds, including small molecule compounds below about MW 800 g/mol, capable of stimulating or modulating a pro-inflammatory response in a patient. In an embodiment, the SMIP compounds are able to stimulate human peripheral blood mononuclear cells to produce cytokines.

[00379] The term "SMIS" refers to small molecule immunosuppressant compounds, including small molecule compounds below about MW 800 g/mol, capable of suppressing or modulating an immune response in a patient. In an embodiment, the SMIS compounds are able to inhibit human peripheral blood mononuclear cell's ability to produce cytokines, chemokines, and/or growth factors. In another embodiment, the SMIS compounds are able to induce TGF-beta production, thereby suppressing an immune response.

[00380] The term "refractory cancer cells" refers to cancer cell lines that are resistant to preexisting therapeutics or treatment regimens, including prescribed dosing schedules.

[00381] The methods of the invention are useful in treating "allergic diseases," that is accomplished in the same way as other immunotherapeutic methods described herein.

[00382] An "allergen" refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander, dust, fungal spores, and drugs (e.g. penicillin).

[00383] "Asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

[00384] The term "leukotriene inhibitor" includes any agent or compound that inhibits, restrains, retards or otherwise interacts with the action or activity of leukotrienes, such as, but not limited to, 5-lipoxygenase ("5-LO") inhibitors, 5-lipoxygenase activating protein ("FLAP") antagonists, and leukotriene D4 ("LTD4 ") antagonists.

[00385] "Modulating" refers to inducing or suppressing.

[00386] "Immune-stimulation" or "immune potentiation" refers to activation of the immune system, including humoral or cellular activation, for example, activation of a cell, such as a killer (T or NK) or dendritic cell of the immune system, for example, causing the increase in cytokine production from a dendritic cell leading to an overall enhancement of host defense (immune response).

[00387] "Modulating an immune response" refers to either immune potentiation or immune suppression as defined herein.

[00388] An "immunogenic composition" refers to a composition capable of modulating the production of cytokines in a subject thereby effecting immune potentiation in the subject.

[00389] "Immune suppression" or "immunosuppression" refers to deactivation of the immune system, for example, preventing or lessening cytokine production from a dendritic cell leading to an overall attenuation of host defense (immune response).

[00390] An "immune-stimulatory effective amount" is an amount effective for activation of the immune system, for example, causing the increase in cytokine production from a dendritic cell leading to an overall enhancement of host defense (immune response).

[00391] "Enhancing the immune response to an antigen" by a compound refers to enhancement of the immune response in comparison to that in the absence of the compound. An enhanced immune-response eliciting composition is a composition generally comprising an antigen and a small molecule immune potentiator compound that elicits an immune response greater that a composition comprising an antigen and not containing one or more small molecule immune potentiator compounds. In this embodiment, the compound acts as an adjuvant, for example for use in vaccine compositions and methods.

[00392] A "disease associated with cellular proliferation" includes, but is not limited to neuro-fibromatosis, atherosclerosis, pulmonary fibrosis, arthritis, psoriasis, glomerulonephritis, restenosis, proliferative diabetic retinopathy (PDR), hypertrophic scar formation, inflammatory bowel disease, transplantation rejection, angiogenesis, and endotoxic shock.

[00393] The term "effective amount" is an amount necessary or sufficient to realize a desired biological effect. For example, an effective amount of a compound to treat an infectious disorder may be an amount necessary to cause an antigen specific immune response upon exposure to an infectious agent. The effective amount may vary, depending, for example, upon the condition treated, weight of the subject and severity of the disease. One of skill in the art can readily determine the effective amount empirically without undue experimentation.

[00394] As used herein "an effective amount for treatment" refers to an amount sufficient to palliate, ameliorate, stabilize, reverse, slow or delay progression of a condition such as a disease state.

[00395] Reference to "metronomic administration" or "administered metronomically" refers to increasingly frequent dosing regimens, at lower drug concentrations, as compared with known dosing regimens for an existing therapeutic. Metronomic administration varies from the typical dosing of cytotoxic drugs, which involves episodic (less frequent) administration at maximum tolerated doses (MTDs).

[00396] A "subject" or "patient" is meant to describe a human or vertebrate animal including a dog, cat, pocket pet, marmoset, horse, cow, pig, sheep, goat, elephant, giraffe, chicken, lion, monkey, owl, rat, squirrel, slender loris, and mouse.

[00397] A "pocket pet" refers to a group of vertebrate animals capable of fitting into a commodious coat pocket such as, for example, hamsters, chinchillas, ferrets, rats, guinea pigs, gerbils, rabbits and sugar gliders.

[00398] As used herein, the term "pharmaceutically acceptable ester" refers to esters, which hydrolyze *in vivo* and include those that break down readily in the human body to leave the parent compound or a salt thereof. Suitable ester groups include, for example, those derived from pharmaceutically acceptable aliphatic carboxylic acids, particularly alkanoic, alkenoic, cycloalkanoic and alkanedioic acids, in which each alkyl or alkenyl moiety advantageously has not more than 6 carbon atoms. Representative examples of particular esters include, but are not limited to, formates, acetates, propionates, butyrates, acrylates and ethylsuccinates.

[00399] The compounds of the present invention can be used in the form of salts as in "pharmaceutically acceptable salts" derived from inorganic or organic acids. These salts include but are not limited to the following: acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, cyclopentanepropionate, dodecylsulfate, ethanesulfonate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, nicotinate, 2-napthalenesulfonate, oxalate, pamoate, pectinate, sulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, p-toluenesulfonate and undecanoate. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides, and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl, and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl bromides, and others. Water or oil-soluble or dispersible products are thereby obtained.

[00400] The term "pharmaceutically acceptable prodrugs" as used herein refers to those prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals with undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention. The term "prodrug" refers to compounds that are rapidly transformed *in vivo* to yield the parent compound of the formula as described herein, for example by hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, Pro-drugs as Novel Delivery Systems, Vol. 14 of the A.C.S. Symposium Series, and

in Edward B. Roche, ed., Bioreversible Carriers in Drug Design, American Pharmaceutical Association and Pergamon Press, 1987. Prodrugs as described in U.S. Patent No. 6,284,772 for example may be used.

[00401] The symbol ~~~ is meant to indicate the point of attachment of an appendage.

[00402] Reference to "halo," "halide," or "halogen" refers to F, Cl, Br, or I atoms.

[00403] The phrase "alkyl" refers to substituted and unsubstituted alky groups such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl and the like. The phrase also includes branched chain isomers of straight chain alkyl groups, including but not limited to, the following which are provided by way of example: $-CH(CH_3)_2$, $-CH(CH_3)(CH_2CH_3)$, $-CH(CH_2CH_3)_2$, $-C(CH_3)_3$, $-C(CH_2CH_3)_3$, $-CH_2CH(CH_3)_2$, $-CH_2CH(CH_3)(CH_2CH_3)$, $-CH_2CH(CH_2CH_3)_2$, $-CH_2C(CH_3)_3$, $-CH_2C(CH_2CHs)_3$, $-CH(CH_3)CH(CH_3)(CH_2CH_3)$, $-CH_2CH_2CH(CH_3)_2$, $-CH_2CH_2CH(CH_3)(CH_2CH_3)$, $-CH_2CH_2CH(CH_2CH_3)_2$, $-CH_2CH_2C(CH_3)_3$, $-CH_2CH_2C(CH_2CH_3)_3$, $-CH(CH_3)CH_2CH(CH_3)_2$, $-CH(CH_3)CH(CH_3)CH(CH_3)_2$, $-CH(CH_2CH_3)CH(CH_3)CH(CH_3)(CH_2CH_3)$, and others. The phrase also includes cyclic alkyl groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl and such rings substituted with straight and branched chain alkyl groups as defined above. The phrase also includes polycyclic alkyl groups such as, but not limited to, adamantyl norbornyl, and bicyclo[2.2.2]octyl and such rings substituted with straight and branched chain alkyl groups as defined above. The phrase alkyl also includes groups in which one or more bonds to a carbon(s) or hydrogen(s) are replaced by a bond to non-hydrogen and non-carbon atoms such as, but not limited to, a halogen atom in halides such as F, Cl, Br, and I; and oxygen atom in groups such as hydroxyl groups, alkoxy groups, aryloxy groups, and ester groups; a sulfur atom in groups such as thiol groups, alkyl and aryl sulfide groups, sulfone groups, sulfonyl groups, and sulfoxide groups; a nitrogen atom in groups such as amines, amides, alkylamines, dialkylamines, arylamines, alkylarylamines, diarylamines, N-oxides, imides, and enamines; a silicon atom in groups such as in trialkylsilyl groups, dialkylarylsilyl groups, alkyldiarylsilyl groups, and triarylsilyl groups; and other heteroatoms in various other groups. Alkyl groups are those limited to having 1 to 20 carbon atoms and as many as 5 additional heteroatoms as described above. More preferred alkyl groups have from 1 to 5 carbon atoms and as many as 2 heteroatoms. The term "$C_{i\_6}$ alkyl" has the same meaning as alkyl, except that it is

limited to alkyl groups of six carbon atoms or less. The term "$C_2$ alkyl" indicates an alkyl group having two carbon atoms.

[00404] The phrase "aryl" refers to substituted and unsubstituted aryl groups that do not contain heteroatoms. Thus the phrase includes, but is not limited to, groups such as phenyl, biphenyl, anthracenyl, naphthenyl by way of example. Aryl groups also include those in which one of the aromatic carbons is bonded to a non-carbon or non-hydrogen atoms described above (in the alkyl definition) and also includes aryl groups in which one or more aromatic carbons of the aryl group is bonded to a substituted and/or unsubstituted alkyl, alkenyl, or alkynyl group as defined herein. This includes bonding arrangements in which two carbon atoms of an aryl group are bonded to two atoms of an alkyl, alkenyl, or alkynyl group to define a fused ring system (e.g. dihydronaphthyl or tetany dronaphthyl). Thus, the phrase "aryl" includes, but is not limited to tolyl, and hydroxyphenyl among others. The term "$C_{6\text{-}10}$ aryl" has the same meaning as aryl, except that it is limited to aryl groups of from six to ten carbon atoms.

[00405] The phrase "alkenyl" refers to straight and branched chain and cyclic groups such as those described with respect to alkyl groups as defined above, except that at least one double bond exists between two carbon atoms. Examples include, but are not limited to vinyl, $-CH=C(H)(CH_3)$, $-CH=C(CH_3)_2$, $-C(CH_3)=C(H)_2$, $-C(CH_3)=C(H)(CH_3)$, $-C(CH_2CH_3)=CH_2$, cyclohexenyl, cyclopentenyl, cyclohexadienyl, butadienyl, pentadienyl, and hexadienyl among others. Included as well are groups in which a non-carbon or non-hydrogen atom is bonded to a carbon double bonded to another carbon and those in which one of the non-carbon or non-hydrogen atoms are bonded to a carbon not involved in a double bond to another carbon. Alkenyl groups are those limited to having 2 to 15 carbon atoms and as many as 4 additional heteroatoms as described above. More preferred alkenyl groups have from 2 to 5 carbon atoms and as many as 2 heteroatoms. The term "$C_{2\text{-}6}$ alkenyl" has the same meaning as alkenyl, except that it is limited to alkenyl groups of from two to six carbon atoms.

[00406] The phrase "alkoxy" refers to substituted or unsubstituted alkoxy groups of the formula -O-alkyl, wherein the point of attachment is the oxy group and the alkyl group is as defined above. Alkoxy groups are those limited to having 1 to 20 carbon atoms and as many as 5 additional heteroatoms, including the oxygen atom. More preferred alkoxy groups have from 1 to 5 carbon atoms and as many as 2 heteroatoms,

including the oxygen atom. The term "Ci $_{.6}$ alkoxy" has the same meaning as alkoxy, except that it is limited to alkoxy groups of six carbon atoms or less.

[00407] The phrase "alkynyl" refers to straight and branched chain groups such as those described with respect to alkyl groups as defined above, except that at least one triple bond exists between two carbon atoms. Examples include, but are not limited to -CsC(H), -C=C($CH_3$), -C^C($CH_2CH_3$), -C($H_2$)CsC(H), -C($H)_2$C^C($CH_3$), and -C$(H)_2$CsC($CH_2CH_3$) among others. Included as well are alkynyl groups in which a non-carbon or non-hydrogen atom is bonded to a carbon triple bonded to another carbon and those in which a non-carbon or non-hydrogen atom is bonded to a carbon not involved in a triple bond to another carbon. Alkynyl groups are those limited to having 2 to 15 carbon atoms and as many as 4 additional heteroatoms as described above. More preferred alkynyl groups have from 2 to 5 carbon atoms and as many as 2 heteroatoms. The term "$C_{2-6}$ alkynyl" has the same meaning as alkynyl, except that it is limited to alkynyl groups of from two to six carbon atoms.

[00408] The phrase "aryloxy" refers to groups having the formula -O-aryl, wherein the point of attachment is the oxy group and the aryl group is as defined above. The phrase "$C_{6-10}$ aryloxy" has the same meaning as aryloxy, except that it is limited to aryloxy groups of six to ten carbon atoms.

[00409] The phrase "trihalomethyl" refers to a methyl group in which the three H atoms of the methyl group are substituted with three halogens which may be same or different. One example of such a group is a -$CF_3$ group in which all three H atoms of the methyl group are substituted with F atoms.

[00410] The phrase "$C_{1-6}$ alkoxy-Ci-$_6$ alkyl" refers to ether groups with as many as 12 carbon atoms. One example of a Ci $_{.6}$ alkoxy-Ci $_{.6}$ alkyl group is -$CH_2$-O-$CH_2CH_3$.

[00411] The phrase "$C_{6-10}$ aryloxy-Ci $_{.6}$ alkyl" refers to aryl ether groups of 16 carbon atoms or less, especially of 10 carbon atoms or less bound at the Ci $_{.6}$ alkyl group. One example of a $C_{6-1}$Oaryloxy-Ci $_{.6}$ alkyl group is propoxybenzene.

[00412] The phrase "$C_{6-1}$Oaryl-Ci $_{.6}$ alkyl" refers to arylalkyl groups of 16 carbon atoms or less, especially of 10 carbon atoms or less bound at the Ci $_{.6}$ alkyl group. One example of a $C_{6-10}$ aryl-C^ alkyl group is toluene.

[00413] The phrase "heterocyclyl" refers to both aromatic and nonaromatic ring compounds including monocyclic, bicyclic, and polycyclic ring compounds such as, but not limited to, quinuclidyl, containing 3 or more ring members of which one or



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more is a heteroatom such as, but not limited to, N, O, and S. Examples of heterocyclyl groups include, but are not limited to: unsaturated 3 to 8 membered rings containing 1 to 4 nitrogen atoms such as, but not limited to pyrrolyl, pyrrolinyl, imidazolyl, pyrazolyl, pyridyl, dihydropyridyl, pyrimidyl, pyrazinyl, pyridazinyl, triazolyl (e.g. 4H-1,2,4-triazolyl, 1H-1,2,3-triazolyl, 2H-1,2,3-triazolyl etc.), tetrazolyl, (e.g. 1H-tetrazolyl, 2H tetrazolyl, etc.); saturated 3 to 8 membered rings containing 1 to 4 nitrogen atoms such as, but not limited to, pyrrolidinyl, imidazolidinyl, piperidinyl, piperazinyl; condensed unsaturated heterocyclic groups containing 1 to 4 nitrogen atoms such as, but not limited to, indolyl, isoindolyl, indolinyl, indolizinyl, benzimidazolyl, quinolyl, isoquinolyl, indazolyl, benzotriazolyl; unsaturated 3 to 8 membered rings containing 1 to 2 oxygen atoms such as, but not limited to furanyl; unsaturated 3 to 8 membered rings containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms such as, but not limited to, oxazolyl, isoxazolyl, oxadiazolyl (e.g. 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, 1,2,5-oxadiazolyl, etc.); saturated 3 to 8 membered rings containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms such as, but not limited to, morpholinyl; unsaturated condensed heterocyclic groups containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, for example, benzoxazolyl, benzoxadiazolyl, benzoxazinyl (e.g. 2H-1,4-benzoxazinyl etc.); unsaturated 3 to 8 membered rings containing 1 to 3 sulfur atoms and 1 to 3 nitrogen atoms such as, but not limited to, thiazolyl, isothiazolyl, thiadiazolyl (e.g. 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,5-thiadiazolyl, etc.); saturated 3 to 8 membered rings containing 1 to 2 sulfur atoms and 1 to 3 nitrogen atoms such as, but not limited to, thiazolodinyl; saturated and unsaturated 3 to 8 membered rings containing 1 to 2 sulfur atoms such as, but not limited to, thienyl, dihydrodithiinyl, dihydrodithionyl, tetrahydrothiophene, tetrahydrothiopyran; unsaturated condensed heterocyclic rings containing 1 to 2 sulfur atoms and 1 to 3 nitrogen atoms such as, but not limited to, benzothiazolyl, benzothiadiazolyl, benzothiazinyl (e.g. 2H-1,4-benzothiazinyl, etc.), dihydrobenzothiazinyl (e.g. 2H-3,4-dihydrobenzothiazinyl, etc.), unsaturated condensed heterocyclic rings containing 1 to 2 oxygen atoms such as benzodioxolyl (e.g. 1,3-benzodioxoyl, etc.); unsaturated 3 to 8 membered rings containing an oxygen atom and 1 to 2 sulfur atoms such as, but not limited to, dihydrooxathiinyl; saturated 3 to 8 membered rings containing 1 to 2 oxygen atoms and 1 to 2 sulfur atoms such as 1,4-oxathiane; unsaturated condensed rings containing 1 to 2 sulfur atoms such as benzothienyl, benzodithiinyl; and

unsaturated condensed heterocyclic rings containing an oxygen atom and 1 to 2 oxygen atoms such as benzoxathiinyl. Heterocyclyl group also include those described above in which one or more S atoms in the ring is double-bonded to one or two oxygen atoms (sulfoxides and sulfones). For example, heterocyclyl groups include tetrahydrothiophene, tetrahydrothiophene oxide, and tetrahydrothiophene 1,1-dioxide. Preferred heterocyclyl groups contain 5 or 6 ring members. More preferred heterocyclyl groups include morphorine, piperazine, piperidine, pyrrolidine, imidazole, pyrazole, 1,2,3-triazole, 1,2,4-triazole, tetrazole, thiomorpholine, thiomorpholine in which the S atom of the thiomorpholine is bonded to one or more O atoms, pyrrole, homopiperazine, oxazolidin-2-one, pyrrolidin-2-one, oxazole, quinuclidine, thiazole, isoxazole, furan, and tetrahydrofuran. "Heterocyclyl" also refers to those groups as defined above in which one of the ring members is bonded to a non-hydrogen atom such as described above with respect to substituted alkyl groups and substituted aryl groups (also referred to herein as "substituted heterocyclyl"). Examples, include, but are not limited to, 2-methylbenzimidazolyl, 5-methylbenzimidazolyl, 5-chlorobenzthiazolyl, 1-methyl piperazinyl, and 2-chloropyridyl among others. Heterocyclyl groups are those limited to having 2 to 15 carbon atoms and as many as 6 additional heteroatoms as described above. More preferred heterocyclyl groups have from 3 to 5 carbon atoms and as many as 2 heteroatoms.

[00414] The term "substituted" as applied to an undefined, yet well known in the art group, such as phenyl, will have the same meaning with respect to the optional appendages as described in the definition of alkyl. Some preferred substitution groups include, for example, hydroxyl, nitro, amino, imino, cyano, halo, thio, thioamido, amidino, imidino, oxo, oxamidino, methoxamidino, imidino, guanidino, sulfonamide, carboxyl, formyl, alkyl, heterocyclyl, aryl, haloalkyl, alkoxy, alkoxyalkyl, alkylcarbonyl, arylcarbonyl, aralkylcarbonyl, alkylthio, aminoalkyl, alkylamino, cyanoalkyl, phosphate, diphosphate, triphosphate and the like. For example, one preferred "substituted $C_{1-6}$ alkyl" is tert-butanol.

[00415] The substitution group can itself be substituted one time. For example, an alkoxy substituent of an alkyl group may be substituted with a halogen, and oxo group, an aryl group, or the like. The group substituted onto the substitution group can be carboxyl, halo, nitro, oxo, amino, cyano, hydroxyl, $C_{1-6}$ alkyl, $C_{1-6}$ alkoxy, $C_{6-}$

io aryl, aminocarbonyl, -SR, thioamido, $-SO_3H$, $-SO_2R$ or cycloalkyl, where R is typically hydrogen, hydroxyl or $C_{i\_6}$ alkyl.

[00416] When the substituted substituent includes a straight chain group, the substitution can occur either within the chain (e.g., 2-hydroxypropyl, 2-aminobutyl, and the like) or at the chain terminus (e.g., 2-hydroxyethyl, 3-cyanopropyl, and the like). Substituted substituents can be straight chain, branched or cyclic arrangements of covalently bonded carbon atoms or heteroatoms.

[00417] Reference to "phosphate" indicates $-O-PO_3$, where the point of attachment is oxo. Where a "phosphate" group is attached at an oxo moiety (e.g. $CH_3$-O-phosphate) the phosphate substituent is $-PO_3$ (e.g. $CH_3$-O-$PO_3$). "Diphosphate" and "triphosphate" groups are respectively 2 and 3 phosphate moieties bound together as in -O-$P(O)_2$-O-$P(O)_2$-O-$PO_3$ for triphophate.

[00418] The term "protected" or a "protecting group" with respect to hydroxyl groups, amine groups, and sulfhydryl groups refers to forms of these functionalities which are protected from undesirable reaction with a protecting group known to those skilled in the art such as those set forth in Protective Groups in Organic Synthesis, Greene, T.W., John Wiley & Sons, New York, NY, (1st Edition, 1981) which can be added or removed using the procedures set forth therein. Examples of protected hydroxyl groups include, but are not limited to, silyl ethers such as those obtained by reaction of a hydroxyl group with a reagent such as, but not limited to, t-butyldimethyl-chlorosilane, trimethylchlorosilane, triisopropylchlorosilane, triethylchlorosilane; substituted methyl and ethyl ethers such as, but not limited to methoxymethyl ether, methythiomethyl ether, benzyloxymethyl ether, t-butoxymethyl ether, 2-methoxyethoxymethyl ether, tetrahydropyranyl ethers, 1-ethoxyethyl ether, allyl ether, benzyl ether; esters such as, but not limited to, benzoylformate, formate, acetate, trichloroacetate, and trifiuoracetate. Examples of protected amine groups include, but are not limited to, benzyl or dibenzyl, amides such as, formamide, acetamide, trifluoroacetamide, and benzamide; imides, such as phthalimide, and dithiosuccinimide; and others. In some embodiments, a protecting group for amines is a benzyl group. Examples of protected sulfhydryl groups include, but are not limited to, thioethers such as S-benzyl thioether, and S-4-picolyl thioether; substituted S-methyl derivatives such as hemithio, dithio and aminothio acetals; and others.

[00419] Within the present invention it is to be understood that a 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, staurosporine analog, derivatized pyridazine, chromen-4-

one, indolinone, quinazoline, nucleoside analog, or other small molecule as described herein may exhibit the phenomenon of tautomerism and that the formulae drawings within this specification can represent only one of the possible tautomeric forms. It is to be understood that the invention encompasses any tautomeric form which possesses immunomodulatory activity and is not to be limited merely to any one tautomeric form utilized within the formulae drawings.

[00420] It is also to be understood that certain 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-diones, staurosporine analogs, derivatized pyridazines, chromen-4-ones, indolinones, quinazolines, nucleoside analogs, or other small molecules as described herein can exist in solvated as well as unsolvated forms such as, for example, hydrated forms. It is to be understood that the invention encompasses all such solvated forms which possess immunomodulatory activity.

[00421] The invention also includes isotopically-labeled compounds, that are structurally identical to those disclosed above, but for the fact that one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, sulfur, fluorine and chlorine, such as $^{2}H$, $^{3}H$, $^{13}C$, $^{14}C$, $^{15}N$, $^{18}O$, $^{17}O$, $^{31}P$, $^{32}P$, $^{35}S$, $^{18}F$ and $^{36}Cl$, respectively. Compounds of the present invention, prodrugs thereof, and pharmaceutically acceptable salts of said compounds and of said prodrugs that contain the aforementioned isotopes and/or other isotopes of other atoms are within the scope of this invention. Certain isotopically labeled compounds of the present invention, for example those into which radioactive isotopes such as $^{3}H$ and $^{14}C$ are incorporated, are useful in drug and/or substrate tissue distribution assays. Tritiated, i.e., $^{3}H$, and carbon-14, i.e., $^{14}C$, isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with heavier isotopes such as deuterium, i.e., $^{2}H$, may afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements and, hence, may be preferred in some circumstances. Isotopically labeled compounds of this invention and prodrugs thereof can generally be prepared by carrying out known or referenced procedures and by substituting a readily available isotopically labeled reagent for a non-isotopically labeled reagent.

[00422] Preferred "cytokines" include IL 1-30 as well as TNF-alpha, TNF-beta, IFN-alpha (family), IFN-beta and IFN-gamma.

[00423] Reference to "IL 1-30" indicates interleukin cytokines selected from the group consisting of ILIA, ILIB, IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, ILIFIO, ILIRI, IL1R2, ILIRAP, ILIRAPLI, IL1RAPL2, ILIRLI, IL1RL2, ILIRN $_3$IL2, IL2RA, IL2RB, IL2RG, IL3, IL3RA, IL4, IL4R, IL5, IL5RA, IL6, IL6R, IL6RL1, IL6ST, IL6ST2, IL6STP, IL7, IL7R, IL8, IL8RA, IL8RB, IL8RBP, IL9, IL9R, IL9RP1, IL9RP2, IL9RP3, IL9RP4, ILIO, ILIORA, ILIORB, ILII, ILIIRA $_3$ILIIRB, IL12A, IL12B, IL12RB1, IL12RB2, IL13, IL13RA1, IL13RA2, IL14, IL15, IL15RA, IL15RB, IL16, IL17, IL17B, IL17C, IL17D, IL17E, IL17F, IL17R, IL17RB, IL17RC, IL17RD, IL17RE, IL18, IL18BP, IL18R1, IL18RAP, IL19, IL20, IL20RA, IL20RB, IL21, IL21R, IL22, IL22RA1, IL22RA2, IL23A, IL24, IL26, IL28A, IL28B, IL28RA, IL29, and IL30. More preferred interleukins include IL-lb, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and IL-13.

[00424] Reference to "chemokines" indicates: CXC chemokines including CXCLI $_3$ CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCLIO, CXCLI 1, CXCL12, CXCL13, CXCL14, CXCL15, and CXCL16; C chemokines including XCLI, and XCL2; $CX_3C$ chemokines including $CX_3CLI$; and CC chemokines including CCLI $_3$CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9/CCL10, CCLII, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, and CCL28.

[00425] "Concomitantly" refers to occurring or existing concurrently or in parallel with another.

[00426] Vaccine compositions contemplated to be within the scope of the several aspects of the present invention may include (an) additional adjuvant(s). Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as, for example (a) MF59™ (WO90/14837), containing 5% squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing MTP-PE) formulated into submicron particles using a microfluidizer, (b) SAF, containing 5% squalene, 0.5% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either

microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox ™); (3) saponin adjuvants, such as QS21 or Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMs may be devoid of additional detergent e.g. WO00/07621; (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (e.g. IL-I, IL-2, IL-4, IL-5, IL-6, IL-7, IL- 12 (WO99/44636), etc.), interferons (e.g. gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) momophosphoryl lipid A (MPL) or 3-0-deacylated MPL (3dMPL), optionally in the substantial absence of alum when used with pneumococcal saccharides e.g. WO00/56358; and RC529 (7) combinations of 3dMPL with, for example, QS21 and /or oil-in-water emulsions e.g. EP-A-0835318; (8) oligonucleotides comprising CpG motifs, i.e. containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (9) a polyoxyethylene ether or a polyoxyethylene ester e.g. WO99/52549; (10) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol (WOO 12 1207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21 152); (11) a saponin and an immunostimulatory oligonucleotide (e.g. a CpG oligonucleotide) (WO00/62800); (12) an immunostimulant and a particle of metal salt e.g WO00/23105; (13) a saponin and an oil-in-water emulsion e.g. WO99/11241; (14) a saponin (e.g. QS21) + 3dMPL +IL- 12 (optionally + a sterol) e.g. WO98/57659; (14) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. In one particular embodiment, Alum (especially aluminium phosphate and/or hydroxide) and MF59 are preferred for use with saccharide antigens.

[00427] In some aspects, the invention is also directed to methods of administering the vaccine composition. The vaccine is administered in an amount effective to stimulate an immune response. The amount that constitutes an effective amount depends, *inter alia,* on the particular vaccine used, the particular adjuvant compound being administered and the amount thereof, the immune response that is to be

enhanced (humoral or cell mediated), the state of the immune system (e.g., suppressed, compromised, stimulated), and the desired therapeutic result. Accordingly it is not practical to set forth generally the amount that constitutes an effective amount of the vaccine. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

[00428] The vaccine compositions of the invention can be administered to animals, e.g., mammals human and non-human, including, for example, pocket pets, fowl, and the like according to conventional methods well known to those skilled in the art (e.g., orally, subcutaneously, nasally, topically).

[00429] Suitable vaccines include, but are not limited to, any material that raises either humoral or cell mediated immune response, or both. Suitable vaccines include live viral and bacterial antigens and inactivated viral, tumor-derived, protozoal, organism-derived, fungal, and bacterial antigens, toxoids, toxins, polysaccharides, proteins, glycoproteins, peptides, and the like. Conventional vaccines, such as those used in connection with BCG (live bacteria), cholera, plague, and typhoid (killed bacteria), hepatitis B, influenza, inactivated polio, and rabies (inactivated virus), measles, mumps, rubella, oral polio, SARS vaccines, and yellow fever (live virus), tetanus and diphtheria (toxoids), hemophilus influenzae b, meningococcal, and pneumococcal (bacterial polysaccharides) also can be used. Any antigen known in the art or disclosed herein may be used.

[00430] Furthermore, it is contemplated that certain currently experimental vaccines, especially materials such as recombinant proteins, glycoproteins, and peptides that do not raise a strong immune response, will also find use in connection with the 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-dione compound. Exemplary experimental subunit antigens include those related to viral disease such as adenovirus, AIDS, chicken pox, cytomegalovirus, dengue, feline leukemia, fowl plague, hepatitis A, hepatitis B, hepatitis C, HSV-I, HSV-2, hog cholera, influenza A, influenza B, Japanese encephalitis, measles, parainfluenza, rabies, respiratory syncytial virus, SARS virus, rotavirus, wart, and yellow fever.

[00431] Specific antigens include: a protein antigen from *N. meningitides* serogroup B (1-7); an outer-membrane vesicle (OMV) preparation from *N. meningitides* serogroup B. (8, 9, 10, 11); a saccharide antigen from *N. meningitides* serogroup A, C W135 and/or Y, such as the oligosaccharide (12) from serogroup C (13); a saccharide antigen *horn Streptococcus pneumoniae* (14, 15, 16); an antigen

from *N. gonorrhoeae* (1, 2, 3); an antigen from *Chlamydia pneumoniae* (17, 18, 19, 20, 21, 22, 23); an antigen from *Chlamydia trachomatis* (24); an antigen from hepatitis A virus, such as inactivated virus (25, 26); an antigen from hepatitis B virus, such as the surface and/or core antigens (e.g. 26, 27); an antigen from hepatitis C virus (28); an antigen from *Bordetella pertussis,* such as petussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis,* optionally also combination with pertactin and/or agglutinogens 2 and 3 (29, 30); a diphtheria antigen, such as a diphtheria toxoid (31:chapter 3) e.g. the $CRM_{197}$ mutant (32); a tetanus antigen, such as a tetanus toxoid (31:chapter 4); a protein antigen from Helicobacter pylori such as CagA (33), VacA (33), NAP (34), HopX (5), HopY (35) and/or urease; a saccharide antigen from Haemophilus influenzae B (13); an antigen from Porphyromonas gingivalis (36); polio antigen(s) (37, 38) such as IPV or OPV; rabies antigen(s) (39) such lyophilized inactivated virus (40, RabAvert™); measles, mumps and/or rubella antigens (31: chapters 9, 10, & 11); influenza antigen(s) (31:chapter 19), such as the haemagglutinin and/or neuraminidase surface proteins; an antigen from Moraxella catarrhalis (41); an antigen from Streptococcus agalactiae (group B streptococcus) (42, 43); an antigen from Streptococcus pyogenes (group A streptococcus) (43, 44, 45); and an antigen from Staphylococcus aureus (46).

[00432] The composition may comprise one or more of the above antigens.

[00433] Where a saccharide or carbohydrate antigen is used, it is preferably conjugated to a carrier protein in order to enhance antigenicity (47-56). Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The $CRM_{197}$ diphtheria toxoid is particularly preferred. Other suitable carrier proteins include the *N. meningitides* outer membrane protein (57), synthetic peptides (58, 59), heat shock proteins (60), pertussis proteins (61, 62), protein D from *H. influenzae* (63), toxin A or B from *C. difficile* (64) etc. Where a mixture comprises capsular saccharides from both serogroups A and C, it is preferred that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (e.g. 2:1, 3:1, 4:4, 5:1, 10:1 or higher). Saccharides from different serogroups of N. meningitides may be conjugated to the same or different carrier proteins.

[00434] Any suitable conjugation reaction can be used, with any suitable linker where necessary. Toxic protein antigens may be detoxified where necessary (e.g. detoxification of pertussis toxin by chemical and/or genetic means (30)). Where a diphtheria antigen is included in the composition it is preferred also to include tetanus

antigens and pertussis antigens. Similar, where a tetanus antigen is include it is preferred also to include diphtheria and pertussis antigens. Similar, where pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

[00435] The pharmaceutical compositions containing the compounds described herein can include additives such as excipients. Suitable pharmaceutically acceptable excipients include processing agents and drug delivery modifiers and enhancers, such as, for example, calcium phosphate, magnesium stearate, talc, monosaccharides, disaccharides, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, dextrose, hydroxypropyl-β-cyclodextrin, polyvinylpyrrolidinone, low melting waxes, ion exchange resins, and the like, as well as combinations of any two or more thereof. Other suitable pharmaceutically acceptable excipients are described in "Remington's Pharmaceutical Sciences," Mack Pub. Co., New Jersey (1991), incorporated herein by reference.

[00436] Pharmaceutical compositions containing the compounds of the invention may be in any form suitable for the intended method of administration, including, for example, a solution, a suspension, or an emulsion. Liquid carriers are typically used in preparing solutions, suspensions, and emulsions. Liquid carriers contemplated for use in the practice of the present invention include, for example, water, saline, pharmaceutically acceptable organic solvent(s), pharmaceutically acceptable oils or fats, and the like, as well as mixtures of two or more thereof. The liquid carrier may contain other suitable pharmaceutically acceptable additives such as solubilizers, emulsifiers, nutrients, buffers, preservatives, suspending agents, thickening agents, viscosity regulators, stabilizers, and the like. Suitable organic solvents include, for example, monohydric alcohols, such as ethanol, and polyhydric alcohols, such as glycols. Suitable oils include, for example, soybean oil, coconut oil, olive oil, saffiower oil, cottonseed oil, and the like. For parenteral administration, the carrier can also be an oily ester such as ethyl oleate, isopropyl myristate, and the like. Compositions of the present invention may also be in the form of microparticles, microcapsules, and the like, as well as combinations of any two or more thereof.

[00437] The compounds and combinations of the present invention can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are fonned by mono- or multilamellar hydrated liquid crystals that are dispersed in an

aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to a compound of the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., *Methods in Cell Biology,* Volume XIV, Academic Press, New York, N.W., p. 33 *et seq* (1976).

**[00438]** Other additives include immunostimulatory agents known in the art or listed herein. Immunostimulatory oligonucleotides and polynucleotides are described in PCT WO 98/55495 and PCT WO 98/16247. U.S. Patent Application No. 2002/0164341 describes adjuvants including an unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant. U.S. Patent Application No. 2002/0197269 describes compositions comprising an antigen, an antigenic CpG-ODN and a polycationic polymer. Other immunostimulatory additives described in the art may be used, for example, as described in U.S. Patent No. 5,026,546; U.S. Patent No. 4,806,352; and U.S. Patent No. 5,026,543. Additionally, SMIP compounds as described in USSN 10/814480 and 60/582654 are contemplated as effective co-administration agents or combination with the compositions of the instant invention.

**[00439]** A controlled release delivery system may be used, such as a diffusion controlled matrix system or an erodible system, as described for example in: Lee, "Diffusion-Controlled Matrix Systems", pp. 155-198 and Ron and Langer, "Erodible Systems", pp. 199-224, in "Treatise on Controlled Drug Delivery", A. Kydonieus Ed., Marcel Dekker, Inc., New York 1992. The matrix may be, for example, a biodegradable material that can degrade spontaneously *in situ* and *in vivo* for, example, by hydrolysis or enzymatic cleavage, *e.g.,* by proteases. The delivery system may be, for example, a naturally occurring or synthetic polymer or copolymer, for example in the form of a hydrogel. Exemplary polymers with cleavable linkages include polyesters, polyorthoesters, polyanhydrides, polysaccharides, poly(phosphoesters), polyamides, polyurethanes, poly(imidocarbonates) and poly(phosphazenes).

**[00440]** The compounds of the invention may be administered enterally, orally, parenterally, sublingually, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. For example, suitable modes of administration

include oral, subcutaneous, transdermal, transmucosal, iontophoretic, intravenous, intramuscular, intraperitoneal, intranasal, subdermal, rectal, and the like. Topical administration may also involve the use of transdermal administration such as transdermal patches or ionophoresis devices. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection, or infusion techniques.

[00441] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-propanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[00442] Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable nonirritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum and release the drug.

[00443] Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e.g., lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

[00444] Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, cyclodextrins, and sweetening, flavoring, and perfuming agents.

[00445] Effective amounts of the compounds of the invention generally include any amount sufficient to detectably treat the disorders described herein.

[00446] Successful treatment of a subject in accordance with the invention may result in the inducement of a reduction or alleviation of symptoms in a subject afflicted with a medical or biological disorder to, for example, halt the further progression of the disorder, or the prevention of the disorder.

[00447] The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy. The therapeutically effective amount for a given situation can be readily determined by routine experimentation and is within the skill and judgment of the ordinary clinician.

Compositions of the invention may be administered in conjunction with one or more antigens for use in therapeutic, prophylactic, or diagnostic methods of the present invention. Preferred antigens include those listed below. Additionally, the compositions of the present invention may be used to treat or prevent infections caused by any of the below-listed microbes.

In addition to combination with the antigens described below, the compositions of the invention may also be combined with an adjuvant as described herein. Antigens for use with the invention include, but are not limited to, one or more of the following antigens set forth below, or antigens derived from one or more of the pathogens set forth below:

A. **Bacterial Antigens**

Bacterial antigens suitable for use in the invention include proteins, polysaccharides, lipopolysaccharides, and outer membrane vesicles which may be isolated, purified or derived from a bacteria. In addition, bacterial antigens may include bacterial lysates and inactivated bacteria formulations. Bacteria antigens may be produced by recombinant expression. Bacterial antigens preferably include epitopes which are exposed on the surface of the bacteria during at least one stage of its life cycle. Bacterial antigens are preferably conserved across multiple serotypes.

Bacterial antigens include antigens derived from one or more of the bacteria set forth below as well as the specific antigens examples identified below.

*Neisseria meningitides: Meningitides* antigens may include proteins (such as those identified in References 1 - 7), saccharides (including a polysaccharide, oligosaccharide or lipopolysaccharide), or outer-membrane vesicles (References 8, 9, 10, 11) purified or derived from *N. meningitides* serogroup A, C, W135, Y, and/or B. Meningitides protein antigens may be selected from adhesions, autotransporters, toxins, Fe acquisition proteins, and membrane associated proteins (preferably integral outer membrane protein).

*Streptococcus pneumoniae: Streptococcus pneumoniae* antigens may include a saccharide (including a polysaccharide or an oligosaccharide) or protein from *Streptococcus pneumoniae*. Saccharide antigens may be selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, HA, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. Protein antigens may be selected from a protein identified in WO 98/18931, WO 98/18930, US Patent No. 6,699,703, US Patent No. 6,800,744, WO 97/43303, and WO 97/37026. Streptococcus pneumoniae proteins may be selected from the Poly Histidine Triad family (PhtX), the Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins, pneumolysin (Ply), PspA, PsaA, Spl28, Spl01, Spl30, Spl25 or Spl33.

*Streptococcus pyogenes (Group A Streptococcus):* Group A Streptococcus antigens may include a protein identified in WO 02/34771 or WO 2005/032582 (including GAS 40), fusions of fragments of GAS M proteins (including those described in WO 02/094851, and Dale, Vaccine (1999) 17:193-200, and Dale, Vaccine 14(10): 944-948), fibronectin binding protein (Sfbl), Streptococcal heme-associated protein (Shp), and Streptolysin S (SagA).

*Moraxella catarrhalis:* Moraxella antigens include antigens identified in WO 02/18595 and WO 99/58562, outer membrane protein antigens (HMW-OMP), C-antigen, and/or LPS.

*Bordetella pertussis:* Pertussis antigens include petussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis,* optionally also combination with pertactin and/or agglutinogens 2 and 3 antigen.

*Staphylococcus aureus:* Staph aureus antigens include *S. aureus* type 5 and 8 capsular polysaccharides optionally conjugated to nontoxic recombinant

*Pseudomonas aeruginosa* exotoxin A, such as StaphVAX™, or antigens derived from surface proteins, invasins (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), carotenoids, catalase production, Protein A, coagulase, clotting factor, and/or membrane-damaging toxins (optionally detoxified) that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin).

*Staphylococcus epidermis: S. epidermidis* antigens include slime-associated antigen (SAA).

*Tetanus:* Tetanus antigens include tetanus toxoid (TT), preferably used as a carrier protein in conjunction/conjugated with the compositions of the present invention.

*Diphtheria:* Diphtheria antigens include diphtheria toxin, preferably detoxified, such as $CRMi_{97}$, additionally antigens capable of modulating, inhibiting or associated with ADP ribosylation are contemplated for combination/co-administration/conjugation with the compositions of the present invention, the diphtheria toxoids are preferably used as carrier proteins.

*Haemophilus influenzae B (Hib):* Hib antigens include a Hib saccharide antigen.

*Pseudomonas aeruginosa:* Pseudomonas antigens include endotoxin A, Wzz protein, *P. aeruginosa* LPS, more particularly LPS isolated from PAO1 (05 serotype), and/or Outer Membrane Proteins, including Outer Membrane Proteins F (OprF) *(Infect Immun.* 2001 May; 69(5): 3510-3515).

*Legionella pneumophila* (Legionnairs' Disease): L. pneumophila antigens may optionally derived from cell lines with disrupted *asd* genes *(Infect Immun.* 1998 May; 66(5): 1898).

*Streptococcus agalactiae (Group B Streptococcus):* Group B Streptococcus antigens include a protein or saccharide antigen identified in WO 02/34771, WO 03/093306, WO 04/041157, or WO 2005/002619 (including proteins GBS 80, GBS 104, GBS 276 and GBS 322, and including saccharide antigens derived from serotypes Ia, Ib, Ia/c, II, III, IV, V, VI, VII and VIII).

*Neiserria gonorrhoeae:* Gonorrhoeae antigens include Por (or porin) protein, such as PorB *(see* Zhu *et al,* Vaccine (2004) 22:660 - 669), a transferring binding protein, such as TbpA and TbpB (See Price *et al,* Infection and Immunity (2004) 71(1):277 - 283), a opacity protein (such as Opa), a reduction-modifiable protein (Rmp), and outer membrane vesicle (OMV) preparations *(see* Plante *et al,* J

Infectious Disease (2000) 182:848 - 855), also see *e.g.* WO99/24578, WO99/36544, WO99/57280, WO02/079243).

*Chlamydia trachomatis:* Chlamydia trachomatis antigens include antigens derived from serotypes A, B, Ba and C are (agents of trachoma, a cause of blindness), serotypes $L_1$, $L_2$ & $L_3$ (associated with Lymphogranuloma venereum), and serotypes, D-K. Chlamydia trachomas antigens may also include an antigen identified in WO 00/37494, WO 03/049762, WO 03/068811, or WO 05/002619.

*Treponema pallidum* (Syphilis): Syphilis antigens include TmpA antigen.

*Haemophilus ducreyi* (causing chancroid): Ducreyi antigens include outer membrane protein (DsrA).

*Enterococcus faecalis or Enterococcus faecium:* Antigens include a trisaccharide repeat or other *Enterococcus* derived antigens provided in US Patent No. 6,756,361.

*Helicobacter pylori:* H pylori antigens include Cag, Vac, Nap, HopX, HopY and/or urease antigen.

*Staphylococcus saprophyticus:* Antigens include the 160 kDa hemagglutinin of *S. saprophyticus* antigen.

*Yersinia enterocolitica* Antigens include LPS *(Infect Immun.* 2002 August; 70(8): 4414).

*E. coli:* E. coli antigens may be derived from enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), and/or enterohemorrhagic *E. coli* (EHEC).

*Bacillus anthracis* (anthrax): *B. anthracis* antigens are optionally detoxified and may be selected from A-components (lethal factor (LF) and edema factor (EF)), both of which can share a common B-component known as protective antigen (PA).

*Yersinia pestis* (plague): Plague antigens include F1 capsular antigen *(Infect Immun.* 2003 Jan; 71(1)): 374-383, LPS *(Infect Immun.* 1999 Oct; 67(10): 5395), Yersinia pestis V antigen *(Infect Immun.* 1997 Nov; 65(1 1): 4476-4482).

*Mycobacterium tuberculosis:* Tuberculosis antigens include lipoproteins, LPS, BCG antigens, a fusion protein of antigen 85B (Ag85B) and/or ESAT-6 optionally formulated in cationic lipid vesicles *(Infect Immun.* 2004 October; 72(10): 6148), Mycobacterium tuberculosis (Mtb) isocitrate dehydrogenase associated antigens

*(Proc Natl Acad Sd USA.* 2004 Aug 24; 101(34): 12652), and/or MPT51 antigens *{Infect Immun.* 2004 July; 72(7): 3829).

*Rickettsia:* Antigens include outer membrane proteins, including the outer membrane protein A and/or B (OmpB) *{Biochim Biophys Acta.* 2004 Nov l;1702(2):145), LPS, and surface protein antigen (SPA) *(J Autoimmun.* 1989 Jun;2 Suppl:81).

*Listeria monocytogenes* : Antigens derived from *L. monocytogenes* are preferably used as carriers/vectors for intracytoplasmic delivery of conjugates/associated compositions of the present invention.

*Chlamydia pneumoniae:* Antigens include those identified in WO 02/02606.

*Vibrio cholerae:* Antigens include proteinase antigens, LPS, particularly lipopolysaccharides of Vibrio cholerae II, O1 Inaba O-specif ic polysaccharides, V. cholera 0139, antigens of IEM108 vaccine *(Infect Immun.* 2003 Oct;71(10):5498-504), and/or Zonula occludens toxin (Zot).

*Salmonella typhi* (typhoid fever): Antigens include capsular polysaccharides preferably conjugates (Vi, i.e. vax-TyVi).

*Borrelia burgdorferi* (Lyme disease): Antigens include lipoproteins (such as OspA, OspB, Osp C and Osp D), other surface proteins such as OspE-related proteins (Erps), decorin-binding proteins (such as DbpA), and antigenically variable VI proteins. , such as antigens associated with P39 and P13 (an integral membrane protein, *Infect Immun.* 2001 May; 69(5): 3323-3334), VlsE Antigenic Variation Protein (J *Clin Microbiol.* 1999 Dec; 37(12): 3997).

*Porphyromonas gingivalis:* Antigens include *P. gingivalis* outer membrane protein (OMP).

*Klebsiella:* Antigens include an OMP, including OMP A, or a polysaccharide optionally conjugated to tetanus toxoid.

Where not specifically referenced, further bacterial antigens of the invention may be capsular antigens, polysaccharide antigens or protein antigens of any of the above. Further bacterial antigens may also include an outer membrane vesicle (OMV) preparation. Additionally, antigens include live, attenuated, split, and/or purified versions of any of the aforementioned bacteria. The bacterial or microbial derived antigens of the present invention may be gram-negative or gram-positive and aerobic or anaerobic.

Additionally, any of the above bacterial-derived saccharides (polysaccharides, LPS, LOS or oligosaccharides) can be conjugated to another agent or antigen, such as a carrier protein (for example $CRM_{197}$). Such conjugation may be direct conjugation effected by reductive animation of carbonyl moieties on the saccharide to amino groups on the protein, as provided in US Patent No. 5,360,897 and *Can JBiochem Cell Biol.* 1984 May; 62(5): 270-5. Alternatively, the saccharides can be conjugated through a linker, such as, with succinamide or other linkages provided in *Bioconjugate Techniques,* 1996 and *CRC, Chemistry of Protein Conjugation and Cross-Linking,* 1993.

**B. Viral Antigens**

Viral antigens suitable for use in the invention include inactivated (or killed) virus, attenuated virus, split virus formulations, purified subunit formulations, viral proteins which may be isolated, purified or derived from a virus, and Virus Like Particles (VLPs). Viral antigens may be derived from viruses propagated on cell culture or expressed recombinantly. Viral antigens preferably include epitopes which are exposed on the surface of the virus during at least one stage of its life cycle. Viral antigens are preferably conserved across multiple serotypes. Viral antigens include antigens derived from one or more of the viruses set forth below as well as the specific antigens examples identified below.

*Orthomyxovirus:* Viral antigens may be derived from an Orthomyxovirus, such as Influenza A, B and C. Orthomyxovirus antigens may be selected from one or more of the viral proteins, including hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (Ml), membrane protein (M2), one or more of the transcriptase components (PBl, PB2 and PA). Preferred antigens include HA and NA.

Influenza antigens may be derived from interpandemic (annual) flu strains. Alternatively influenza antigens may be derived from strains with the potential to cause pandemic a pandemic outbreak (i.e., influenza strains with new haemagglutinin compared to the haemagglutinin in currently circulating strains, or influenza strains which are pathogenic in avian subjects and have the potential to be transmitted horizontally in the human population, or influenza strains which are pathogenic to humans).

*Paramyxoviridae* viruses: Viral antigens may be derived from Paramyxoviridae viruses, such as Pneumoviruses (RSV), Paramyxoviruses (PIV) and Morbilliviruses (Measles).

*Pneumovirus:* Viral antigens may be derived from a Pneumovirus, such as Respiratory syncytial virus (RSV), Bovine respiratory syncytial virus, Pneumonia virus of mice, and Turkey rhinotracheitis virus. Preferably, the Pneumovirus is RSV. Pneumovirus antigens may be selected from one or more of the following proteins, including surface proteins Fusion (F), Glycoprotein (G) and Small Hydrophobic protein (SH), matrix proteins M and M2, nucleocapsid proteins N, P and L and nonstructural proteins NS1 and NS2. Preferred Pneumovirus antigens include F, G and M. See e.g., *J Gen Virol.* 2004 Nov; 85(Pt 11):3229). Pneumovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV.

*Paramyxovirus:* Viral antigens may be derived from a Paramyxovirus, such as Parainfluenza virus types 1- 4 (PIV), Mumps, Sendai viruses, Simian virus 5, Bovine parainfluenza virus and Newcastle disease virus. Preferably, the Paramyxovirus is PIV or Mumps. Paramyxovirus antigens may be selected from one or more of the following proteins: Hemagglutinin -Neuraminidase (HN), Fusion proteins F1 and F2, Nucleoprotein (NP), Phosphoprotein (P), Large protein (L), and Matrix protein (M). Preferred Paramyxovirus proteins include HN, F1 and F2. Paramyxovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV. Commercially available mumps vaccines include live attenuated mumps virus, in either a monovalent form or in combination with measles and rubella vaccines (MMR).

*Morbillivirus:* Viral antigens may be derived from a Morbillivirus, such as Measles. Morbillivirus antigens may be selected from one or more of the following proteins: hemagglutinin (H), Glycoprotein (G), Fusion factor (F), Large protein (L), Nucleoprotein (NP), Polymerase phosphoprotein (P), and Matrix (M). Commercially available measles vaccines include live attenuated measles virus, typically in combination with mumps and rubella (MMR).

*Picornavirus:* Viral antigens may be derived from Picornaviruses, such as Enteroviruses, Rhinoviruses, Heparnavirus, Cardioviruses and Aphthoviruses. Antigens derived from Enteroviruses, such as Poliovirus are preferred.

*Enterovirus:* Viral antigens may be derived from an Enterovirus, such as Poliovirus types 1, 2 or 3, Coxsackie A virus types 1 to 22 and 24, Coxsackie B virus types 1 to 6, Echovirus (ECHO) virus) types 1 to 9, 11 to 27 and 29 to 34 and Enterovirus 68 to 71. Preferably, the Enterovirus is poliovirus. Enterovirus antigens are preferably selected from one or more of the following Capsid proteins VP1, VP2, VP3 and VP4. Commercially available polio vaccines include Inactivated Polio Vaccine (IPV) and Oral poliovirus vaccine (OPV).

*Heparnavirus:* Viral antigens may be derived from an Heparnavirus, such as Hepatitis A virus (HAV). Commercially available HAV vaccines include inactivated HAV vaccine.

*Togavirus:* Viral antigens may be derived from a Togavirus, such as a Rubivirus, an Alphavirus, or an Arterivirus. Antigens derived from Rubivirus, such as Rubella virus, are preferred. Togavirus antigens may be selected from E1, E2, E3, C, NSP-I, NSPO-2, NSP-3 or NSP-4. Togavirus antigens are preferably selected from E1, E2 or E3. Commercially available Rubella vaccines include a live cold-adapted virus, typically in combination with mumps and measles vaccines (MMR).

*Flavivirus:* Viral antigens may be derived from a Flavivirus, such as Tick-borne encephalitis (TBE), Dengue (types 1, 2, 3 or 4), Yellow Fever, Japanese encephalitis, West Nile encephalitis, St. Louis encephalitis, Russian spring-summer encephalitis, Powassan encephalitis. Flavivirus antigens may be selected from PrM, M, C, E, NS-I, NS-2a, NS2b, NS3, NS4a, NS4b, and NS5. Flavivirus antigens are preferably selected from PrM, M and E. Commercially available TBE vaccine include inactivated virus vaccines.

*Pestivirus:* Viral antigens may be derived from a Pestivirus, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).

*Hepadnavirus:* Viral antigens may be derived from a Hepadnavirus, such as Hepatitis B virus. Hepadnavirus antigens may be selected from surface antigens (L, M and S), core antigens (HBc, HBe). Commercially available HBV vaccines include subunit vaccines comprising the surface antigen S protein.

*Hepatitis C virus:* Viral antigens may be derived from a Hepatitis C virus (HCV). HCV antigens may be selected from one or more of E1, E2, E1/E2, NS345 polyprotein, NS 345-core polyprotein, core, and/or peptides from the nonstructural regions (Houghton et al., *Hepatology* (1991) 14:381).

*Rhabdovirus:* Viral antigens may be derived from a Rhabdovirus, such as a Lyssavirus (Rabies virus) and Vesiculovirus (VSV). Rhabdovirus antigens may be selected from glycoprotein (G), nucleoprotein (N), large protein (L), nonstructural proteins (NS). Commercially available Rabies virus vaccine comprise killed virus grown. on human diploid cells or fetal rhesus lung cells.

*Caliciviridae;* Viral antigens may be derived from Calciviridae, such as Norwalk virus.

*Coronavirus:* Viral antigens may be derived from a Coronavirus, SARS, Human respiratory coronavirus, Avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible gastroenteritis virus (TGEV). Coronavirus antigens may be selected from spike (S), envelope (E), matrix (M), nucleocapsid (N), and Hemagglutinin-esterase glycoprotein (HE). Preferably, the Coronavirus antigen is derived from a SARS virus. SARS viral antigens are described in WO 04/92360;

Retrovirus: Viral antigens may be derived from a Retrovirus, such as an Oncovirus, a Lentivirus or a Spumavirus. Oncovirus antigens may be derived from HTLV-I, HTLV-2 or HTLV-5. Lentivirus antigens may be derived from HIV-I or HIV-2. Retrovirus antigens may be selected from gag, pol, env, tax, tat, rex, rev, nef, vif, vpu, and vpr. HIV antigens may be selected from gag (p24gag and p55gag), env (gpl60 and gp41), pol, tat, nef, rev vpu, miniproteins, (preferably p55 gag and gpl40v delete). HIV antigens may be derived from one or more of the following strains: ffIV πb, HIVsF$_2$, HIVLAV, HIV $_{LA}$I, HIVMN, HIV-1 $_{C}$M235, HIV-1 $_{US4}$.

*Reovirus:* Viral antigens may be derived from a Reovirus, such as an Orthoreo virus, a Rotavirus, an Orbivirus, or a Coltivirus. Reovirus antigens may be selected from structural proteins λl, λ2, λ3, μ*l*, μ*2*, σl, σ*2*, or σ3, or nonstructural proteins σNS, μNS, or σls. Preferred Reovirus antigens may be derived from a Rotavirus. Rotavirus antigens may be selected from VPl, VP2, VP3, VP4 (or the cleaved product VP5 and VP8), NSP 1, VP6, NSP3, NSP2, VP7, NSP4, or NSP5. Preferred Rotavirus antigens include VP4 (or the cleaved product VP5 and VP8), and VP7.

*Parvovirus:* Viral antigens may be derived from a Parvovirus, such as Parvovirus B19. Parvovirus antigens may be selected from VP-I, VP-2, VP-3, NS-I and NS-2. Preferably, the Parvovirus antigen is capsid protein VP-2.

*Delta hepatitis virus (HDV):* Viral antigens may be derived HDV, particularly δ-antigen from HDV (see, e.g., U.S. Patent No. 5,378,814).

*Hepatitis E virus (HEV):* Viral antigens may be derived from HEV.

*Hepatitis G virus (HGV):* Viral antigens may be derived from HGV.

*Human Herpesvirus:* Viral antigens may be derived from a Human Herpesvirus, such as Herpes Simplex Viruses (HSV), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8). Human Herpesvirus antigens may be selected from immediate early proteins (α), early proteins (β), and late proteins (γ). HSV antigens may be derived from HSV-I or HSV-2 strains. HSV antigens may be selected from glycoproteins gB, gC, gD and gH, fusion protein (gB), or immune escape proteins (gC, gE, or gI). VZV antigens may be selected from core, nucleocapsid, tegument, or envelope proteins. A live attenuated VZV vaccine is commercially available. EBV antigens may be selected from early antigen (EA) proteins, viral capsid antigen (VCA), and glycoproteins of the membrane antigen (MA). CMV antigens may be selected from capsid proteins, envelope glycoproteins (such as gB and gH), and tegument proteins

*Papovaviruses:* Antigens may be derived from Papovaviruses, such as Papillomaviruses and Polyomaviruses. Papillomaviruses include HPV serotypes 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 and 65. Preferably, HPV antigens are derived from serotypes 6, 11, 16 or 18. HPV antigens maybe selected from capsid proteins (L1) and *(LT),* or E1 - E7, or fusions thereof. HPV antigens are preferably formulated into virus-like particles (VLPs). Polyomyavirus viruses include BK virus and JK virus. Polyomavirus antigens may be selected from VP1, VP2 or VP3.

Further provided are antigens, compositions, methods, and microbes included in *Vaccines,* 4th Edition (Plotkin and Orenstein ed. 2004); *Medical Microbiology* 4th Edition (Murray et al. ed. 2002); *Virology,* 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology,* 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), which are contemplated in conjunction with the compositions of the present invention.

**Fungal** Antigens

In some embodiments compositions of the present invention are combined with fungal antigens for use in methods of the present invention, including treatment or prevention of mycoses. Fungal antigens for use herein, associated with vaccines include those described in: U.S. Pat. Nos. 4,229,434 and 4,368,191 for prophylaxis

and treatment of trichopytosis caused by Trichophyton mentagrophytes; U.S. Pat. Nos. 5,277,904 and 5,284,652 for a broad spectrum dermatophyte vaccine for the prophylaxis of dermatophyte infection in animals, such as guinea pigs, cats, rabbits, horses and lambs, these antigens comprises a suspension of killed *T. equinum,* T. mentagrophytes (var. granulare), *M. canis* and/or *M. gypseum* in an effective amount optionally combined with an adjuvant; U.S. Pat. Nos. 5,453,273 and 6,132,733 for a ringwoπn vaccine comprising an effective amount of a homogenized, formaldehyde-killed fungi, i.e., *Microsporum canis* culture in a carrier; U.S. Pat. No. 5,948,413 involving extracellular and intracellular proteins for pythiosis. Additional antigens identified within antifungal vaccines include Ringvac bovis LTF-130 and Bioveta.

Further, fungal antigens for use herein may be derived from Dermatophytres, including: *Epidermophytonfloccusum, Microsporum audouini, Microsporum canis, Microsporum distortum, Microsporum equinum, Microsporum gypsum, Microsporum nanum, Trichophyton concentricum, Trichophyton equinum, Trichophyton gallinae, Trichophyton gypseum, Trichophyton megnini, Trichophyton mentagrophytes, Trichophyton quinckeanum, Trichophyton rubrum, Trichophyton schoenleini, Trichophyton tonsurans, Trichophyton verrucosum, T. verrucosum* var. album, var. discoides, var. ochraceum, *Trichophyton violaceum,* and/or *Trichophyton faviforme.*

Fungal pathogens for use as antigens or in derivation of antigens in conjunction with the compositions of the present invention comprise *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Aspergillus sydowi, Aspergillus flavatus, Aspergillus glaucus, Blastoschizomyces capitatus, Candida albicans, Candida enolase, Candida tropicalis, Candida glabrata, Candida krusei, Candida par apsïlosis, Candida stellatoidea, Candida kusei, Candida parakwsei, Candida lusitaniae, Candida pseudotropicalis, Candida guilliermondi, Cladosporium carrionii, Coccidioides immitis, Blastomyces dermatidis, Cryptococcus neoformans, Geotrichum clavatum, Histoplasma capsulatum, Klebsiella pneumoniae, Paracoccidioides brasiliensis, Pneumocystis carinii, Pythiumn insidiosum, Pityrosporum ovale, Sacharomyces cerevisae, Saccharomyces boulardii, Saccharomyces pombe, Scedosporium apiosperum, Sporothrix schenckii, Trichosporon beigelii, Toxoplasma gondii, Penicillium marneffei,* Malassezia spp., Fonsecaea spp., Wangiella spp., Sporothrix spp., Basidiobolus spp., Conidiobolus spp., Rhizopus spp, Mucor spp, Absidia spp, Mortierella spp, Cunninghamella spp, and Saksenaea spp.

Other fungi from which antigens are derived include Alternaria spp, Curvularia spp, Helminthosporium spp, Fusarium spp, Aspergillus spp, Penicillium spp, Monolinia spp, Rhizoctonia spp, Paecilomyces spp, Pithomyces spp, and Cladosporium spp.

Processes for producing a fungal antigens are well known in the art (see US Patent No. 6,333,164). In a preferred method a solubilized fraction extracted and separated from an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that the process comprises the steps of: obtaining living fungal cells; obtaining fungal cells of which cell wall has been substantially removed or at least partially removed; bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; obtaining an insoluble fraction; and extracting and separating a solubilized fraction from the insoluble fraction.

**STD** Antigens

Embodiments of the invention include compositions and methods related to a prophylactic and therapeutic treatments for microbes that can be neutralized prior to infection of a cell. In particular embodiments, microbes (bacteria, viruses and/or fungi) against which the present compositions and methods can be implement include those that cause sexually transmitted diseases (STDs) and/or those that display on their surface an antigen that can be the target or antigen composition of the invention. In a preferred embodiment of the invention, compositions are combined with antigens derived from a viral or bacterial STD. Antigens derived from bacteria or viruses can be administered in conjunction with the compositions of the present invention to provide protection against at least one of the following STDs, among others: chlamydia, genital herpes, hepatitis (particularly HCV), genital warts, gonorrhea, syphilis and/or chancroid (See, WO00/15255).

In another embodiment the compositions of the present invention are co-administered with an antigen for the prevention or treatment of an STD.

Antigens derived from the following viruses associated with STDs, which are described in greater detail above, are preferred for co-administration with the

compositions of the present invention: hepatitis (particularly HCV), HPV, HIV, or HSV.

Additionally, antigens derived from the following bacteria associated with STDs, which are described in greater detail above, are preferred for co-administration with the compositions of the present invention: *Neiserria gonorrhoeae, Chlamydia pneumoniae, Chlamydia trachomatis, Treponemapallidum, ox Haemophilus ducreyi.*

Respiratory **Antigens**

The invention provides methods of preventing and/or treating infection by a respiratory pathogen, including a virus, bacteria, or fungi such as respiratoiy syncytial virus (RSV), PIV, SARS virus, influenza, *Bacillus anthracis,* particularly by reducing or preventing infection and/or one or more symptoms of respiratory virus infection. A composition comprising an antigen described herein, such as one derived from a respiratory virus, bacteria or fungus is administered in conjunction with the compositions of the present invention to an individual which is at risk of being exposed to that particular respiratory microbe, has been exposed to a respiratory microbe or is infected with a respiratory virus, bacteria or fungus. The composition(s) of the present invention is/are preferably co-administered at the same time or in the same formulation with an antigen of the respiratory pathogen. Administration of the composition results in reduced incidence and/or severity of one or more symptoms of respiratory infection.

**Tumor Antigens**

One embodiment of the present involves a tumor antigen or cancer antigen in conjunction with the compositions of the present invention. Tumor antigens can be, for example, peptide-containing tumor antigens, such as a polypeptide tumor antigen or glycoprotein tumor antigens. A tumor antigen can also be, for example, a saccharide-containing tumor antigen, such as a glycolipid tumor antigen or a ganglioside tumor antigen. The tumor antigen can further be, for example, a polynucleotide-containing tumor antigen that expresses a polypeptide-containing tumor antigen, for instance, an RNA vector construct or a DNA vector construct, such as plasmid DNA.

Tumor antigens appropriate for the practice of the present invention

encompass a wide variety of molecules, such as (a) polypeptide-containing tumor antigens, including polypeptides (which can range, for example, from 8-20 amino acids in length, although lengths outside this range are also common), lipopolypeptides and glycoproteins, (b) saccharide-containing tumor antigens, including poly-saccharides, mucins, gangliosides, glycolipids and glycoproteins, and (c) polynucleotides that express antigenic polypeptides.

The tumor antigens can be, for example, (a) full length molecules associated with cancer cells, (b) homologs and modified forms of the same, including molecules with deleted, added and/or substituted portions, and (c) fragments of the same. Tumor antigens can be provided in recombinant form. Tumor antigens include, for example, class I-restricted antigens recognized by CD8+ lymphocytes or class II-restricted antigens recognized by CD4+ lymphocytes.

Numerous tumor antigens are known in the art, including: (a) cancer-testis antigens such as NY-ESO-I, SSX2, SCPI as well as RAGE, BAGE, GAGE and MAGE family polypeptides, for example, GAGE-I, GAGE-2, MAGE-I, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, and MAGE-12 (which can be used, for example, to address melanoma, lung, head and neck, NSCLC, breast, gastrointestinal, and bladder tumors), (b) mutated antigens, for example, p53 (associated with various solid tumors, e.g., colorectal, lung, head and neck cancer), p21/Ras (associated with, e.g., melanoma, pancreatic cancer and colorectal cancer), CDK4 (associated with, e.g., melanoma), MUMl (associated with, e.g., melanoma), caspase-8 (associated with, e.g., head and neck cancer), CIA 0205 (associated with, e.g., bladder cancer), HLA-A2-R1 701, beta catenin (associated with, e.g., melanoma), TCR (associated with, e.g., T-cell non-Hodgkins lymphoma), BCR-abl (associated with, e.g., chronic myelogenous leukemia), triosephosphate isomerase, KIA 0205, CDC-27, and LDLR-FUT, (c) over-expressed antigens, for example, Galectin 4 (associated with, e.g., colorectal cancer), Galectin 9 (associated with, e.g., Hodgkin's disease), proteinase 3 (associated with, e.g., chronic myelogenous leukemia), WT 1 (associated with, e.g., various leukemias), carbonic anhydrase (associated with, e.g., renal cancer), aldolase A (associated with, e.g., lung cancer), PRAME (associated with, e.g., melanoma), HER-2/neu (associated with, e.g., breast, colon, lung and ovarian cancer), alpha-fetoprotein (associated with, e.g., hepatoma), KSA (associated with, e.g., colorectal cancer), gastrin (associated with, e.g., pancreatic and gastric cancer), telomerase catalytic protein, MUC-I (associated with, e.g., breast and ovarian cancer), G-250

(associated with, e.g., renal cell carcinoma), p53 (associated with, e.g., breast, colon cancer), and carcinoembryonic antigen (associated with, e.g., breast cancer, lung cancer, and cancers of the gastrointestinal tract such as colorectal cancer), (d) shared antigens, for example, melanoma-melanocyte differentiation antigens such as MART-1/Melan A, gplOO, MClR, melanocyte-stimulating hormone receptor, tyrosinase, tyrosinase related protein- l/TRPl and tyrosinase related protein-2/TRP2 (associated with, e.g., melanoma), (e) prostate associated antigens such as PAP, PSA, PSMA, PSH-Pl, PSM-Pl, PSM-P2, associated with e.g., prostate cancer, (f) immunoglobulin idiotypes (associated with myeloma and B cell lymphomas, for example), and (g) other tumor antigens, such as polypeptide- and saccharide-containing antigens including (i) glycoproteins such as sialyl Tn and sialyl $Le^x$ (associated with, e.g., breast and colorectal cancer) as well as various mucins; glycoproteins may be coupled to a carrier protein (e.g., MUC-I may be coupled to KLH); (ii) lipopolypeptides (e.g., MUC-I linked to a lipid moiety); (iii) polysaccharides (e.g., Globo H synthetic hexasaccharide), which may be coupled to a carrier proteins (e.g., to KLH), (iv) gangliosides such as GM2, GM12, GD2, GD3 (associated with, e.g., brain, lung cancer, melanoma), which also may be coupled to carrier proteins (e.g., KLH). Additional tumor antigens which are known in the art include p i 5, Hom/Mel-40, H-Ras, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, EBNA, human papillomavirus (HPV) antigens, including E6 and E7, hepatitis B and C virus antigens, human T-cell lymphotropic virus antigens, TSP-1 80, pl85erbB2, pl80erbB-3, c-met, mn-23Hl, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, p 16, TAGE, PSCA, CT7, 43-9F, 5T4, 791 Tgp72, beta-HCG, BCA225, BTAA, CA 125, CA 15-3 (CA 27.29\BCAA), CA 195, CA 242, CA-50, CAM43, CD68\KP1, CO-029, FGF-5, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-Ag, MOVl 8, NB/70K, NY-CO-I, RCASl, SDCCAG 16, TA-90 (Mac-2 binding proteMcyclophilin C-associated protein), TAAL6, TAG72, TLP, TPS, and the like. These as well as other cellular components are described for example in United States Patent Application 20020007173 and references cited therein.

Polynucleotide-containing antigens in accordance with the present invention typically comprise polynucleotides that encode polypeptide cancer antigens such as those listed above. Preferred polynucleotide-containing antigens include DNA or RNA vector constructs, such as plasmid vectors (e.g., pCMV), which are capable of expressing polypeptide cancer antigens *in vivo*.

Tumor antigens may be derived, for example, from mutated or altered cellular components. After alteration, the cellular components no longer perform their regulatory functions, and hence the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras, p53, Rb, altered protein encoded by the Wilms' tumor gene, ubiquitin, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor. These as well as other cellular components are described for example in U.S. Patent No. 5,693,522 and references cited therein.

Additionally, bacterial and viral antigens, may be used in conjunction with the compositions of the present invention for the treatment of cancer. In particular, carrier proteins, such as $CRMi_{97}$, tetanus toxoid, or *Salmonella typhimurium* antigen can be used in conjunction/conjugation with compounds of the present invention for treatment of cancer. The cancer antigen combination therapies will show increased efficacy and bioavailability as compared with existing therapies.

Additional information on cancer or tumor antigens can be found, for example, in

Moingeon P, "Cancer vaccines," Vaccine, 2001, 19:1305-1326; Rosenberg SA, "Progress in human tumor immunology and immunotherapy," Nature, 2001, 411:380-384; Dermine, S. et al, "Cancer Vaccines and Immunotherapy," British Medical Bulletin, 2002, 62, 149-162; Espinoza-Delgado L, "Cancer Vaccines," The Oncologist, 2002, 7(suppl3):20-33; Davis, LD. et al., "Rational approaches to human cancer immunotherapy," Journal of Leukocyte Biology, 2003, 23: 3-29; Van den Eynde B, et al., "New tumor antigens recognized by T cells," Curr. Opin. Immunol., 1995, 7:674-81; Rosenberg SA, "Cancer vaccines based on the identification of genes encoding cancer regression antigens, Immunol. Today, 1997, 18: 175-82; Offringa R et al., "Design and evaluation of antigen-specific vaccination strategies against cancer," Current Opin. Immunol., 2000, 2:576-582; Rosenberg SA, "A new era for cancer immunotherapy based on the genes that encode cancer antigens," Immunity, 1999, 10:281-7; Sahin U et al., "Serological identification of human tumor antigens," Curr. Opin. Immunol., 1997, 9:709-16; Old LJ et al., "New paths in human cancer serology," J. Exp. Med., 1998, 187:1 163-7; Chaux P, et al., "Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes," J. Exp.

Med., 1999, 189:767-78; GoldP, et al., "Specific carcinoembryonic antigens of the human digestive system," J. Exp. Med., 1965, 122:467-8; Livingston $PO_3$ et al., Carbohydrate vaccines that induce antibodies against cancer: Rationale," Cancer Immunol. Immunother., 1997, 45:1-6; Livingston PO, et al., Carbohydrate vaccines that induce antibodies against cancer: Previous experience and future plans," Cancer Immunol. Immunother., 1997, 45:10-9; Taylor-Papadimitriou J, "Biology, biochemistry and immunology of carcinoma-associated mucins," Immunol. Today, 1997, 18:105-7; Zhao X-J et al., "GD2 oligosaccharide: target for cytotoxic T lymphocytes," J. Exp. Med., 1995, 182:67-74; Theobald M, et al., "Targeting p53 as a general tumor antigen," Proc. Natl. Acad. Sci. USA, 1995, 92:1 1993-7; Gaudernack G, "T cell responses against mutant ras: a basis for novel cancer vaccines," Immunotechnology, 1996, 2:3-9; WO 91/02062; U.S. Patent No. 6,015,567; WO 01/08636; WO 96/30514; U.S. Patent No. 5,846,538; and U.S. Patent No. 5,869,445.

**Pediatric/Geriatric Antigens**

In one embodiment the compositions of the present invention are used in conjunction with an antigen for treatment of a pediatric population, as in a pediatric antigen. In a more particular embodiment the pediatric population is less than about 3 years old, or less than about 2 years, or less than about 1 years old. In another embodiment the pediatric antigen (in conjunction with the composition of the present invention) is administered multiple times over at least 1, 2, or 3 years.

In another embodiment the compositions of the present invention are used in conjunction with an antigen for treatment of a geriatric population, as in a geriatric antigen.

**Other Antigens**

Other antigens for use in conjunction with the compositions of the present include hospital acquired (nosocomial) associated antigens.

In another embodiment, parasitic antigens are contemplated in conjunction with the compositions of the present invention. Examples of parasitic antigens include those derived from organisms causing malaria and/or Lyme disease.

In another embodiment, the antigens in conjunction with the compositions of the present invention are associated with or effective against a mosquito born illness. In another embodiment, the antigens in conjunction with the compositions of the

present invention are associated with or effective against encephalitis. In another embodiment the antigens in conjunction with the compositions of the present invention are associated with or effective against an infection of the nervous system.

In another embodiment, the antigens in conjunction with the compositions of the present invention are antigens transmissible through blood or body fluids.

**Antigen Formulations**

In other aspects of the invention, methods of producing microparticles having adsorbed antigens are provided. The methods comprise: (a) providing an emulsion by dispersing a mixture comprising (i) water, (ii) a detergent, (iii) an organic solvent, and (iv) a biodegradable polymer selected from the group consisting of a poly(α-hydroxy acid), a polyhydroxy butyric acid, a polycaprolactone, a polyorthoester, a polyanhydride, and a polycyanoacrylate. The polymer is typically present in the mixture at a concentration of about 1% to about 30% relative to the organic solvent, while the detergent is typically present in the mixture at a weight-to-weight detergent-to-polymer ratio of from about 0.00001:1 to about 0.1:1 (more typically about 0.0001:1 to about 0.1:1, about 0.001:1 to about 0.1:1, or about 0.005:1 to about 0.1:1); (b) removing the organic solvent from the emulsion; and (c) adsorbing an antigen on the surface of the microparticles. In certain embodiments, the biodegradable polymer is present at a concentration of about 3% to about 10% relative to the organic solvent.

Microparticles for use herein will be formed from materials that are sterilizable, non-toxic and biodegradable. Such materials include, without limitation, poly(α-hydroxy acid), polyhydroxybutyric acid, polycaprolactone, polyorthoester, polyanhydride, PACA, and polycyanoacrylate. Preferably, microparticles for use with the present invention are derived from a poly(α-hydroxy acid), in particular, from a poly(lactide) ("PLA") or a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly(D,L-lactide-co-glycolide) ("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials which have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide ratios, the selection of which will be largely a matter of choice, depending in part on the coadministered macromolecule. These parameters are discussed more fully below.

Further antigens may also include an outer membrane vesicle (OMV) preparation.

Additional formulation methods and antigens (especially tumor antigens) are provided in U.S. Patent Serial No. 09/581,772.

Antigen References

The following references include antigens useful in conjunction with the compositions and methods of the present invention:

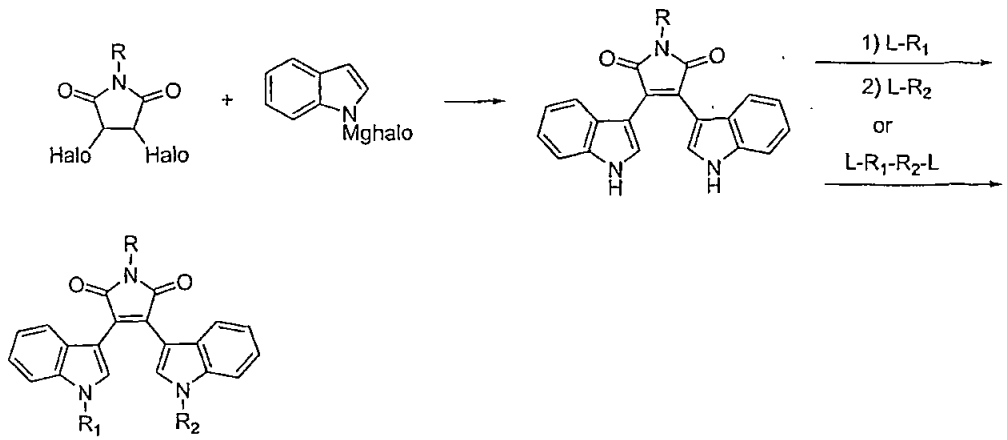

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37 Sutter et al. (2000) Pediatr Clin North Am 47:287-308.
38 Zimmerman & **Spam** (1999) Am Fan Physician 59:113-118, 125-126.
39 Dreensen (1997) Vaccine 15 Suppl"S2-6.
40 MMWR Morb Mortal Wkly rep 1998 Jan 16:47(1):12, 9.
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59 European patent application 0427347.
60 International patent application WO93/17712.
61 International patent application WO98/58668.
62 European patent application 0471 177.
63 International patent application WO00/56360.
64 International patent application WO00/67 161.

**[00448]** It is contemplated that the invention encompasses all possible combinations of the embodiments described herein.

## EXAMPLES

### Preparation of Quinazolines

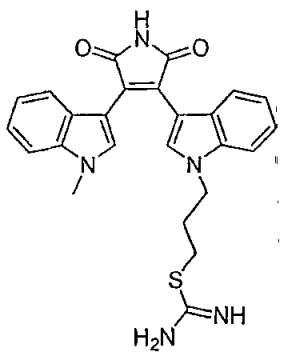
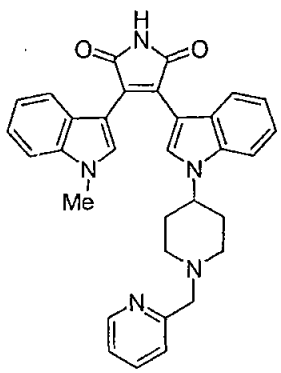
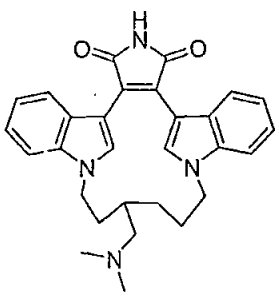


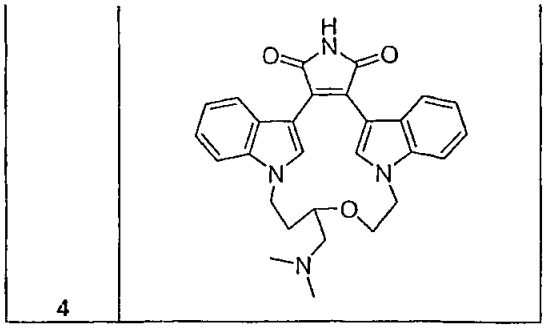

[00449] In the above reaction, halo is preferably chloro, bromo, or iodo. The reaction is a Grignard, carried out in an inert organic solvent, such as toluene, at a temperature between room temperature and the reflux temperature of the reaction mixture. Most significantly, the reaction is dependent on solvent conditions. When carried out in a Toluene:THF:ether solvent system, the reaction provides the product in high yield. The product is precipitated from the reaction mixture with ammonium chloride ($NH_4Cl$). The resulting bis-3,4(3'-indolyl)-lN-pyrrole-2,5-dione product, may be isolated by standard techniques.

[00450] In the next step, L is a good leaving group such as chloro, bromo, iodo, mesyl, tosyl, and the like. L may also be a hydroxy or other precursor that may be readily converted to a good leaving group by techniques known in the art. For example, the hydroxy may be readily converted to a sulfonic ester such as mesyl by reacting the hydroxy with methanesulfonyl chloride to produce the mesylate leaving group. The reaction is accomplished by any of the known methods of preparing N-substituted indoles. This reaction usually involves approximately equimolar amounts of the two reagents, although other ratios, especially those wherein the alkylating reagent is in excess, are operative. The reaction is best carried out in a polar aprotic solvent employing an alkali metal salt or other such alkylation conditions as are appreciated in the art. When the leaving group is bromo or chloro, a catalytic amount of iodide salt, such as potassium iodide may be added to speed the reaction. Reaction conditions include the following: Potassium hexamethyldisilazide in dimethylformamide or tetrahydrofuran, sodium hydride in dimethylformamide.

[00451] Preferably, the reaction is carried out under slow reverse addition with cesium carbonate in either acetonitrile, dimethylformamide (DMF), or tetrahydrofuran (THF). The temperature of the reaction is preferably from about ambient temperature to about the reflux temperature of the reaction mixture.

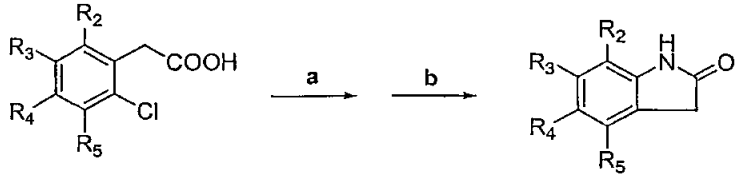
**Table 1:** 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione compounds

| Example | Structure |
|---|---|
| 1 |  |
| 2 |  |
| 3 |  |



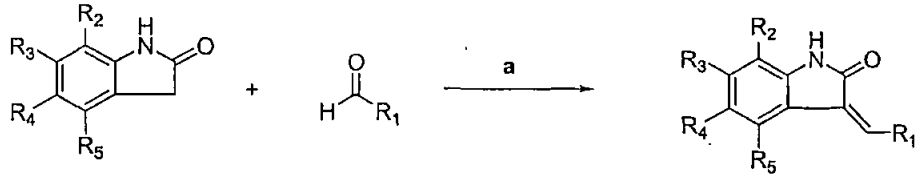

**Preparation of Indolinones**

Scheme 1:




[00452] Scheme 1 is performed as a one pot procedure, with reagents in step **a** being $NH_4OH$, CuCl in $H_2O$. Followed by addition of aq. HCl in step b. $R_2$-$R_5$ are as defined herein.

Scheme 2:

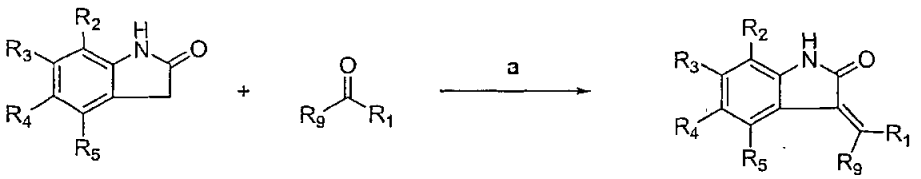



**[00453]** In Scheme 2, the reagents are stirred in EtOH in the presence of piperidine (a) to afford the final product. As will be apparent to a skilled artisan, the reaction may be heated to enhance the yield, depending on reactivity of the particular starting materials.

Scheme 3:



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[00454] In Scheme 3, the reagents are refluxed in EtOH in the presence of NaOBu-t (a) to afford the final product. $R_9$ as shown in scheme 3 is H, -OH, -CN, alkyl, aryl, heterocyclyl, alkoxy, or $-NR_aR_b$ as defined herein. It is contemplated that the above structure may replace Formula I to allow substitution at Rg, whereby all other substituents are as defined herein.

Scheme 4 (Synthesis of Example 1):

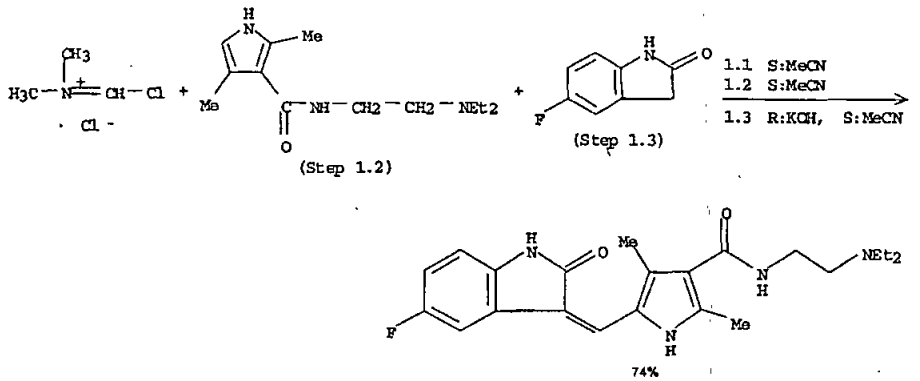
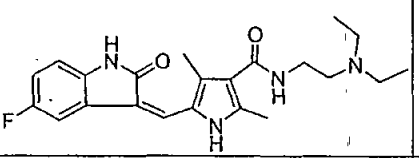
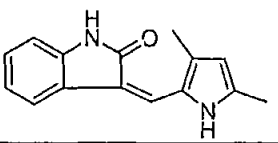
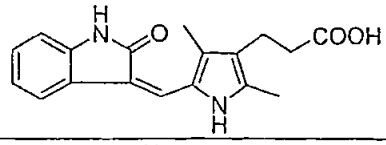
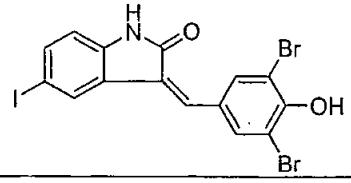
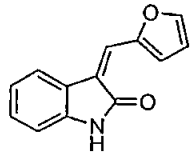
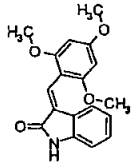
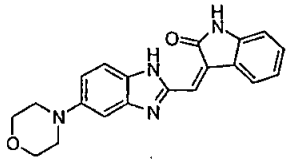



Table 1: Indolinone Compounds

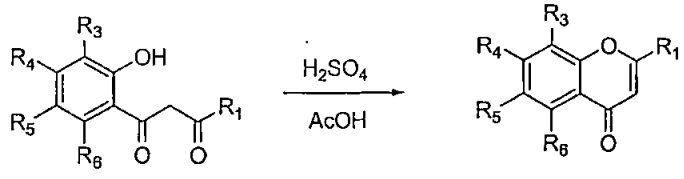
| Example | Name | Structure | Reference |
|---|---|---|---|
| 1 | N-(2-(dimethylamino)ethyl)-5-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2,4-diethyl-1H-pyrrole-3-carboxamide |  | WO 01/60814 |
| 2 | 3-((3,5-dimethyl-1H-pyrrol-2-yl)methylene)indolin-2-one |  | US 5,883,113<br>WO 99/61422 |

| | | | |
|---|---|---|---|
| 3 | 3-(2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl)-1H-pyrrol-3-yl)propanoic acid |  | US 5,883,113 WO 99/61422 |
| 4 | 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodoindolin-2-one |  | Lackey, K., et al. 2000 *Bioorg. Med. Chem. Lett.* 10. 223 |

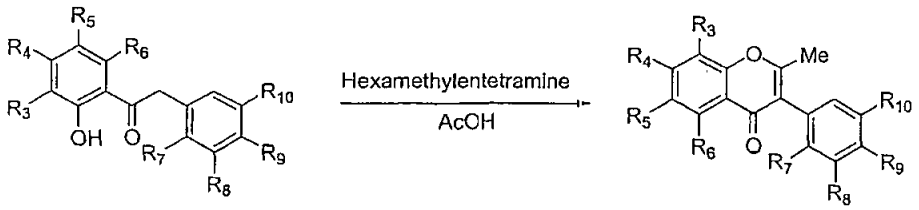
| Example | Name | Structue | MH+ |
|---|---|---|---|
| 5 | 3-((furan-2-yl)methylene)indolin-2-one |  | 212.2 |
| 6 | 3-(2,4,6-trimethoxybenzylidene)indolin-2-one |  | 312.3 |
| 7 | 3-((5-morpholino-1H-benzo[d]imidazol-2-yl)methylene)indolin-2-one |  | 347.4 |

[00455] Preparation of Example Chromen-4-ones

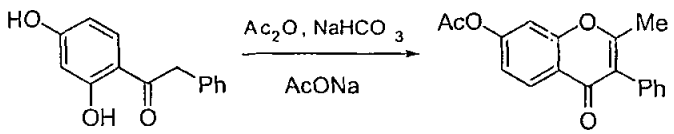
Scheme 1: Synthesis of general flavones



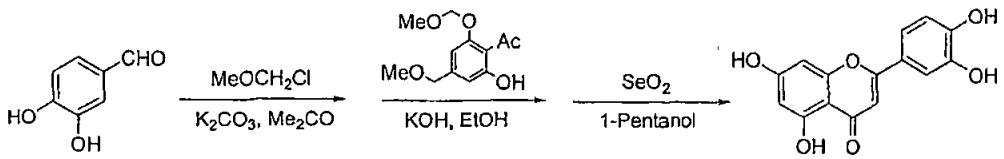

Scheme 2: Synthesis of general isoflavones




**Scheme 3**: Synthesis of 2-methyl-4-oxo-3-phenyl-4H-chromen-7-yl acetate

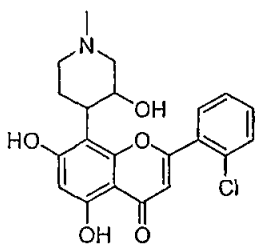
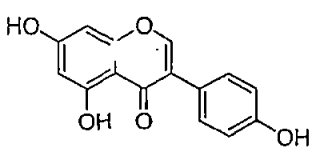



**Scheme 4**: Synthesis of 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one




**[00456]** Further, it will become apparent to one skilled in the art that many chromen-4-ones, particularly flavones and isoflavones, are commercially available, including many of the Examples disclosed herein, as well as starting materials that can be functionalized to arrive at the desired final product.

**Table 1**: Chromen-4-one SMIPs

| Example | Structure | Name | Patent |
|---|---|---|---|
| 1 |  | 2-(2-chlorophenyl)-2,3-dihydro-5,7-dihydroxy-8-(3-hydroxy-1-methylpiperidin-4-yl)chromen-4-one | WO 97/42949 WO 98/13444 |
| 2 |  | 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one | US 5,554,519 |

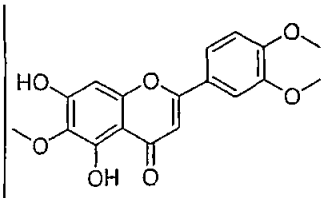
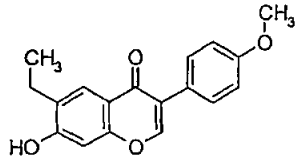
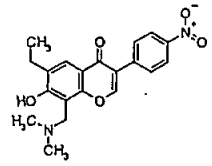
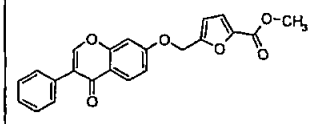
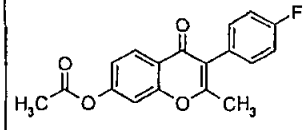
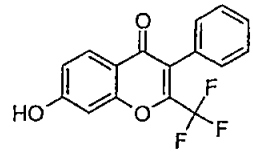
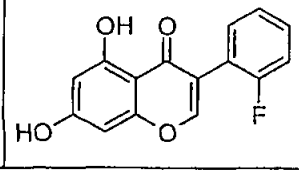
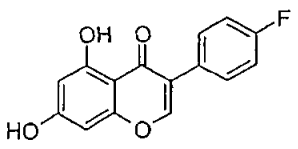
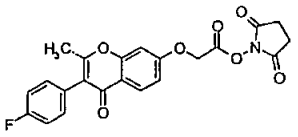
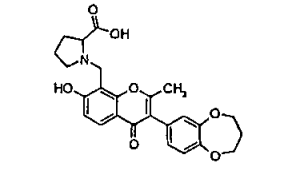
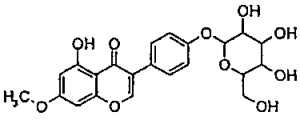
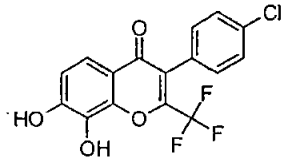
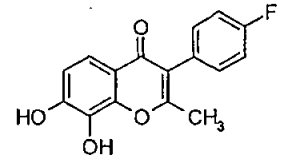
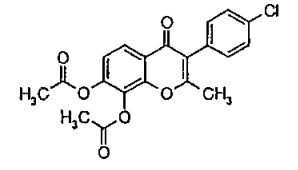
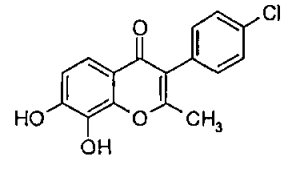
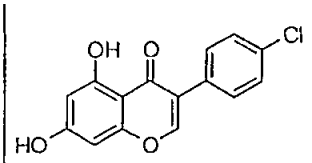
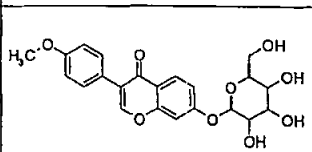
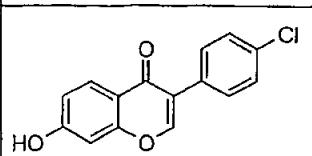
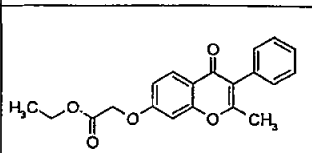
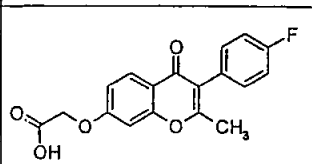
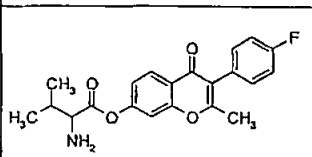
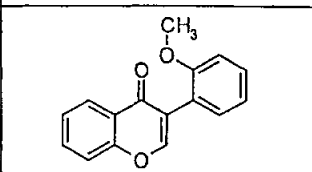
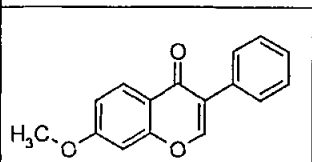
| | | | |
|---|---|---|---|
| 3 |  | 2,3-dihydro-5,7-dihydroxy-6-methoxy-2-(3,4-dimethoxyphenyl)chromen-4-one | WO 98/04541 US 6,025,387 |

Table 2: Examples 4-49 3-phenylchromen-4-one (Isoflavone) SMIPs

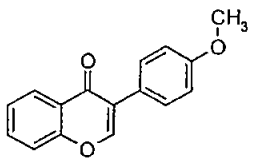
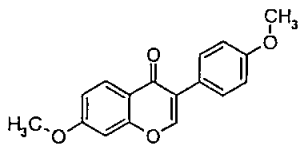
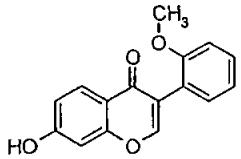
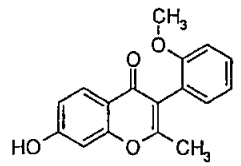
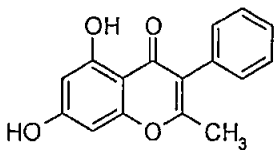
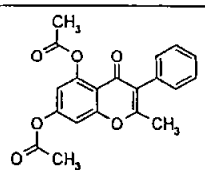
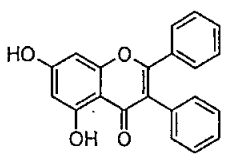
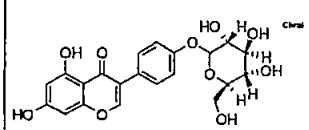
| Example | Structure | MH+ |
|---|---|---|
| 4 |  | 297.3 |
| 5 |  | 369.4 |
| 6 |  | 377.4 |
| 7 |  | 313.3 |
| 8 |  | 307.2 |
| 9 |  | 273.2 |

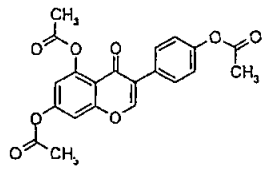
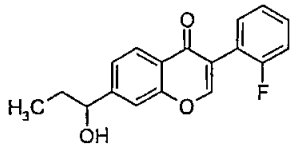
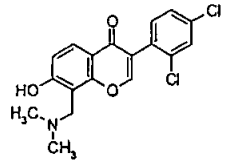
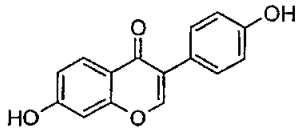
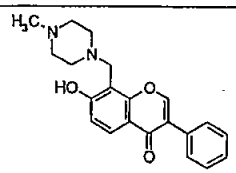
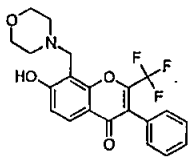
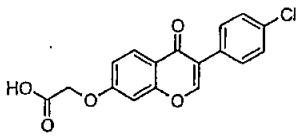
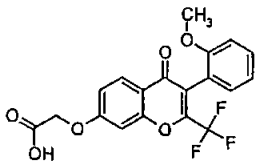
| | | |
|---|---|---|
| 10 |  | 273.2 |
| 11 |  | 426.4 |
| 12 |  | 452.5 |
| 13 |  | 447.4 |
| 14 |  | 357.7 |
| 15 |  | 287.3 |
| 16 |  | 387.8 |
| 17 |  | 303.7 |

| | | |
|---|---|---|
| 18 |  | 289.7 |
| 19 |  | 431.4 |
| 20 |  | 273.7 |
| 21 |  | 339.4 |
| 22 |  | 329.3 |
| 23 |  | 370.4 |
| 24 |  | 253.3 |
| 25 |  | 253.3 |



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| | | |
|---|---|---|
| 26 |  | 253.3 |
| 27 |  | 283.3 |
| 28 |  | 269.3 |
| 29 |  | 283.3 |
| 30 |  | 269.3 |
| 31 |  | 353.3 |
| 32 |  | 331.3 |
| 33 |  | 433.4 |

| | | |
|---|---|---|
| 34 |  | 397.3 |
| 35 |  | 299.3 |
| 36 |  | 365.2 |
| 37 |  | 255.2 |
| 38 |  | 351.4 |
| 39 |  | 406.4 |
| 40 |  | 331.7 |
| 41 |  | 395.3 |



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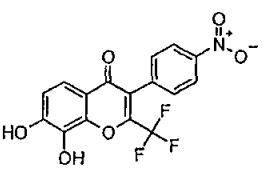
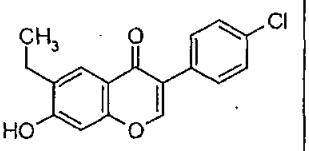
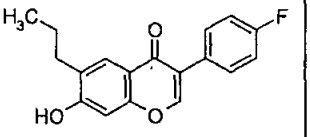
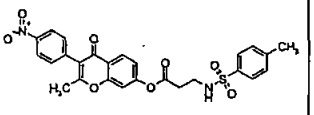
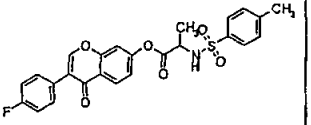
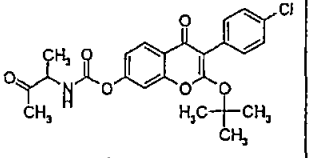
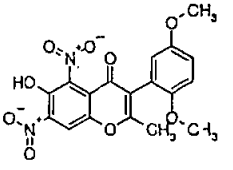
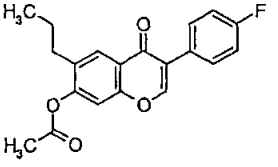
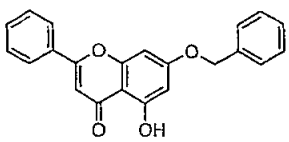
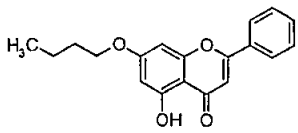
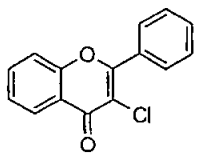
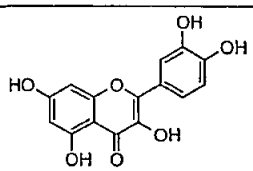
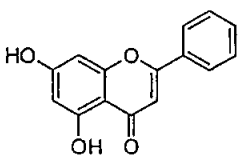
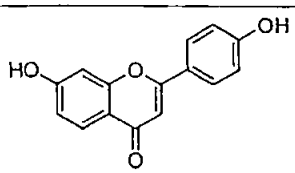
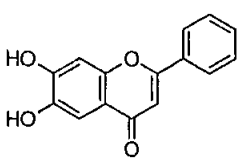
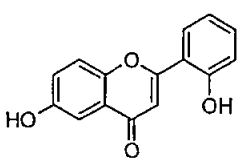
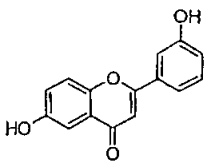
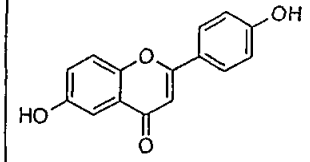
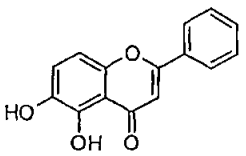
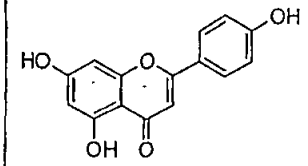
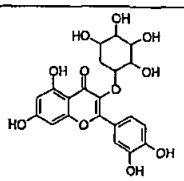
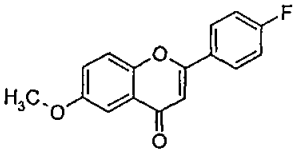
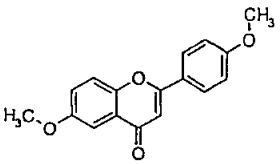
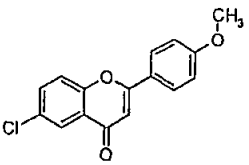
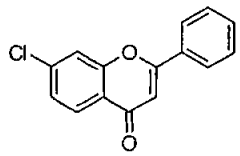
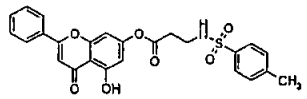
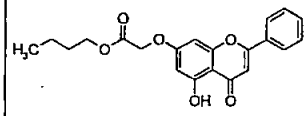
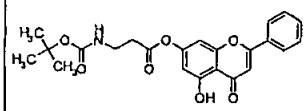
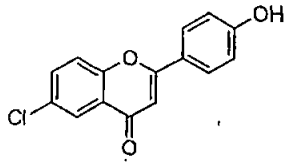
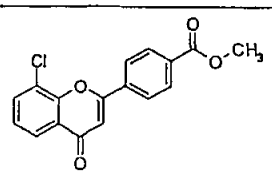
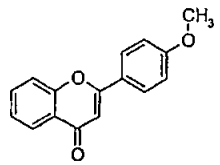
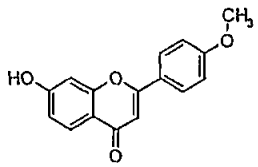
| | | |
|---|---|---|
| 42 |  | 368.2 |
| 43 |  | 301.7 |
| 44 |  | 299.3 |
| 45 |  | 523.5 |
| 46 |  | 482.5 |
| 47 |  | 458.9 |
| 48 |  | 403.3 |
| 49 |  | 341.3 |

Table 3: Examples 50-100 2-Phenylchromen-4-one (Flavone) SMIPs

| Example | Structure | MH+ |
|---|---|---|

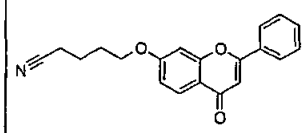
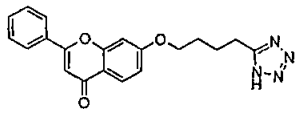
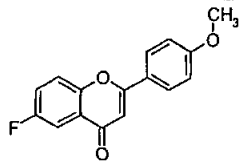
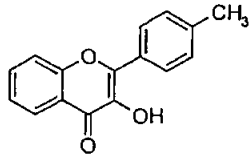
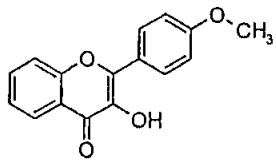
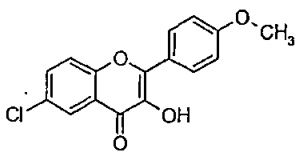
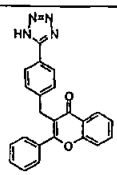
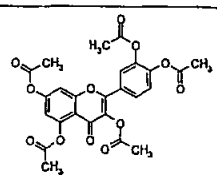
| | | |
|---|---|---|
| 50 |  | 345.4 |
| 51 |  | 311.3 |
| 52 |  | 257.7 |
| 53 |  | 303.2 |
| 54 |  | 255.2 |
| 55 |  | 255.2 |
| 56 |  | 255.2 |
| 57 |  | 255.2 |

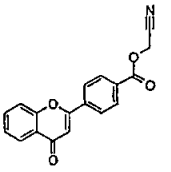
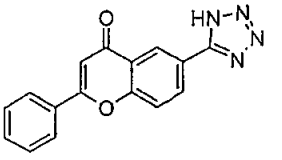
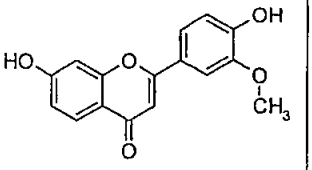
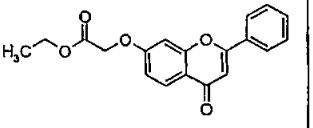
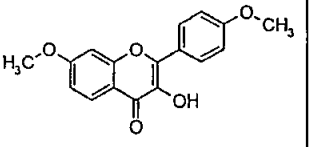
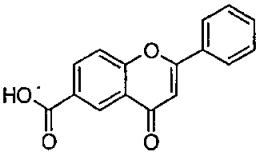
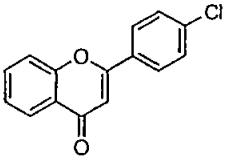
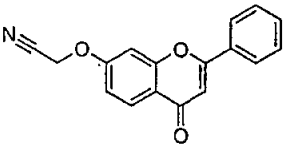
| | | |
|---|---|---|
| 58 |  | 255.2 |
| 59 |  | 255.2 |
| 60 |  | 255.2 |
| 61 |  | 271.2 |
| 62 |  | 449.4 |
| 63 |  | 271.3 |
| 64 |  | 283.3 |
| 65 |  | 287.7 |

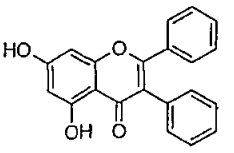
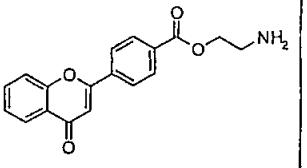
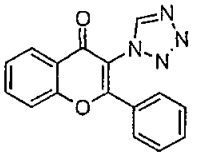
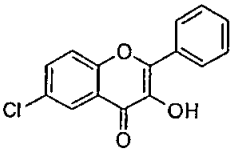
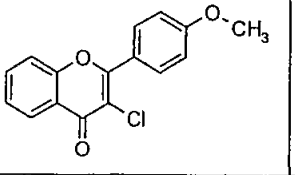
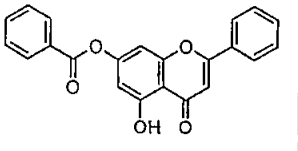
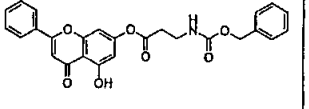
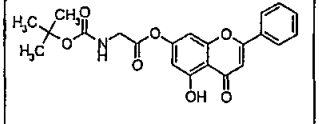
| | | |
|---|---|---|
| 66 |  | 257.7 |
| 67 |  | 480.5 |
| 68 |  | 369.4 |
| 69 |  | 426.4 |
| 70 |  | 273.7 |
| 71 |  | 315.7 |
| 72 |  | 253.3 |
| 73 |  | 269.3 |

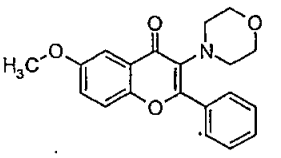
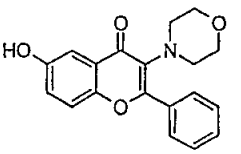
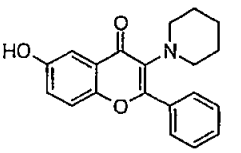


223

| | | |
|---|---|---|
| 74 |  | 320.4 |
| 75 |  | 363.4 |
| 76 |  | 271.3 |
| 77 |  | 253.3 |
| 78 |  | 269.3 |
| 79 |  | 303.7 |
| 80 |  | 381.4 |
| 81 |  | 513.4 |

| | | |
|---|---|---|
| 82 |  | 306.3 |
| 83 |  | 291.3 |
| 84 |  | 285.3 |
| 85 |  | 325.3 |
| 86 |  | 299.3 |
| 87 |  | 267.3 |
| 88 |  | 257.7 |
| 89 |  | 278.3 |

| | | |
|---|---|---|
| 90 |  | 331.3 |
| 91 |  | 310.3 |
| 92 |  | 291.3 |
| 93 |  | 273.7 |
| 94 |  | 287.7 |
| 95 |  | 359.3 |
| 96 |  | 460.5 |
| 97 |  | 412.4 |

| | | |
|---|---|---|
| 98 |  | 338.4 |
| 99 |  | 324.3 |
| 100 |  | 322.4 |

**[00457]** **Preparation of Derivatized** Pyridazines

**[00458]**

**[00459]** Preparation of the following Examples 60-68 were described in US 6,258,812, which also includes other reaction schemes that may be helpful in synthesizing the compounds of the present invention.

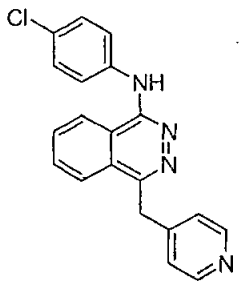
Example 60:

**[00460]** 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine dihydrochloride

**[00461]** A mixture of 15.22 g (59.52 mmol) 1-chloro-4-(4-pyridylmethyl)phthalazine (for preparation see German Auslegeschriftno. 1 061 788 published Jul. 23, 1959]), 7.73 g (60.59 mmol) 4-chloroaniline and 200 ml 1-butanol is heated for 2 h under reflux. The crystallizate which is obtained when the mixture slowly cools to 5° C. is then filtered off and washed with 1-butanol and ether. The filter residue is dissolved in about 200 ml hot methanol, the solution is treated with 0.75 g activated carbon and filtered Via a Hyflo Super Cel, and the pH of the filtrate is adjusted to about 2.5 with 7 ml 3N methanolic HCl. The filtrate is evaporated to about half the original volume and ether added until slight turbidity occurs; cooling then leads to the precipitation of crystals. The crystallizate is filtered off, washed with a mixture of methanol/ether (1:2) as well as ether, dried for 8 h at 110° C. under HV, and equilibrated for 72 h at 20° C. and in room atmosphere. In this way, the title

compound is obtained with a water content of 8.6%; m.p. >270° C ; $^1$H NMR (DMSO-d 6 ) 11.05-12.20 (br), 9.18-9.23 (m, IH), 8. 88 (d, 2H), 8.35-8.40 (m, IH), 8.18-8.29 (m, 2H), 8.02 (d, 2H), 7.73 (d, 2H), 7.61 (d, 2H), 5.02 (s, 2H); ESI-MS: $(M+H)^+$=347.

Example 61:




**[00462]** 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine hydrochloride

**[00463]** A mixture of 0.972 g (3.8 mmol) 1-chloro-4-(4- pyridylmethyl)phthalazine, 0.656 g (4 mmol) 4-chloroaniline hydrochloride (Research Organics, Inc., Cleveland, Ohio, USA) and 20 ml ethanol is heated for 2 h under reflux. The reaction mixture is cooled in an ice bath, filtered, and the crystallizate washed with a little ethanol and ether. After drying under HV for 8 h at 110° C. and for 10 h at 150° C , the title compound is obtained as a result of thermal removal of HCl; m.p. >270° C ; ' H NMR (DMSO-d 6 ) 9.80-11.40 (br), 8.89-8.94 (m, IH), 8.67 (d, 2H), 8.25-8.30 (m, IH), 8.06-8.17 (m, 2H), 7.87 (d, 2H), 7. 69 (d, 2H), 7.49 (d, 2H), 4.81 (s, 2H); ESI-MS: $(M+H)^+$=347.

Example 62

**[00464]** 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine hydrochloride

**[00465]** A mixture of 1.28 g (5 mmol) 1-chloro-4-(4-pyridylmethyl)phthalazine, 0.67 g (5.25 mmol) 4-chloroaniline and 15 ml 1-butanol is heated for 0.5 h at 100 C while stirring in a nitrogen atmosphere. The mixture is then cooled to RT, filtered, and the filtrate washed with 1-butanol and ether. For purification, the crystallizate is dissolved in 40 ml of hot methanol, the solution treated with activated carbon, filtered via Hyflo Super Cel, and the filtrate evaporated to about half its original volume, resulting in the formation of a crystalline precipitate. After cooling to 0° C , filtration, washing of the filter residue with ether, and drying under HV for 8 h at 130° C , the title compound is

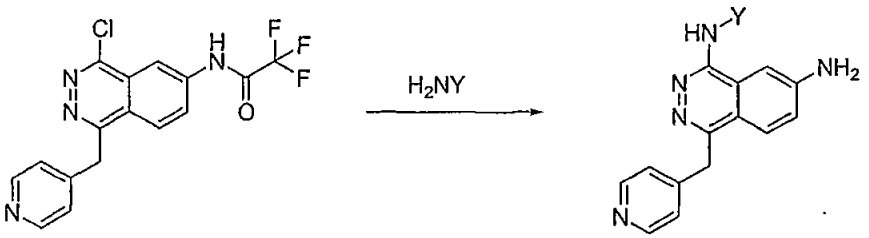
obtained; m.p. >270° C ; $^1$H NMR (DMSO-d 6 ) 9.80-1 1.40 (br), 8.89-8.94 (m, IH), 8.67 (d, 2H)$_5$ 8.25-8.30 (m, IH), 8.06-8.17 (m, 2H), 7.87 (d, 2H), 7.69 (d, 2H)$_5$ 7. 49 (d, 2H), 4.81 (s, 2H); ESI-MS: $(M+H)^+$=347.

Example 63

**[00466]** 1-(4-Chloroanilino)-4-(4-pyridylmethyl)phthalazine

**[00467]** A mixture of 14.19 g (0.1 mol) phosphorus pentoxide, 13.77 g (0.1 mol) triethylamine hydrochloride and 12.76 g (0.1 mol) 4-chloroaniline is heated and stirred in a nitrogen atmosphere at 200° C. until a homogeneous melt has formed (about 20 min). To the melt, 5.93 g (0.025 mol) 4-(4-pyridylmethyl)-1 (2H)-phthalazinone (for preparation see German Auslegeschrift no. 1 061 788 [published 23.07.1959]) is added, and the reaction mixture is stirred for 3 h at 200° C. After the reaction mixture has cooled to about 100° C , 200 ml of water is added. Stirring is continued until the temperature reaches about 30° C , and then 20 ml cone. ammonia (30% aqueous ammonium hydroxide solution) and 900 ml chloroform are added consecutively. As soon as a diphasic mixture has formed, the organic phase is separated off, dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated on a RE to a volume of about 50 ml, to which 100 ml acetate is then added, and the mixture is cooled in an ice bath. The crystallizate obtained is filtered off and washed with acetate and ether. After recrystallization from methanol and drying under HV for 8 h at 120° C , the title compound is obtained; m.p. 194-195° C ; ESI-MS: $(M+H)^+$=347.

Example 64




**[00468]** A: 7-Amino- 1-(4-chloroanilino)-4-(4-pyridylmethyl)phthalazine hydrochloride

**[00469]** Under exclusion of air, 381 mg (0.77 mmol) 7-trifluoroacetamino-l- chloro-4-(4-pyridylmethyl)phthalazine is heated to 100° C for 5 h in 3.1 ml n-butanol with

295 mg (2.31 mmol) 4-chloroaniline. After cooling, the dark suspension is filtered off, washed with n-butanol and ethanol, and dried to obtain title compound: m.p.: >300° C ; HPLC: t Ret (Grad 5-40 )=12.9; FAB MS (M+H) $^+$=362. With DIPE, further product may be precipitated out from the filtrate. The starting material is prepared as follows:

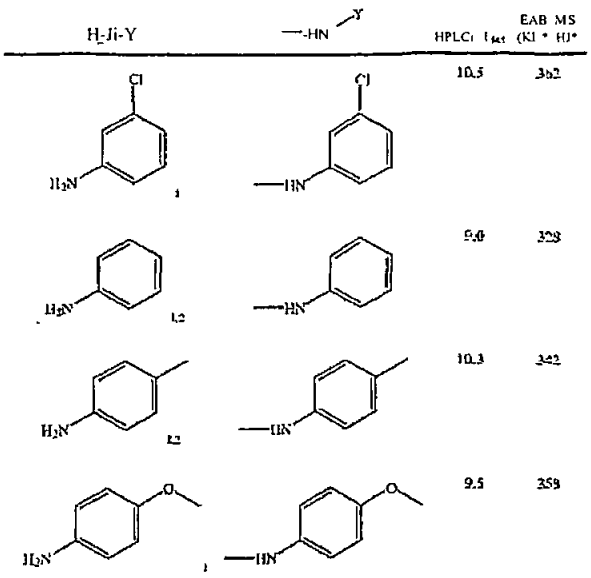
**[00470]** 64A. 1) 7-Trifluoroacetamino-4-(4-pyridylmethyl)-l(2H)-phthalazinone hydrochloride

**[00471]** A suspension of 500 mg (1.98 mmol) 7-amino-4-(4-pyridylmethyl)-l(2H)-phthalazinone [=l-oxo-4-[pyridyl-(4')-methylq-7-amino-l,2- dihydrophthalazine (for preparation see German Auslegeschrft no. 1061788 [published 23. 7.1959]) in 1.65 ml (11.88 mmol) trifiuoroacetic acid anhydride is stirred over the weekend at RT. Addition of water and sonication yield a suspension which can be filtered and washed out with water. The crystals are suspended in 15 ml acetic acid. When 2.47 ml of a 2.4 M solution of HCl in dioxane is added, the suspension dissolves, and scraping eventually leads to renewed crystallization. Filtering and washing with ethyl acetate yield the title compound; HPLC: t Ret (Grad 5- 40 )=1 1.3; FAB MS (M+H) $^+$=349.

**[00472]** 64A.2) 7-Trifluoroacetamino- 1-chloro-4-(4-pyridylmethyl)phthalazme

**[00473]** Under $N_2$ atmosphere, 552 mg (1.44 mmol) 7-trifiuoroacetamino-4-(4-pyridylmethyl)-l (2H)-phthalazinone hydrochloride is added to 4.2 ml acetonitrile 0.328 ml (3.58 mmol) phosphoroxychloride and heated for 4 h to 100° C. This is then cooled to 10° C , and 1.4 g $NaHCO_3$ in 7 ml water is added. After ethyl acetate is added, a reddish suspension forms which is filtered and washed out. Drying under HV yields the title compound: HPLC: t Ret (Grad 5-40 )=12.1; FAB MS (M+H) $^+$=367.

**[00474]** The following compounds are prepared in the same manner:



| H-Ji-Y | —HN—Y | HPLC | EAB MS (M+H)+ |
|---|---|---|---|
| | | 10.5 | 362 |
| | | 9.0 | 328 |
| | | 10.3 | 342 |
| | | 9.5 | 358 |

Example **65**

**[00475]** 5-(4-Chloroanilino)-8-(4-pyridylmethyl)pyrido[2.3-d]pyridazine

**[00476]** Under $N_2$ atmosphere, a mixture of 1.19 g (8.38 mmol) phosphorus pentoxide, 1.156 g (8.4 mmol) triethylamine hydrochloride, and 1.072 g (8. 4 mmol) 4-chloroaniline is heated for 5 min to 200° C. Then 0.50 g (2.1 mmol) 8-(4-pyridylmethyl)-.6H.-pyrido[2,3-.d.]pyridazin-5one is added to the melt, and this is stirred for 3 h at 200° C. After cooling, the melt is taken up in 25 ml dichloromethane, 10 ml water, and 5 ml sat. NH 3 solution, and the organic phase is separated off, dried (Na 2 SO 4 ), and concentrated by evaporation. Column chromatography ($SiO_2$; acetate/ClH-jOH 50: l→ 25:1) and crystallization from acetonitrile/methanol yields the title compound: m.p.: 220-222° C ; Anal. calc. C 65.61%, H 4.06%, N 20.14%; found C 65.7%, H 4.1%, N 20.1%; FAB MS $(M+H)^+$=348.

The starting material is prepared as follows:

**[00477]** 65.1) 6-(Pyridin-4-yl)-l pyridin-5,7-dione

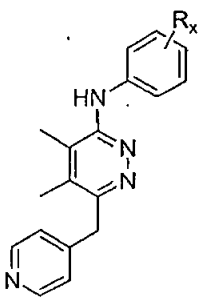
**[00478]** To a suspension of 20.27 g (150 mmol) furo[3,4-b]pyridin-5(7h)-one (for preparation see Synthesis 1997,113) and 14.13 ml (150 mmol) 4-pyridinecarbaldehyde in 120 ml methanol and 75 ml ethyl propionate, 27.8 ml (150 mmol) of a 5.4M solution of sodium methylate in methanol is added dropwise under ice cooling (and $N_2$ atmosphere). The mixture is heated for 15 min to RT and then for 2 h to reflux temperature. The suspension temporarily goes into solution before a solid

forms again. After cooling, 120 ml water is added, before stirring, filtering and washing the product with water. Further product is obtainable from the filtrate by acidification with acetic acid: FAB MS $(M+H)^+$=225.

**[00479]** **65.2)** 8-(4-Pyridylmethyl)-.6H.-pyrido[2,3-.d.]pyridazin-5-one **(A)** and **5-(4-**pyridylmethyl)-.7H.-pyrido[2,3-.d.]pyridazin-8-one **(B)**

**[00480]** A suspension of 8.7 g (38.8 mmol) 6-(pyridin-4-yl)-[ll]pyridin-5,7- dione in 40 ml hydrazine hydrate is heated for 4 h to reflux. The suspension goes into solution temporarily, then once again a solid precipitates out, which is filtered off after cooling to RT, washed with water and ether, and dried. Fractionated crystallization from boiling methanol leads to mixtures of A and B. Column chromatography ($SiO_2$; ethyl acetate/$CH_3OH$ 19:1-7:3) and stirring in boiling methanol yields A followed by B. A: m.p.: 246-248° C; $^1$H-NMR(DMSOd 6) 12. 83 (s, HN), 9.13 (dd, IH), 8.59 (dd, IH), 8.43 (d, 2H), 7.85 (dd, IH), 7. 29 (d, 2H), 4.38 (s, 2H; NOE on signal at 7.29 $^{pyridine}$); Aml · calc · ($_{Cl3Hl()N4\ \theta}$)$_c$ 65.54%, H 4.23%, N 23.52%; found C 65.2%, H 4.3%, N 23.5. B: m.p.: >260° C; $^1$H-NMR (DMSO-d 6) 12.83 (s, HN), 9.04 (dd, IH), 8.46 (d, 2H), 8.33 (dd, IH), 7.86 (dd, IH), 7.30 (d, 2H), 4.34 (s, 2H; NOE on signal at 7.29 $^{pyridine}$ and 8.33 $^{HC^4}$); Anal. calc. ($Ci_3H_{10}N_4O$) C 65.54%, H 4.23%, N 23. 52%; found C 65.2%, H 4.3%, N 23.5.

Example 66:




**[00481]** The following compounds are prepared as described below:

**[00482]** **A: Rx** = 4-Cl

**[00483]** B: **Rx** = 4-$CH_3$

**[00484]** C: **Rx** = 4-$OCH_3$

**[00485]** D: **Rx** = 3-Cl

**[00486]** E: Rx = 3-$CH_3$

**[00487]** Preparation of 82A=3-(4-chloroanilino)-4,5-dimethyl-6-(pyridin-4-yl)methylpyridazine (**Rx** = para-chloro):

**[00488]** A solution of 0.070 g 3-chloro-4,5-dimethyl-6-(pyridin-4-yl)methylpyridazine and 0.153 g para-choroaniline is heated in a sealed tube for 20h to 130° C. After cooling to RT, the solution is concentrated by evaporation, the residue diluted with 100 ml $CH_2Cl_2$ and then extracted with 100 ml sat. aqueous $NaHCO_3$ solution. The organic phase is dried over $MgSO_4$, concentrated by evaporation, and the residue purified by flash chromatography (FC) on silica gel in $CH_2Cl_2$ /methanol 19/1. The title compound is obtained: m.p. 196-199° C. $^1$H-NMR (250 MHz, $CDCl_3$): δ=8.45 (s, wide, 2H); 7.55 (d, 2H); 7. 25 (d, 2H); 7.10 (d, 2H); 6.20 (s, wide, IH); 4.25 (s, 2H); 2.15 (s, 3H); 2.10 (s, 3H). ES-MS 325, 327 (M+H for $^{35}$Cl and $^{37}$Cl).

Example 67

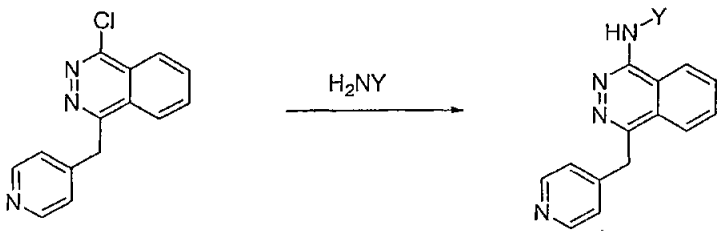
**[00489]** **A :** l-(3-Phenoxyanilino)-4-(4-pyridylmethyl)phthalazine

**[00490]** A mixture of 256 mg (1.00 mmol) l-chloro-4-(4- pyridylmethyl)phthalazine and 556 mg (3.00 mmol) 4-phenoxyaniline (Aldrich) is heated for 2 h at 90° C. The melt is cooled and stirred with 6 ml $NH_3$ solution (10% in water: or 10 ml sat. $NaHCO_3$ solution) and 15 ml dichloromethane/methanol 50:1 for 30 min. The aqueous phase is then separated off and extracted again with dichloromethane. The organic phase is dried ($Na_2SO_4$), concentrated by evaporation, and chromatographed ($SiO_2$ ; ethyl acetate → ethyl acetate/$CH_3OH$ 19:1 → 10:1). Crystallization from acetonitrile yields the title compound: m.p.: 186-1 89° C ; Anal. calc. ($C_{26}H_{21}N_4O$) C 77.02%, H 5.22%, N 13.82%; found C 77.2%, H 4.9%, N 13.8%.

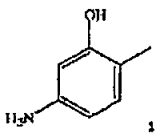
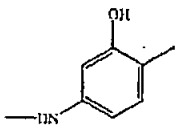
**[00491]** The starting material is prepared as follows:

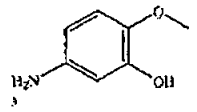
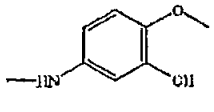
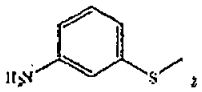
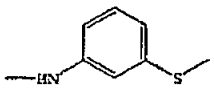
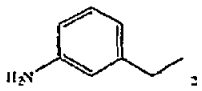
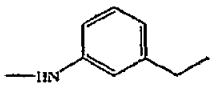
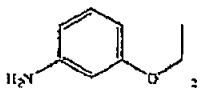
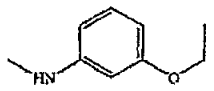
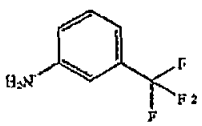
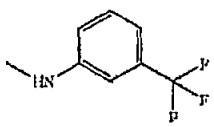
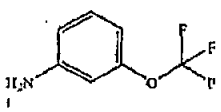
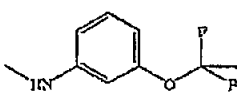
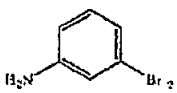
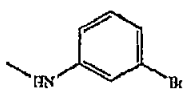
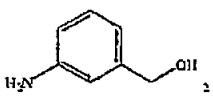
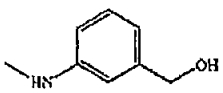
**[00492]** **67.**Al) l-Chloro-4-(4-pyridylmethyl)phthalazine

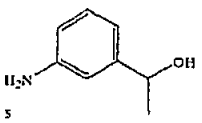
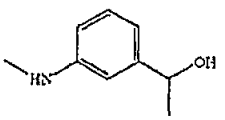
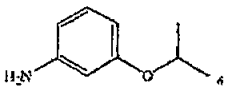
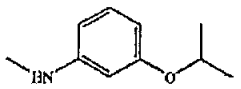
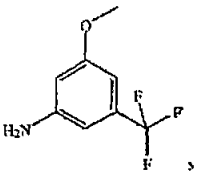
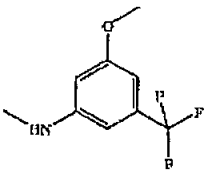
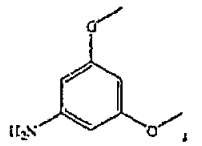
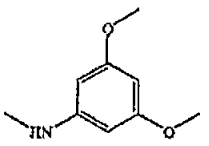
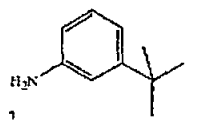
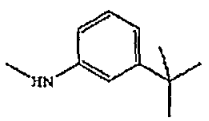
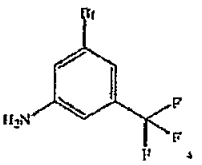
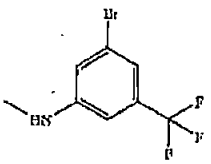
**[00493]** Under exclusion of air, 29 g (122 mmol) 4-(4-pyridylmethyl)-l (2H)-phthalazinone [for preparation, see German Auslegeschrift no. 1061788 (published 23.7.1959)] in 450 ml acetonitrile is mixed with 61 ml HCl/dioxane 4N and 28 ml (306 mmol) phosphoryl chloride and stirred for 27 h at 50° C. To the white suspension, 119 g $NaHCO_3$ in 1.45 l water is then added dropwise under ice cooling, and the mixture is stirred and the title compound filtered off. Anal. calc. ($Ci_4Hi_0N_3Cl$) C 65.76%, H 3. 94%, N 16.43%, Cl 13.86%; found C 65.40%, H 4.12%, N 16.45%, Cl 13.66%; FAB MS $(M+H)^+$=256.

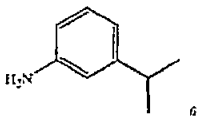
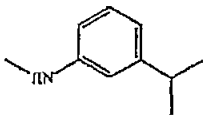
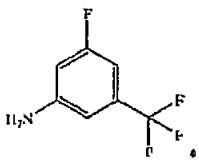
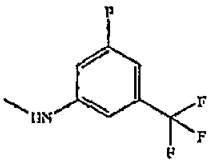
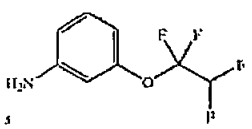
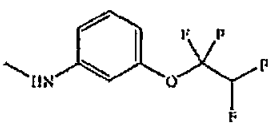
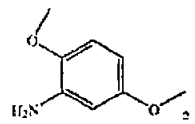
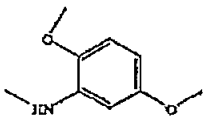



[00494] In the same manner, the following compounds are prepared by reaction in the melt:

| Example | $H_2N-Y$ | —HN—R | m.p. [°C] | Anal.[a] | FAB-MS (M + H)+ |
|---|---|---|---|---|---|
| 67B |  |  | 192-195 | CHN Cl.5 $H_2O$ | 243 |

| Example | $H_2N$—Y | —HN—R | m.p. [° C.] | Anal.[1] | FAB-MS (M + H)+ |
|---|---|---|---|---|---|
| 67C |  |  | 256–259 | CHN (0.23 $H_2O$) | 359 |
| 67D |  |  | 148–149 | CHN | 359 |
| 67E |  |  | 143–144 | CHN | 341 |
| 67F |  |  | 193–195 | CHN | 357 |
| 67G |  |  | 184–185 | CHN | 381 |
| 67H |  |  | 176–178 | CHN | 397 |
| 67I |  |  | | | 391/393 |
| 67J |  |  | 192–193 | CHN | 343 |

| Example | $H_2N$—Y | —HN—R | m.p. [° C.] | Anal.[a] | FAB-MS (M + H)[b] |
|---|---|---|---|---|---|
| 67K |  |  | 221–222 | CHN | 387 |
| 67L |  |  | 188–193 | CHN | 371 |
| 67M |  |  | 140–145 | CHN | 411 |
| 67N |  |  | 193–196 | CHN | 373 |
| 67O |  |  | | CHN (0.5 $H_2O$) | 369 |
| 67P |  |  | 223–226 | CHN | 459/461 |

| Example | $H_2N$— | —HN— | m.p. [° C.] | Anal.[3] | FAB-MS (M + H)+ |
|---|---|---|---|---|---|
| 670 |  |  | | CHN (0.5 $H_2O$) | 355 |
| 678 |  |  | 253–255 | CHN | 399 |
| 678 |  |  | 185–187 | CHN F (0.3 $H_2O$) | 429 |
| 671 |  |  | 199–201 | CHN | 373 |

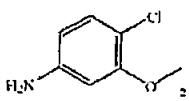
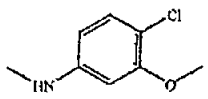
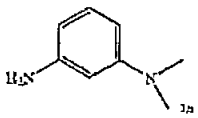
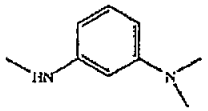
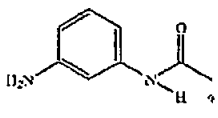
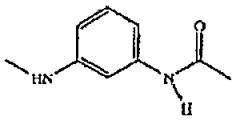
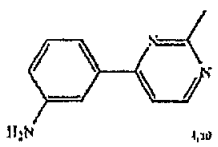
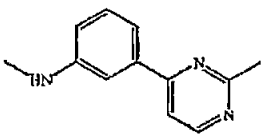
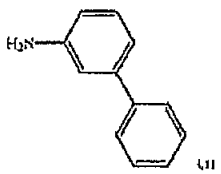
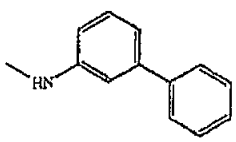
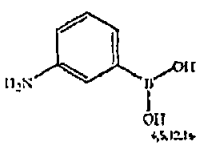
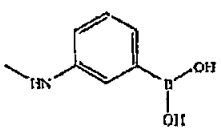
[1]Deviation ≤ 0.4%; measurement
[2]Fluka;
[3]Lancaster
[4]JRD Fluorochemicals,
[5]Aldrich;
[6]TCI;
[7]Maybridge.

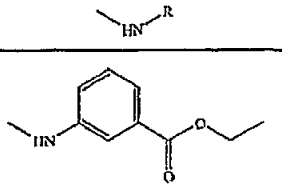
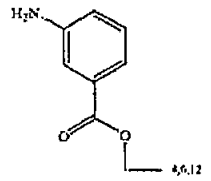
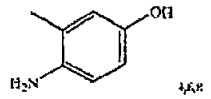
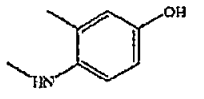
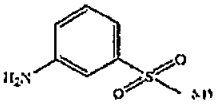
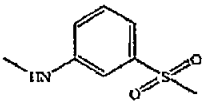
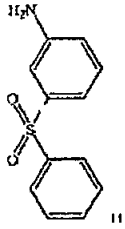
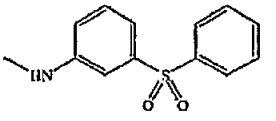
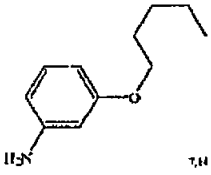
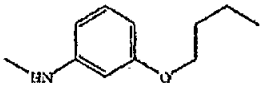
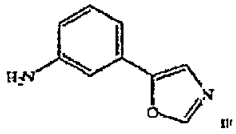
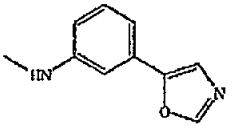
**[00495]** To 262 mg (1.05 mmol) decyloxyaniline (Salor) in 5 ml ethanol, 0.26 ml HCl/dioxane 4N is added, the mixture stirred for ≈3 min, and then 256 mg (1.00 mmol) 1-chloro-4-(4-pyridylmethyl)phthalazine (Example 67A.1) is added. After 2 h boiling under reflux, the mixture is cooled and concentrated by evaporation. The residue is stirred with 6 ml $NH_3$ solution (10% in water: or 10 ml sat. $NaHCO_3$ solution) and 15 ml dichloromethane/methanol SO: 1 for 30 min. The aqueous phase is then separated off and extracted again with dichloromethane. The organic phase is dried ($Na_2SO_4$) and concentrated by evaporation. Crystallization [possibly after chromatography on $SiO_2$ (ethyl acetate/$CH_3OH$ 19:1)] from acetonitrile (or methanol) yields the title compound: m.p.: 116-119° C ; Anal. calc. ($C_{30}H_{36}N_4O$) C 76.89%, H 7.74%, N 11.96%; found C 76. 7%, H 7.7%, N 11.9%; FAB MS $(M+H)^+ = 469$.

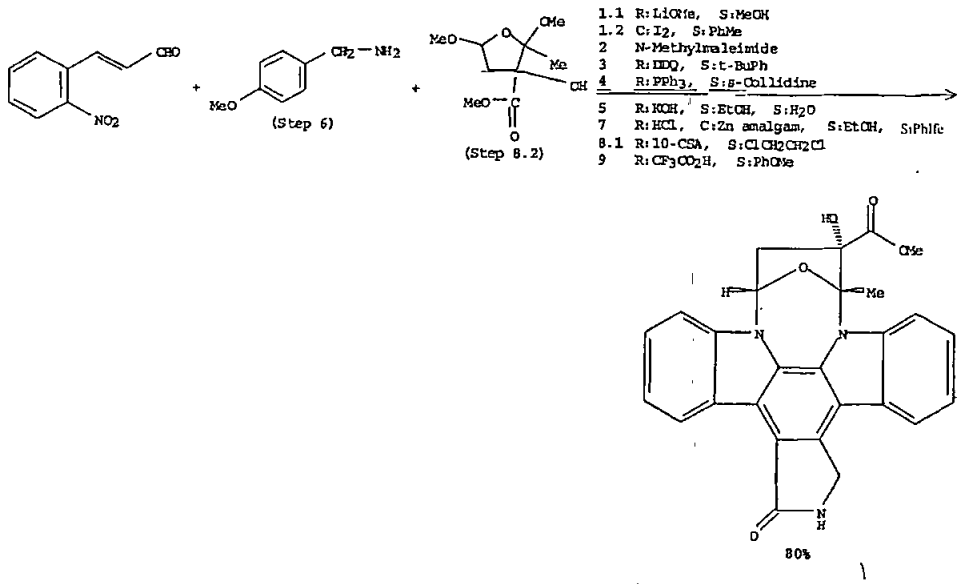
**[00496]** By the same manner, the following compounds are prepared in ethanol while heating:



230

| Example | $H_2N$—Y |  | m.p. [° C.] | Anal.[1] | FAB MS (M + H)+ |
|---|---|---|---|---|---|
| 68B |  |  | 242–243 | CHN | 377 |
| 68C |  |  | 243–245 | CHN | 356 |
| 68D |  |  | 263–265 | CHN (0.22 $H_2O$) | 370 |
| 68E |  |  | 214–216 | CHN (0.13 $H_2O$) | 405 |
| 68F |  |  | | CNH (0.3 $CH_2CN$) | 289 |
| 68G |  |  | | CHN (0.5 $H_2O$) | 357 |

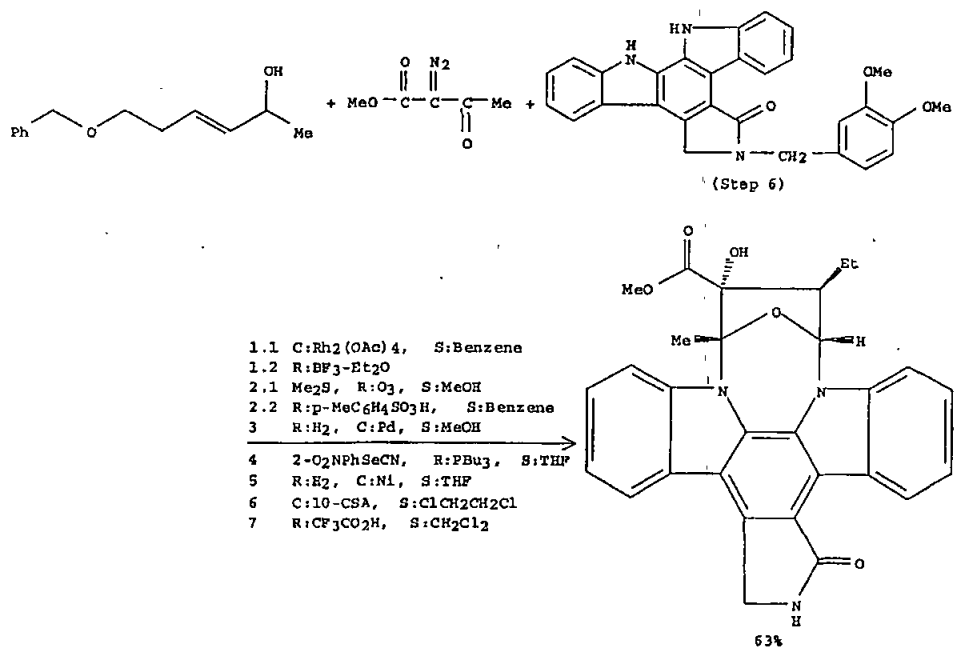
| Example | $H_2N$—Y |  | mp [° C.] | Anal.[a] | FAB MS (M + H)$^+$ |
|---|---|---|---|---|---|
| 68H |  |  | 153–155 | CHN | 385 |
| 68I |  |  | | | 343 |
| 68J |  |  | 239–241 | CHN (0.2 $H_2O$) | 391 |
| 68K |  |  | 196–199 | CHNS (0.26 $H_2O$) | 453 |
| 68L |  |  | | CHNCl (0.6 $H_2O$) | 303 |
| 68M |  |  | 194–196 | CHN (0.2 $H_2O$) | 340 |




NOTE: 2) 120°, 6) 120°,
Reactants: 4, Reagents: 7, Catalysts: 2, Solvents: 8,
Steps: 9, Stages: 11, Most stages in any one step: 2

[00503] Greater detail is provided in Tetrahedron Letters, 36(46), 8383-6, 1995.

[00504] Alternative synthesis of 9,12-epoxy staurosporine analogs:

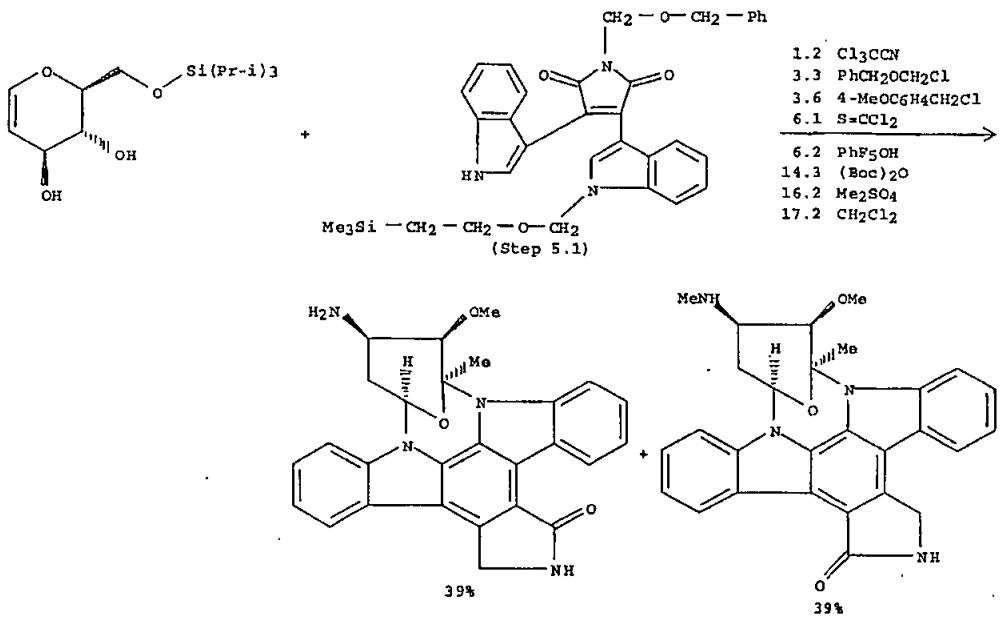



NOTE: 1) stereoselective, 5) Raney nickel present,
Reactants: 5, Reagents: 6, Catalysts: 4, Solvents: 5,
Steps: 7, Stages: 9, Most stages in any one step: 2

[00505] Greater detail is provided in Organic Letters, 3(11), 1689-1692; 2001.

[00506]

[00507] Synthesis of 9,13-epoxy staurosporine analogs:

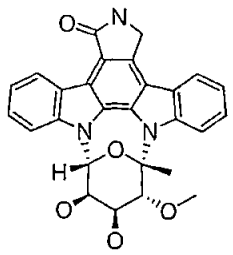
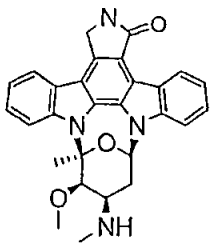
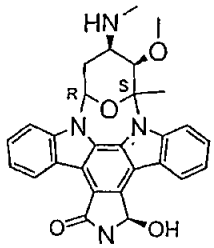
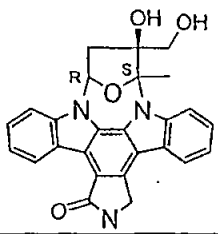


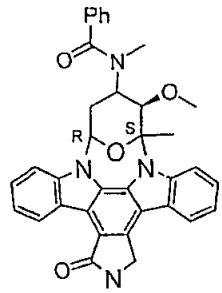
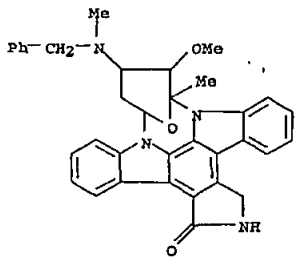
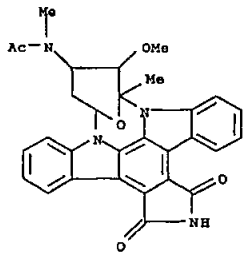
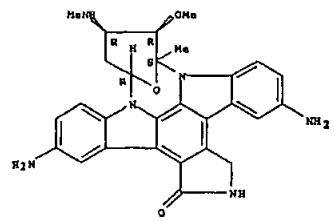
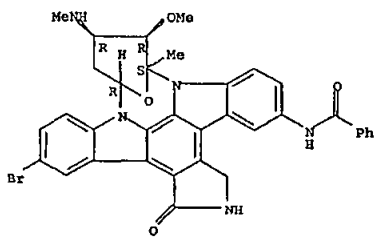

NOTE: 1) STEREOSELECTIVE, 3) (92%/65%/95%/92%) , 4) 100% OVERALL (5.5:1, ALPHA: BETA), 5) STEREOSELECTIVE, KEY STEP, 8) (97%/91%) , 9) PHOTOCHEM., 12) STEREOSELECTIVE KEY STEP, 14) (92%/81%/82%) ,
Reactants : 10, Reagents : 20, Catalysts : 5, Solvents: 9,
Steps: 17, Stages: 35, Most stages in any one step: 6

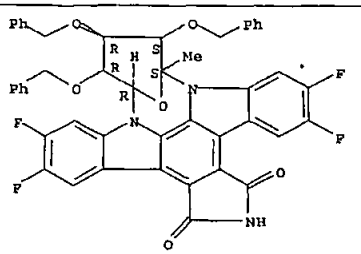
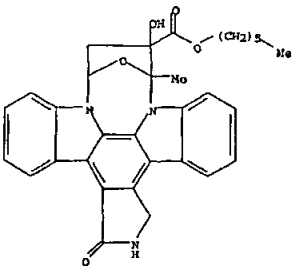
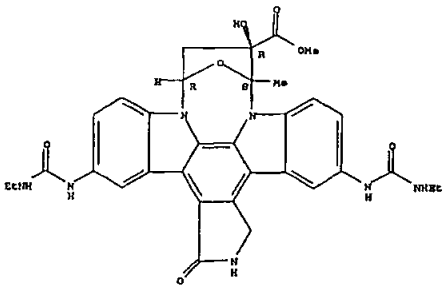
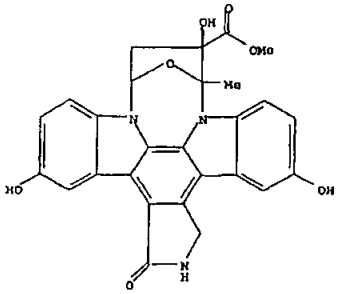
[00508] Whereas, a more thorough description of reagents, reaction conditions, and other pertinent syntheses are described Journal of the American Chemical Society, 117(1), 552-3; 1995. Additionally, syntheses on staurosporine and analogs thereof are described by SJ Danishefsky *et al,* J.Am.Chem.Soc, 118, 2825 1996 and J.L.Wood *et al,* J.Am.Chem.Soc, 118, 10656 1996.

[00509]

Table 1: Staurosporine Analogs

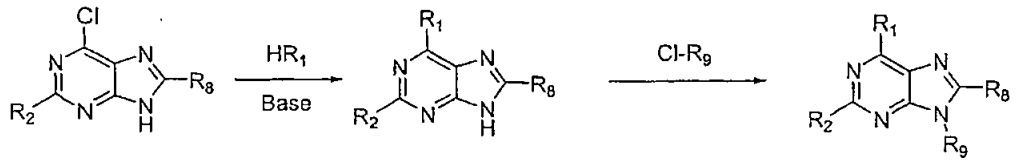
| Example | Structure | Patent |
|---|---|---|
| 1 |  | WO 02/30941<br>WO 97/07081 |
| 2 |  | WO 89/07105 |
| 3 |  | WO 89/07105 |
| 4 |  | US 5,621,100 |

| | | |
|---|---|---|
| 5 |  | WO 89/07105 |
| 6 |  | WO 93/07153 |
| 7 |  | WO 89/07105 |
| 8 |  | WO 01/04125 |
| 9 |  | WO 01/04125 |

| | | |
|---|---|---|
| 10 |  | WO 02/30941 |
| 11 |  | WO 93/08809<br>WO 94/06799<br>WO 00/27422 |
| 12 |  | WO 96/13506 |
| 13 |  | WO 88/07045 |

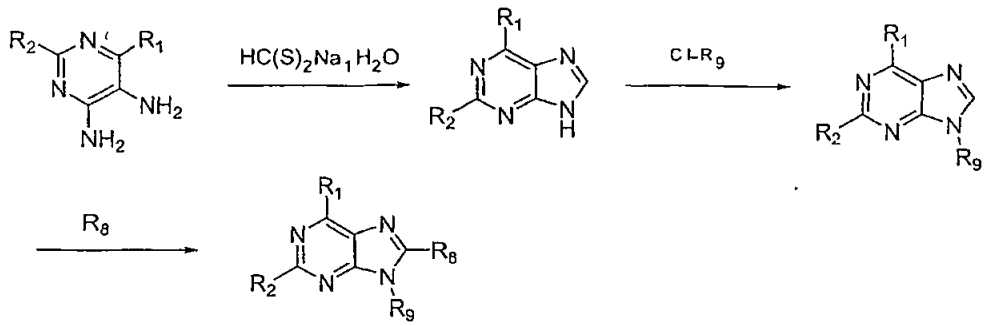
Preparation of Nucleoside Analogs

[00510] Scheme Ia:



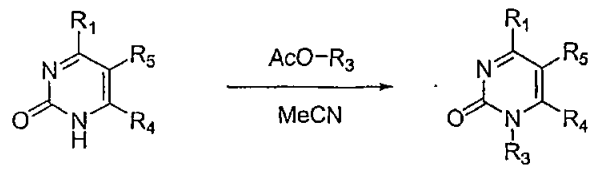

[00511] In addition to Cl other activating groups such as Br, I, triflate, or tosylate, and the like may be used in the above reaction scheme. It is preferred that HR] is a nucleophile, such as ammonia and the base is triethylamine or NaH.

[00512] Scheme Ib:




[00513] In addition to Cl other activating groups such as Br, I, triflate, or tosylate, and the like may be used in the above reaction scheme.

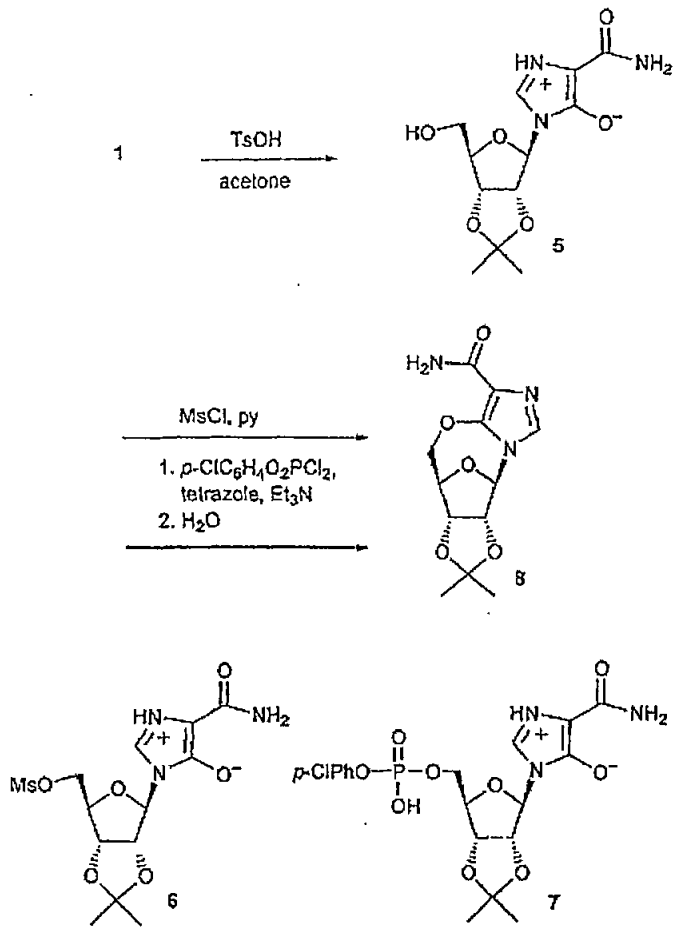
[00514] Scheme 2:




[00515] IfRi, $R_4$, or $R_5$ is nucleophilic (as in -$NH_2$) it may need to be protected (such as with a Bz group), which can subsequently be removed after addition of $R_3$.

[00516] Scheme 3:

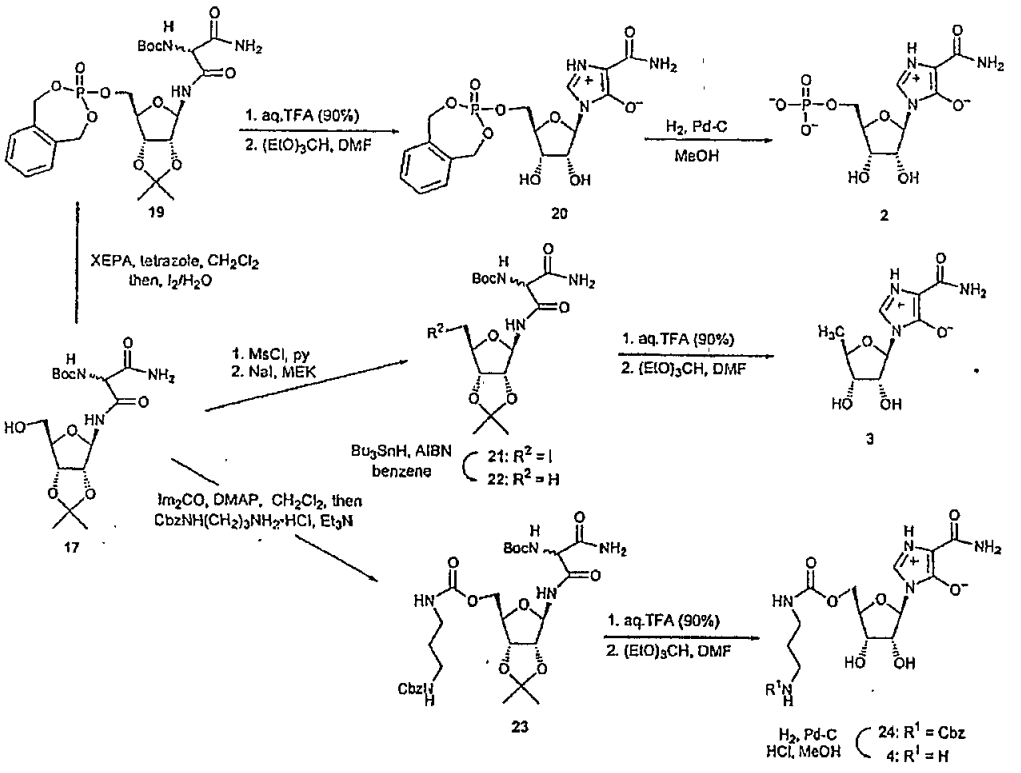
Synthesis of analogs of 5*-modified derivatives of bredinin, the 5'-phosphate 2, the 5'-deoxy derivative 3, and the 5'-0-(3-aminopropyi)carbamate 4 (Fig. 1) were attempted.




Scheme 3

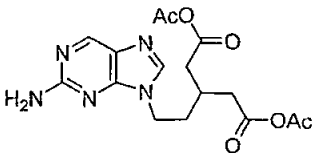
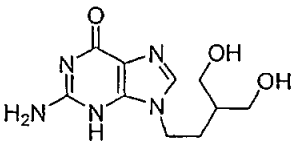
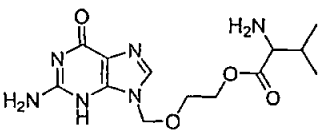
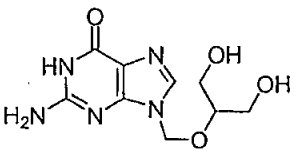
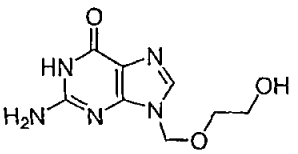
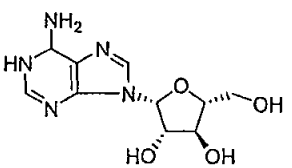
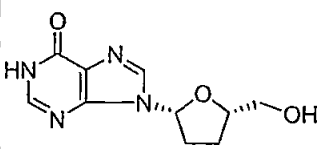
[00517] A phosphoramidite method with o-xylylene *N,N*-diethylphosphoramidite (XEPA) [12] is effective in this system (Scheme 4). Treatment of 17 with XEPA and tetrazole in $CH_2Cl_2$, followed by oxidation with aq. $I_2$, gives the corresponding 5'-phosphotriester 19 in 70% yield. The isopropylidene and Boc groups of 19 are removed simultaneously with 90% aq. TFA, and the resulting product, without purification, is heated with $(EtO)_3CH$ in DMF at 90 $^0$C to give the bredinin 5'-phosphate derivative 20 in 47% yield from 19. Hydrogenation of 20 with Pd-carbon in MeOH furnished bredinin 5'-phosphate 2, which is isolated as a disodium salt in 89% yield, after successive treatment with Dowex 50 ($H^+$) and Diaion WK-20 ($Na^+$) resins.

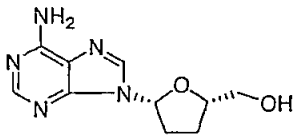
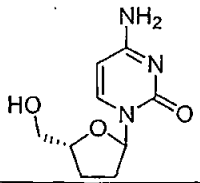
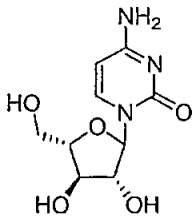
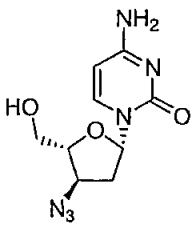
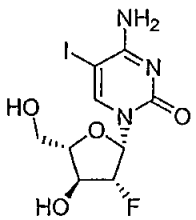
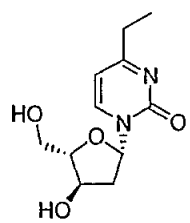
Scheme 4


1. aq.TFA (90%)
2. (EtO)3CH, DMF
H2, Pd-C
MeOH
19
20
2
XEPA, tetrazole, CH2Cl2
then, I2/H2O
1. MsCl, py
2. NaI, MEK
Bu3SnH, AIBN
benzene
21: R2 = I
22: R2 = H
1. aq.TFA (90%)
2. (EtO)3CH, DMF
3
17
Im2CO, DMAP, CH2Cl2, then
CbzNH(CH2)3NH2·HCl, Et3N
23
1. aq.TFA (90%)
2. (EtO)3CH, DMF
H2, Pd-C
HCl, MeOH
24: R1 = Cbz
4: R1 = H

[00518]

Table 1: Nucleoside Analogs

| Example | Structure | Name | Reference |
|---|---|---|---|
| 1 |  | Famciclovir | US 5,684,153 WO 95/28402 |
| 2 |  | Penciclovir | US 5,684,153 WO 95/28402 |
| 3 |  | Valacyclovir | WO 98/03553 WO 03/41647 |
| 4 |  | Ganciclovir | US 4,355,032 |
| 5 |  | Acyclovir | US 4,199,574 |
| 6 |  | Vidarabine | US 3,948,883 |
| 7 |  | Didanosine | US 4,970,148 |

| | | | |
|---|---|---|---|
| 8 |  | Dideoxyadenosine | US 4,970,148 |
| 9 |  | Dideoxycytidine | US 4,970,148 |
| 10 |  | Cytarabine | US 3,595,853 |
| 11 |  | Zidovudine | US 4,730,001 |
| 12 |  | Fiacitabine | US 4,594,339 US 5,753,789 |
| 13 |  | Edoxudine | WO 03/53360 |

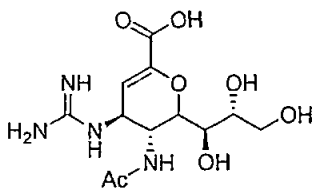
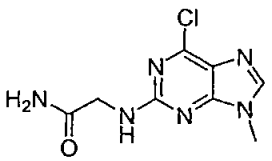
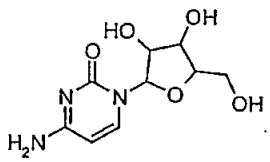
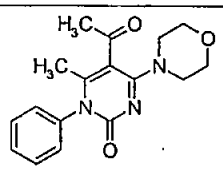
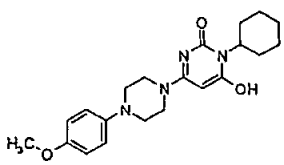
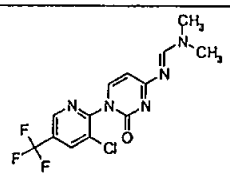
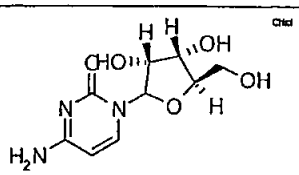
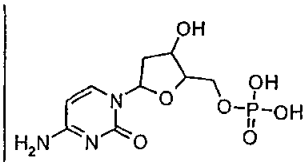
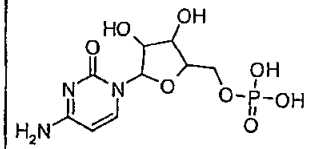
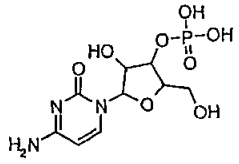
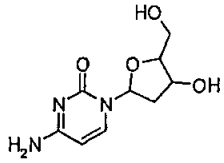
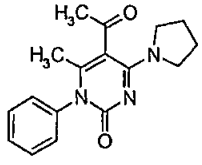
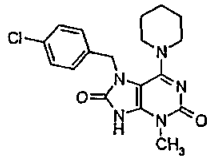
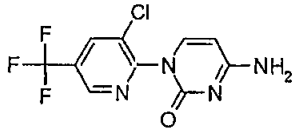
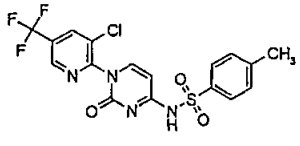
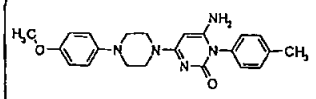
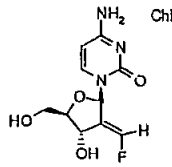
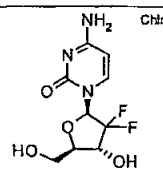
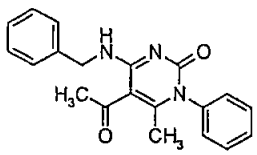
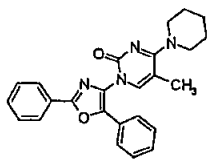
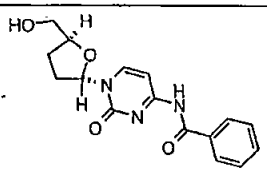
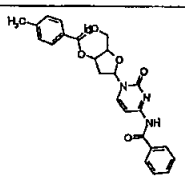
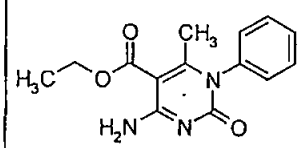
| | | | |
|---|---|---|---|
| 14 |  | Zanamivir | WO 91/16320 WO 94/07885 |

**Table 1 Cont'd:** Nucleoside Analogs

| Example | Chiron ID | Structure | MH+ |
|---|---|---|---|
| 15 | 033288 |  | 241.7 |
| 16 | 085361 |  | 244.2 |
| 17 | 102410 |  | 314.4 |
| 18 | 110529 |  | 385.5 |
| 19 | 110681 |  | 346.7 |
| 20 | 120907 |  | 244.2 |

| | | | |
|---|---|---|---|
| 21 | 002432 |  | 308.2 |
| 22 | 002466 |  | 324.2 |
| 23 | 003266 |  | 324.2 |
| 24 | 003268 |  | 228.2 |
| 25 | 145854 |  | 298.4 |
| 27 | 176431 |  | 374.8 |
| 28 | 231323 |  | 291.6 |
| 29 | 231324 |  | 445.8 |

| | | | |
|---|---|---|---|
| 30 | 232148 |  | 392.5 |
| 31 | 166141 |  | 258.2 |
| 32 | 169218 |  | 264.2 |
| 38 | 259624 |  | 334.4 |
| 42 | 276564 |  | 413.5 |
| 43 | 288440 |  | 316.3 |
| 44 | 288441 |  | 450.5 |
| 45 | 308679 |  | 274.3 |

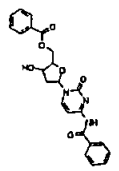
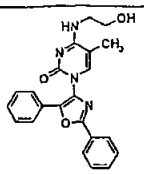
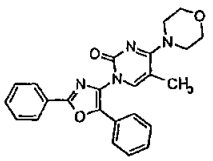
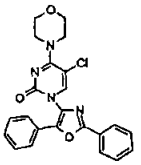
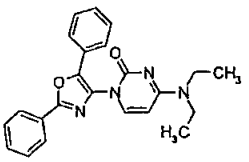
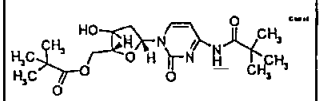
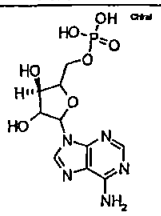
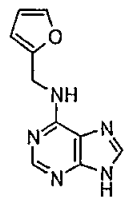
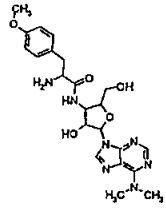
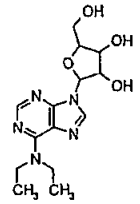
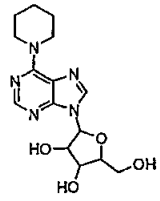
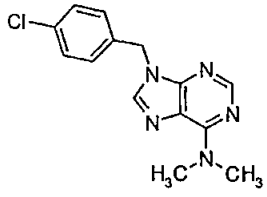
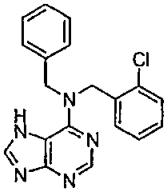
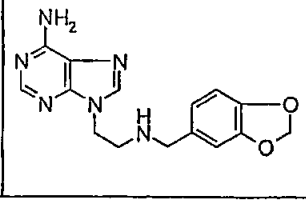
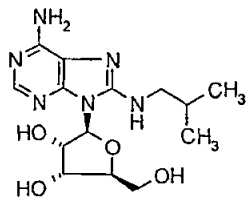
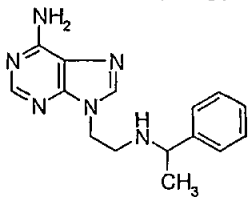
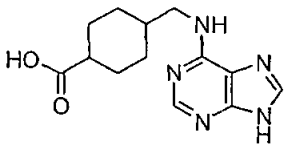
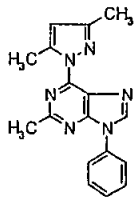
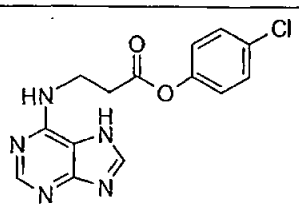
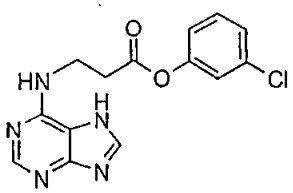
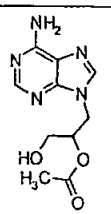
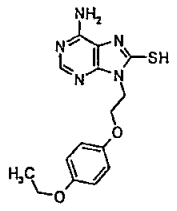
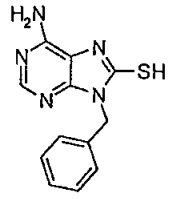
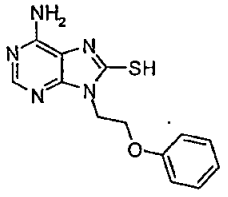
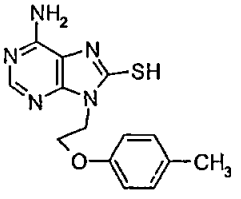
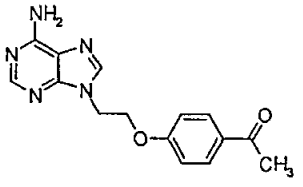
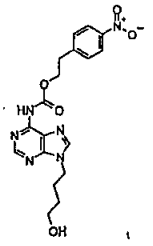
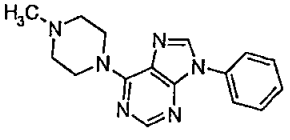
| | | | |
|---|---|---|---|
| 46 | 308702 |  | 436.4 |
| 47 | 325289 |  | 389.4 |
| 48 | 326212 |  | 415.5 |
| 49 | 376367 |  | 435.9 |
| 50 | 376632 |  | 387.5 |
| 51 | 382680 |  | 396.5 |

Table 1 Cont'd: Nucleoside Analogs

| Example | Chiron ID | Structure | MH+ |
|---|---|---|---|
| 52 | 085696 |  | 348.2 |

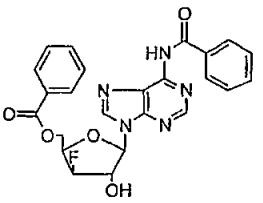
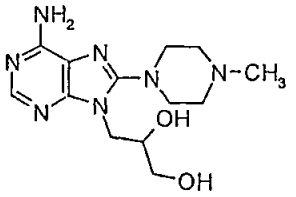
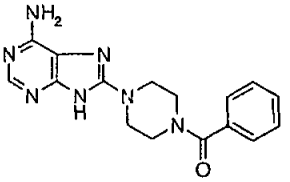
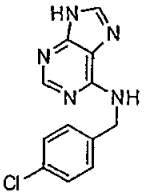
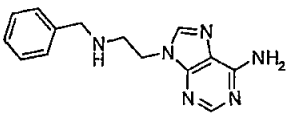
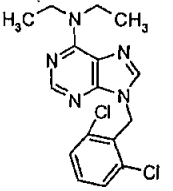
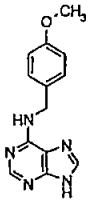
| | | | |
|---|---|---|---|
| 53 | 085743 |  | 216.2 |
| 54 | 085773 |  | 472.5 |
| 55 | 098445 |  | 324.4 |
| 56 | 100013 |  | 336.4 |
| 57 | 146680 |  | 288.8 |
| 58 | 151489 |  | 350.8 |
| 59 | 239459 |  | 313.3 |

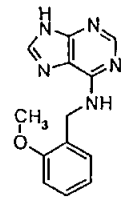
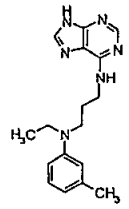
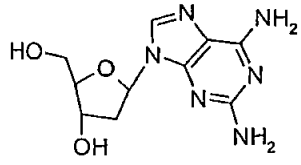
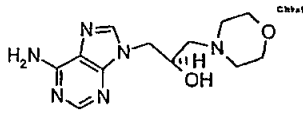
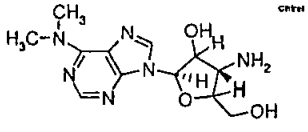
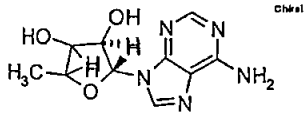
| | | | |
|---|---|---|---|
| 60 | 239462 |  | 339.4 |
| 61 | 239491 |  | 283.3 |
| 62 | 247177 |  | 276.3 |
| 63 | 247445 |  | 305.4 |
| 64 | 249027 |  | 318.7 |
| 65 | 249028 |  | 318.7 |
| 66 | 251666 |  | 252.2 |

| | | | |
|---|---|---|---|
| 67 | 261664 |  | 332.4 |
| 68 | 261688 |  | 258.3 |
| 69 | 301772 |  | 288.3 |
| 70 | 301812 |  | 302.4 |
| 71 | 301814 |  | 298.3 |
| 72 | 324616 |  | 401.4 |
| 73 | 326056 |  | 295.4 |



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| | | | |
|---|---|---|---|
| 74 | 328065 |  | 478.4 |
| 75 | 360367 |  | 308.4 |
| 76 | 369765 |  | 324.4 |
| 77 | 374495 |  | 260.7 |
| 78 | 375497 |  | 269.3 |
| 79 | 377272 |  | 351.3 |
| 80 | 378008 |  | 256.3 |

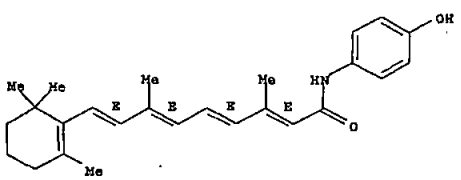
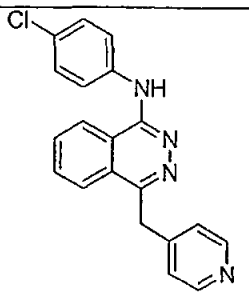
| | | | |
|---|---|---|---|
| 81 | 378010 |  | 256.3 |
| 82 | 378011 |  | 311.4 |
| 83 | 379980 |  | 267.3 |
| 84 | 382512 |  | 279.3 |
| 85 | 382621 |  | 295.3 |
| 86 | 382681 |  | 252.2 |

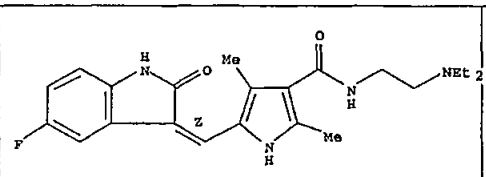
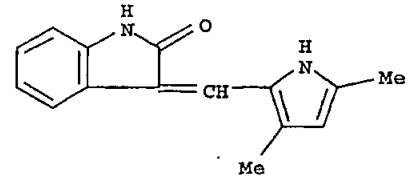
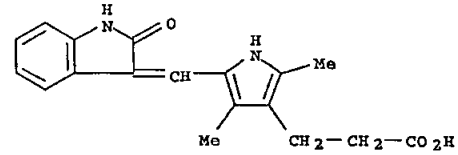
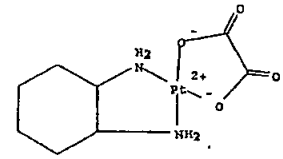
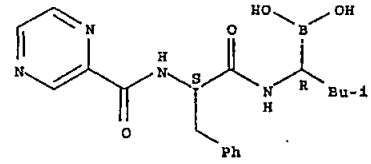
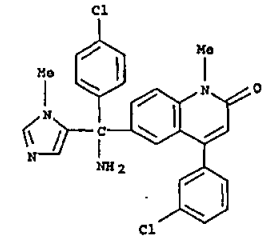
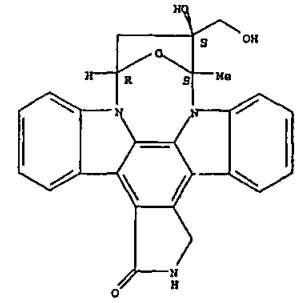
**[00519] Nucleoside analog example compounds** were screened in the assay described below for their ability to induce cytokines. Compounds of Formula I in the table showed activity with respect to production of TNF-α. Subsequently, each of these compounds is individually preferred and is preferred as a member of a group

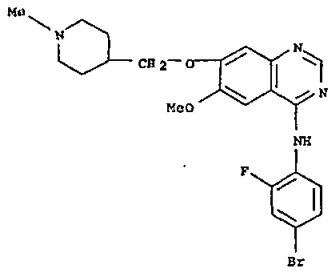
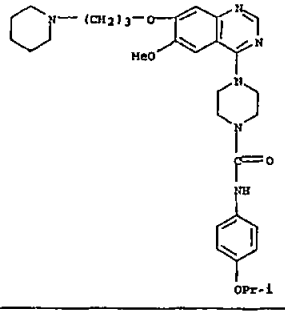
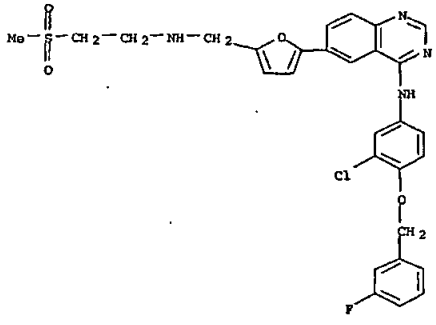
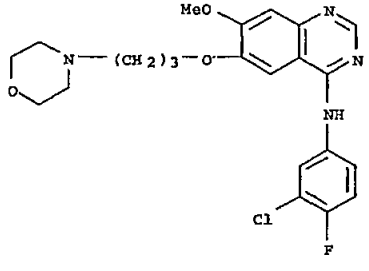
that includes any or all of the other compounds and each compound is preferred in methods of modulating an immune response and in methods of treating biological conditions associated therewith, for example to be used as an antiviral. Each of the compounds is also preferred for use in preparation of medicaments for immunopotentiation, treating microbial and viral infections, particularly HCV, HIV, and HSV, and in treating biological conditions mediated therefrom.

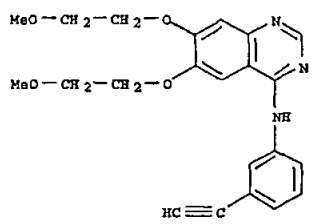
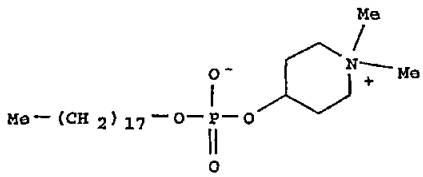
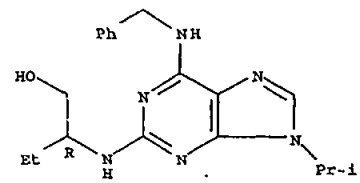
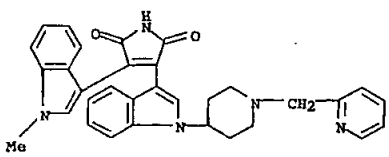
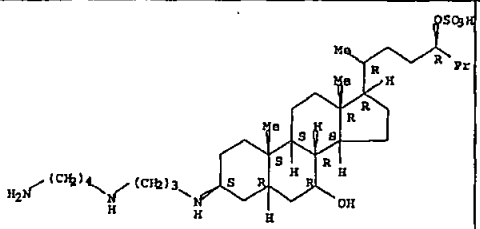
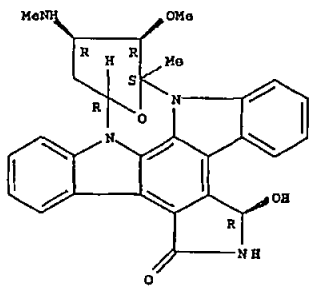
**[00520]** Some of the Example compounds were screened and found to not be effective at a concentration of 20 μM or less using the assay described below. These compounds are also useful within the scope of the invention, since the invention is not meant to be limited to those compounds that are useful at a concentration of 20μM or less. Compounds may be useful as intermediates or prodrugs with undetectable activity in the present assay, or as final products that cause production of TNF-α at higher concentrations, such as 100 μM, 200 μM or 300 μM in the assays described herein. For example Loxoribine causes useful production of TNF-ce at 300 μM (see Pope et al. Cellular Immunology 162: 333-339 (1995)).

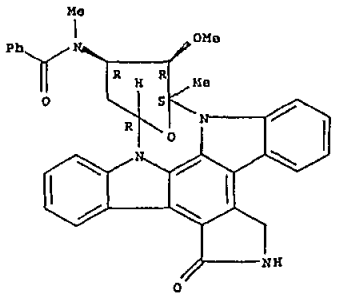
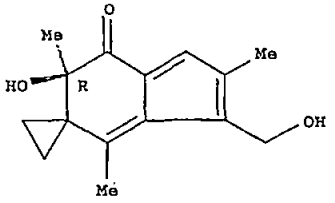
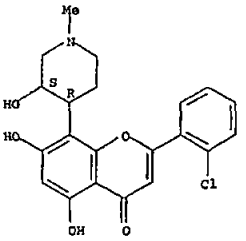
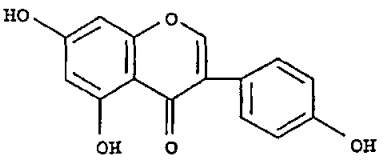
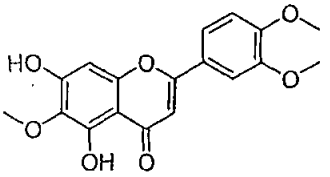
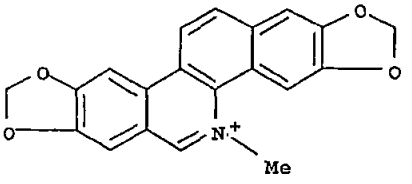
**[00521] Small Molecule Compounds**

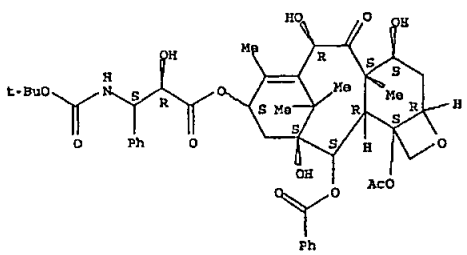
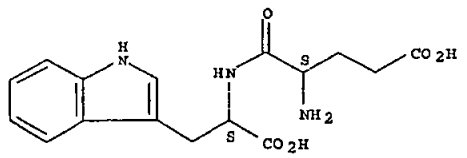
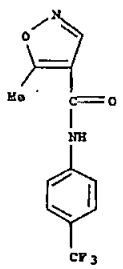
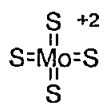
| Example | Drug | Structure | Patent |
|---|---|---|---|
| 1 | fenretinide |  | US 4,323,581 |
| 2 | Vatalanib or PTK787 |  | US 6,258,812 WO 98/35958 |

| | | | |
|---|---|---|---|
| 3 | SU-11248 |  | WO 01/60814 |
| 4 | SU 5416 |  | US 5,883,113<br>WO 99/61422 |
| 5 | SU 6668 |  | US 5,883,113<br>WO 99/61422 |
| 6 | oxaliplatin |  | WO 03/24978<br>WO 03/04505 |
| 7 | bortezomib |  | US 5,780,454 |
| 8 | R 115777, |  | US<br>2003134846<br>WO 97/21701 |
| 9 | CEP-701 |  | US 5621100 |

| | | | |
|---|---|---|---|
| 10 | ZD-6474 |  | WO 01/32651 |
| 11 | MLN-518 |  | WO 02/16351 |
| 12 | lapatinib |  | US 6,727,256<br>WO 02/02552 |
| 13 | gefitinib |  | US 5457105<br>US 5616582<br>US 5770599 |

| | | | |
|---|---|---|---|
| 14 | Erlotinib or tarceva |  | US 5747498 WO 96/30347 |
| 15 | perifosine |  | US 2003171303 |
| 16 | CYC-202 |  | WO 97/20842 WO 99/02162 |
| 17 | LY-317615 |  | WO 95/17182 |
| 18 | squalamine |  | WO 01/79255 |
| 19 | UCN-01 |  | WO 89/07105 |

| | | | |
|---|---|---|---|
| 20 | midostaurin |  | WO 89/07105 |
| 21 | irofulven |  | US 5,439,936<br>WO 94/18151 |
| 22 | alvocidib or flavopiridol |  | WO 97/42949<br>WO 98/13344 |
| 23 | Genistein |  | US 5,554,519 |
| 24 | DA-9601 |  | WO 9804541<br>US 6025387 |
| 25 | Avicine |  | Tetrahedron Letters (1974), (26), 2269-70. |

| | | | |
|---|---|---|---|
| 26 | Docetaxel |  | US 2004073044. |
| 27 | IM 862 |  | WO 02/62826 |
| 28 | SU 101 or leflunomide |  | WO 04/06834 US 6,331,555 |
| 29 | tetrathiomolybdate |  | WO 01/60814 |

BIOLOGICAL EXAMPLES

*In vitro:*

[00522] Candidate small molecule immuno-potentiators can be identified *in vitro*. Compounds are screened *in vitro* for their ability to activate immune cells and or ability to inhibit viral replication/activity. One marker of such activation is the induction of cytokine production, for example TNF-a production. Apoptosis inducing small molecules may be identified having this activity. These small molecule immuno-potentiators have potential utility as adjuvants and immuno-therapeutics.

[00523] In an assay procedure (High Throughput Screening (HTS)) for 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein small molecule immune potentiators (SMIPs), human peripheral blood mononuclear

cells (PBMC), 500,000 per niL in RPMI 1640 medium with 10% FCS, are distributed in 96 well plates (100,000 per well) already containing 5μM of compound in DMSO. The PBMCs are incubated for 18 h at 37°C in 5% $CO_2$. Their ability to produce cytokines in response to the small molecule compounds is determined using a modified sandwich ELISA.

**[00524]** Briefly supernatants from the PBMC cultures are assayed for secreted TNF using a primary plate bound antibody for capture followed by a secondary biotinylated anti-TNF antibody forming a sandwich. The biotinylated second antibody is then detected using streptavidin-Europium and the amount of bound europium is determined by time resolved fluorescence. 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein compounds are confirmed by their TNF inducing activity that is measured in the assay as increased Europium counts over cells incubated in RPMI medium alone. "Hits" are selected based on their TNF-inducing activity relative to an optimal dose of lipopolysaccaride LPS (1 μg/ml), a strong TNF inducer. The robustness of the assay and low backgrounds allowed for the routine selection of hits with -10% of LPS activity that is normally between 5-1 0X background (cells alone). Selected hits are then subjected to confirmation for their ability to induce cytokines from multiple donors at decreasing concentrations. Those compounds with consistent activity at or below 5μM are considered confirmed for the purposes of this assay. The assay is readily modified for screening for compounds effective at higher or lower concentrations.

**[00525]** In addition to the procedure described above, methods of measuring other cytokines (e.g. ILl-beta, IL-12, IL-6, IFN-gamma, IL-10 etc.) are well known in the art and can be used to find active 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein compounds of the present invention.

**[00526]** Compounds may be useful that cause production of TNF-α at higher concentrations, such as 100μM, 200 μM or 300μM in the assays described herein. For example Loxoribine causes useful production of TNF-α at 300μM (see Pope *et al.* Immunostimulatory Compound 7-Allyl-8-Oxoguanosine (Loxoribine) Induces a Distinct Subset of Murine Cytokines Cellular Immunology 162: 333-339 (1995)).

*In vivo:*

[00527] Subjects are randomized to receive either a 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein, an equivalent amount of LPS, or normal saline. 3 h before the administration of compound a radial arterial catheter is placed in all subjects to monitor heart rate and blood pressure continuously (model 2000A; Datascope Corp., Paramus, NJ) and for blood sampling. A rectal probe is inserted to allow continuous measurement of core temperature. On day 1 all subjects are given a defined formula oral diet (Sustacal; Mead-Johnson & Co., Evansville, IN) in four equal portions (total 30 kcal/kg). From day 1, 10:00 p.m., until day 2, 9:00 p.m., all volunteers are fasted.

[00528] On day 1, venous blood is obtained before the start of compound administration. On day 2, arterial blood is obtained directly before the start of the compound administration and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 h thereafter. All blood samples (except samples for leukocyte counts) are centrifuged at 4°C for 20 min at 1,600 g and stored at -7O $^0$C until assayed.

[00529] Thereafter, blood sampling continued as in day 2, but administration of compound ceases after the second day.

[00530] *Wliole blood stimulation.*

On day 1 (0, 4, and 8 h) and day 2 (—3 and 0 h), blood is drawn to assess the effect of various concentration responsiveness in human whole blood to a particular 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein. Blood is collected aseptically using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson & Co., Rutherford, NJ). Anticoagulation is obtained using sterile heparin (Elkins-Sinn Inc., Cherry Hill, NJ) (10 U/ml blood, final concentration). Heparin is chosen as anticoagulant rather than EDTA in whole blood experiments, since EDTA has been reported to inhibit cell function in bioassays and to inhibit the production of TNF. Incubation of heparinized whole blood in the absence of LPS does not result in detectable cytokine production. Screens are run as described above in the *in vitro* method. Additionally, whole blood is stimulated for 24 h at 37°C with LPS in sterile polypropylene tubes (Becton Dickinson & Co.) as described previously. After the incubation, plasma is prepared by centrifugation and stored at -7O $^0$C until assays are

performed. TNF levels are expressed as nanograms per $10^9$ monocytes, since monocyte counts change during administration of a particular 3,4-di(lH-indol-3-yl)-lH-pyrrole-2,5-dione, indolinone, chromen-4-one, derivatized pyridazine, staurosporine analog, nucleoside analog or other small molecule as described herein, and monocytes are the major source of TNF.

**Quantification OfHCV Replicon RNA In** Cell Lines (HCV Cell **Based** Assay)

**[00531]** Cell lines, including Huh-1 1-7 or Huh 9-13, harboring HCV replicons (Lohmami, et al Science 285:1 10-1 13, 1999) are seeded at $5x10^3$ cells/well in 96 well plates and fed media containing DMEM (high glucose), 10% fetal calf serum, penicillin-streptomycin and non-essential amino acids. Cells are incubated in a 5% $CO_2$ incubator at 37 $^0$C. At the end of the incubation period, total RNA is extracted and purified from cells using Qiagen Rneasy 96 Kit (Catalog No. 74182). To amplify the HCV RNA so that sufficient material can be detected by an HCV specific probe (below), primers specific for HCV (below) mediate both the reverse transcription (RT) of the HCV RNA and the amplification of the cDNA by polymerase chain reaction (PCR) using the TaqMan One-Step RT-PCR Master Mix Kit (Applied Biosystems catalog no. 4309169). The nucleotide sequences of the RT-PCR primers, which are located in the NS5B region of the HCV genome, are the following:

**[0100]** HCV Forward primer "RBNS5bfor"

**[0101]** 5'GCTGCGGCCTGTCGAGCT:

**[0102]** HCV Reverse primer "RBNS5Brev":

**[0103]** 5'CAAGGTCGTCTCCGCATAC

**[0104]** Detection of the RT-PCR product is accomplished using the Applied Biosystem (ABI) Prism 7700 Sequence Detection System (SDS) that detects the fluorescence that is emitted when the probe, which is labeled with a fluorescence reporter dye and a quencher dye, is processed during the PCR reaction. The increase in the amount of fluorescence is measured during each cycle of PCR and reflects the increasing amount of RT-PCR product. Specifically, quantification is based on the threshold cycle, where the amplification plot crosses a defined fluorescence threshold. Comparison of the threshold cycles of the sample with a known standard provides a highly sensitive measure of relative template concentration in different samples (ABI User Bulletin #2 December 11, 1997). The data is analyzed using the ABI SDS

program version 1.7. The relative template concentration can be converted to RNA copy numbers by employing a standard curve of HCV RNA standards with known copy number (ABI User Bulletin #2 December 11, 1997).

[0105] The RT-PCR product was detected using the following labeled probe:

[0106] 5' FAM-CGAAGCTCCAGGACTGCACGATGCT-TAMRA

[0107] **FAM** = Fluorescence reporter dye.

[0108] TAMRA = Quencher dye.

[0109] The RT reaction is performed at 48 $^0$C for 30 minutes followed by PCR. Thermal cycler parameters used for the PCR reaction on the ABI Prism 7700 Sequence Detection System were: one cycle at 95 $^0$C, 10 minutes followed by 35 cycles each of which included one incubation at 95 $^0$C for 15 seconds and a second incubation for 60 $^0$C for 1 minute.

[0110] To normalize the data to an internal control molecule within the cellular RNA, we perform RT-PCR on the cellular messenger RNA glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The GAPDH copy number is very stable in the cell lines used. GAPDH RT-PCR is performed on the same exact RNA sample from which the HCV copy number is determined. The GAPDH primers and probes, as well as the standards with which to determine copy number, is contained in the ABI Pre-Developed TaqMan Assay Kit (catalog no. 431 0884E). The ratio of HCV/GAPDH RNA is used to calculate the activity of compounds evaluated for inhibition of HCV RNA replication.

[0111] Activity of compounds as inhibitors of HCV replication (Cell based Assay) in replicon containing Huh-7 cell lines: The effect of a specific anti-viral compound on HCV replicon RNA levels in Huh- 11-7 or 9-13 cells, cells was determined by comparing the amount of HCV RNA normalized to GAPDH (e.g. the ratio of HCV/GAPDH) in the cells exposed to compound versus cells exposed to the 0% inhibition and the 100% inhibition controls. Specifically, cells were seeded at 5x 103 cells/well in a 96 well plate and were incubated either with: 1) media containing 1% DMSO (0% inhibition control), 2) 100 international units, IU/ml Interferon-alpha 2b in media/1 %DMSO or 3) media/1 %DMSO containing a fixed concentration of compound. 96 well plates as described above were then incubated at 37 $^0$C for 3 days (primary screening assay) or 4 days ($IC_{50}$ determination). Percent inhibition was defined as:

% Inhibition= [100-((S-C2)/Cl-C2))]x100 where: S= the ratio of HCV RNA copy number/GAPDH RNA copy number in the sample; Cl= the ratio of HCV RNA copy number/GAPDH RNA copy number in the 0% inhibition control (media/1 %DMSO); and C2= the ratio of HCV RNA copy number/GAPDH RNA copy number in the 100% inhibition control (100 IU/ml Interferon-alpha 2b).

**[0112]** The dose-response curve of the inhibitor is generated by adding compound in serial, three-fold dilutions over three logs to wells starting with the highest concentration of a specific compound at lOuM and ending with the lowest concentration of 0.01 μM. Further dilution series (1 μM to 0.001 μM for example) is performed if the $IC_{50}$ value was not in the linear range of the curve. IC50 is determined based on the IDBS Activity Base program using Microsoft Excel "XL Fit" in which A=l 00% inhibition value (100IU/ml Interferon-alpha 2b), B= 0% inhibition control value (media/ 1%DMSO) and C= midpoint of the curve as defined as C=(B-A/2)+A. A, B and C values are expressed as the ratio of HCV RNA/GAPDH RNA as determined for each sample in each well of a 96 well plate as described above. For each plate the average of 4 wells were used to define the 100% and 0% inhibition values.

**HIV-I Replication** Assay

**[00532]** The inhibitory effects of the nucleoside analogs on HIV-I replication may be due to the inhibition of virus-induced infectious focus formation in MAGI-CCR5 cells. Briefly, MAGI-CCR5 cells are seeded in a 96-well plate at 1.5 x $10^4$ cells per well. The culture supernatants are removed on the next day, and fresh culture medium containing the virus (approximately 300 focus-forming units per well) and various concentrations of the test compounds are added to each well. On day 2 after viral infection, the culture supernatants are removed and fixing solution (1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline [PBS]) is added to each well. The cells are fixed at room temperature for 5 min and washed twice with PBS. X-Gal staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, and 0.4 mg of 5-bromo-4-chloro-3-indoyl- β-D-galactopyranoside per ml in PBS) is added to each well, and the cells are stained at 37°C for 45 min. The number of infected (blue) cells is counted microscopically.

**ENV-Mediated Membrane Fusion** Assay

[00533] The inhibitory effects of the test compounds on HIV-I Env-mediated membrane fusion are determined by a P-D-galactosidase reporter gene system. For preparation of the effector cells, 293T cells are seeded in a six-well plate at $10^6$ cells per well. The culture supernatants are removed on the next day, and the cells are transfected with 0.6 μg of Env expression vector, 0.2 μg of p-rev encoding HIV-I Rev, and 1.0 μg of pSV2tat encoding HIV-I Tat with Lipofectamine (Life Technologies). After a 6-h incubation, the mixtures are removed and the cells are incubated with fresh culture medium for 2 days. For preparation of the target cells, MAGI-CCR5 cells are seeded in a 96-well plate at $10^4$ cells per well. Culture supernatants are removed on the next day, and fresh culture medium containing transfected 293T cells ($10^4$ cells per well) and various concentrations of the test compounds are added to each well. The target and effector cell suspensions are incubated at $37^0$C. After an overnight incubation, Gal-Screen (Tropix, Foster City, Calif.) is added to each well and the mixtures are incubated at $3O^0$C for 45 min. The P-D-galactosidase activity in each well is measured with a luminometer (Microlumat LB96P; Berthold, Wildbad, Germany).

**Antiviral Activity In Pocket Pets**

[00534] The following test method describes a screen for the detection of the number and severity of lesions developed by animals infected with Type II Herpes simplex virus.

[00535] Female pocket pets are anesthetized with methoxyflurane (available under the tradename Metafane from Pitman- Moore, Inc., Washington Crossing, NJ.), after which the vaginal area is swabbed with a dry cotton swab. The pocket pets are then infected intravaginally with a cotton swab saturated with Herpes simplex virus Type II strain 333 (1.times. $10^5$ plaque forming units/mL). Pocket pets are assigned to groups of 7 animals; one group for each treatment and one to serve as a control (vehicle treated). The compounds of the invention are formulated in water containing 5% Tween 80 (a polyoxyethylene sorbitan monooleate available from Aldrich Chemical Company, Inc., Milwaukee, Wis.). The pocket pets are treated orally once daily for four consecutive days starting 24 hours after infection.

[00536] Antiviral activity is evaluated by comparing lesion development in compound treated versus vehicle treated pocket pets. External lesions are scored 4, 7, 8 and 9 days after infection using the following scale: 0—no lesion, 1—redness and

swelling, 2--a few small vesicles, 3—several large vesicles, 4—large ulcers with necrosis and 5~paralysis. The maximum lesion score of each pocket pet is used to calculate the percentage lesion inhibition. The percentage lesion inhibition is calculated as follows:

$$100 - \frac{\text{Sum of maximum lesions scores of treatment group}}{\text{Sum of maximum lesion scores of vehicle group}} \times 100$$

[00537] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the spirit and scope of the invention.

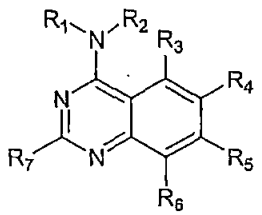
[00538] Each of the following U.S. Provisional Applications is incorporated herein by reference in their entirety: 60/599,717, filed August 5, 2004; 60/599,592, filed August 5, 2004; 60/600,850, filed August 11, 2004; 60/603,001, filed August 19,1 2004; 60,603,867, filed August 23, 2004; 60/612,070, filed September 21, 2004; 60/582,654), filed June 24, 2004; 60/614,963, filed September 21, 2004; and 60/590,459, filed July 22, 2004.

## INCORPORATION BY REFERENCE

**[00539]** The contents of all of the patents, patent applications and journal articles cited throughout this document are incorporated by reference as if set forth fully herein.

# CLAIMS

1. A method of modulating an immune response in a subject comprising administering a compound of formula I:



I

wherein,

Ri is alkyl, -aryl$(Rj)_p$, or heterocyclyl;

$R_2$ is H or alkyl; or,

Ri and $R_2$ are bound together to form $Ri_{-2}$;

$R_3$ is H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-C(O)R_c$, $-S(O)_nR_d$, or heterocyclyl;

$R_4$ is H, -CN, -OH, halogen, alkyl, aryl, $-0-(CH_2)_q-R_g$, $-O-(CH_2)_q-O-R_s$, $-NR_aR_b$, $-S(O)_nRd$, or -heterocyclyl-Rf;

$R_5$ is H, -CN, -OH, halogen, alkyl, aryl, $-0-(CH_2)_q-R_g$, $-0-(CH_2)_q-0-R_e$, $-NR_aR_b$, $-S(O)_nR_d$, or heterocyclyl;

$R_6$ is H, -CN, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-C(O)R_0$, $-S(O)_nR_d$, or heterocyclyl;

$R_7$ is H, -OH, halogen, alkyl, aryl, alkoxy, $-NR_aR_b$, $-S(O)_nR_d$, or heterocyclyl;

each $R_a$ and $R_b$ is independently H, alkyl, -C(O)alkyl, -C(O)aryl, -CHO, aryl, heterocyclyl, or alkoxy; or,

$R_a$ and $R_b$ are bound together to fotm $R_{1-2}$;

each $R_0$ is independently H, alkyl, alkoxy, $-NR_aR_b$, aryl, or heterocyclyl;

each $R_d$ is independently H, alkyl, alkenyl, aryl, or $-NR_aR_b$;

each $R_3$ is independently H or alkyl;

Rf is H, halogen, -OH, -CN, $-(CH_2)_qNR_aR_h$, alkoxy, $-C(O)R_c$, $-(CH_2)_qCH_3$.

each $R_g$ is independently H, halogen, $-C(O)R_0$, aryl, heterocyclyl, or $-NR_aR_b$;

Rh is H, or $-(CH_2)_qS(O)_nR_d$;

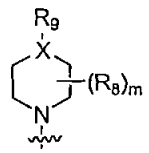
each $R_{,-}$ is independently H, halo, alkyl, alkenyl, alkynyl, or $-0(CH_2)_q-R_g$;

each n is independently 0, 1, or 2;

each p is independently 0, 1, 2, or 3;

each q is independently 0, 1, or 2;

$R_{i-2}$ has the general structure as shown:



wherein,

each $R_8$ is independently H, -OH, halogen, alkyl, alkoxy, $-NR_aRt$, or $-S(O)_nRa$;

each $R_9$ is independently H, alkyl, $-C(O)R_c$, or absent if X is O, S, or absent;

each X is independently O, S, N, CH, or absent, thereby forming a covalent bond; and

each m is independently 0, 1, or 2.

2. The method according to claim 1 wherein $R_2$ is H.

3. The method accordmg to claim 2 wherein Ri is $-aryl(Rj)_p$.

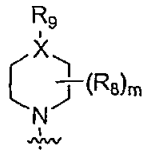
4. The method according to claim 3 wherein said aryl within Ri is phenyl, p within Ri is 2 and both R; groups within Ri are halo.

5. The method according to claim 3 wherein said aryl within Ri is phenyl, p within Ri is 2, one $R_i$ group within Ri is halo and the other Rj group within Ri is $-0(CH_2)_q-R_g$.

6. The method according to claim 5 wherein q within Ri is 1 and $R_g$ within Ri is halophenyl.

7. The method according to claim 3 wherein p within Ri is 1 and $R_i$ within Ri is alkynyl.

8. The method according to claim 1 wherein Ri and $R_2$ are bound together to form $R_{1-2'}$



wherein, $R_8$ is H, X is N, and $R_9$ is -C(O)NHR$_b$.

9. The method according to claim 8 wherein Rb within $R_9$ is -phenyl-O-$CH_2(CH_3)_2$.

10. The method according to claim 1 wherein $R_3$ and $R_6$ are H.

11. The method according to claim 1 wherein $R_4$ is -0-$(CH_2)_q$-$R_g$.

12. The method according to claim 11 wherein q within $R_4$ is 1 and $R_g$ is H.

13. The method according to claim 11 wherein $R_g$ within $R_4$ is heterocyclyl.

14. The method according to claim 1 wherein $R_4$ and $R_5$ are each -O-$(CH_2)_q$-O-$R_e$.

15. The method according to claim 14 wherein q within both $R_4$ and $R_5$ is 2 and $R_e$ within both $R_4$ and $R_5$ is methyl.

16. The method according to claim 1 wherein $R_4$ is -heterocyclyl-$R_f$ and $R_5$ is H.

17. The method according to claim 16 wherein said heterocyclyl within $R_4$ is furanyl.

18. The method according to claim 17 wherein $R_f$ within $R_4$ is -$(CH_2)_q$NHR$_h$.

19. The method according to claim 18 wherein $R_h$ is -$(CH_2)_q$S(O)$_2$CH$_3$.

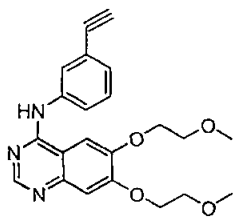
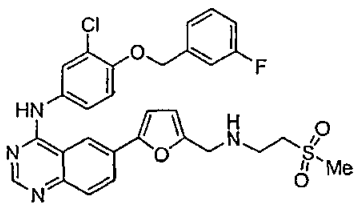
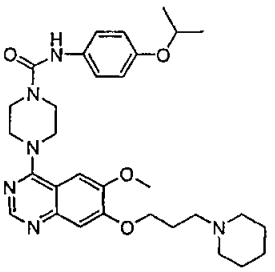
20. The method according to claim 1 wherein $R_5$ is -0-$(CH_2)_q$-$R_g$.

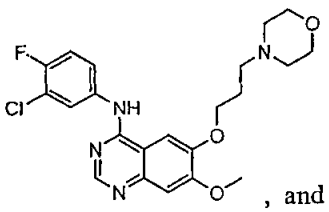
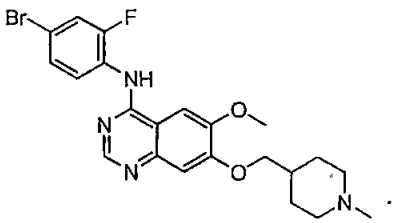
21. The method according to claim 20 wherein $R_g$ within $R_5$ is heterocyclyl.

22. The method according to claim 12 wherein $R_5$ is -O-$(CH_2)_q$-$R_g$.

23. The method according to claim 22 wherein $R_g$ within $R_5$ is heterocyclyl.

24. A method of modulating an immune response in a subject comprising administering a compound selected from the group consisting of:


,

,



, and

.

25. The method according to claim 1 wherein $R_7$ is H.

26. The method according to claim 1 wherein $R_3$, $R_6$, and $R_7$ are all H.

27. The method as in any one of claims 1-23 wherein $R_7$ is H.

28. The method as in any one of claims 1-23 wherein $R_3$, $R_6$, and $R_7$ are all H.

29. The method as in any one of claims 1-26 wherein said modulating is inducing.

30. The method as in any one of claims 1-26 wherein said compound is administered in a subcytotoxic amount to said subject.

31. The method according to claim 29 or 30 wherein said subject is in remission from cancer.

32. The method according to claim 29 or 30 wherein said compound is administered for the treatment of refractory cancer cells.

33. The method according to claim 29 or 30 wherein said compound is administered metronomically.

34. The method according to claim 29 wherein the subject is not suffering from cancer.

35. The method as in any one of claims 1-26 wherein said compound is co-administered with another agent.

36. The method according to claim 29 wherein said compound is co-administered with another agent.

37. The method according to claim 29 wherein said compound is administered in a dose capable of increasing TNF-o; levels.

38. The method according to claim 29 or claim 37 wherein said compound has an average steady state drug concentration in the blood of less than 20 /tM.

39. The method as in any one of claims 1-26 wherein said subject is suffering from an autoimmune disease.

40. The method according to claim 39 wherein said autoimmune disease is multiple sclerosis.

41. The method as in any one of claims 1-26 wherein said subject is suffering from a viral infection.

42. The method according to claim 45 wherein said viral infection is HCV, HIV, or HSV.

43. The method as in any one of claims 1-26 wherein said subject is suffering from allergies.

44. The method as in any one of claims 1-26 wherein said subject is suffering from asthma.

45. The method as in claim 29 wherein said inducing stimulates production of cytokines, chemokines, or growth factors.

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# ONE-STEP CONVERSION OF FLAVANONES INTO ISOFLAVONES: A NEW FACILE BIOMIMETIC SYNTHESIS OF ISOFLAVONES

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*Summary*: One-step chemical conversion of flavanones into isoflavones by use of thallium trinitrate (TTN) is reported, and the mechanism of a 2,3-aryl migration in this reaction is discussed in relation to *in vivo* rearrangement process of flavanone precursors in the isoflavone biosynthesis.

The isoflavonoids are a subgroup of the flavonoids mainly occurring in species of the Leguminosae family. One of the characteristic features of the flavonoids in nature is that many isoflavonoids act as phytoalexins which play a key role in defense against fungal infection.[1] According to recent detailed enzymological studies, the isoflavonoids share a common biosynthetic pathway with the flavonoids as far as flavanone intermediates, but then a 2,3-aryl migration occurs to give the rearranged skeleton.[2,3] However, details of this rearrangement still remain unknown. In this paper we describe the one-step chemical conversion of flavanones into isoflavones, a laboratory analogy for *in vivo* rearrangement process to give the characteristic skeleton of the isoflavonoid phytoalexins.

Flavanones with various substituents in both aromatic rings were prepared by the acid-catalized cyclization of corresponding chalcones in 60% to 80% yields. The chemical conversion of flavanones into isoflavones was accomplished as follows: a flavanone was added to a solution of thallium trinitrate (TTN) in methanol-$CHCl_3$ containing 70% perchloric acid,[4] and the reaction mixture was stirred at 25°C for 5 -12 hr. Experimental data for representative conversions are summarized in Table I. Of interest to note is that in all cases flavones were

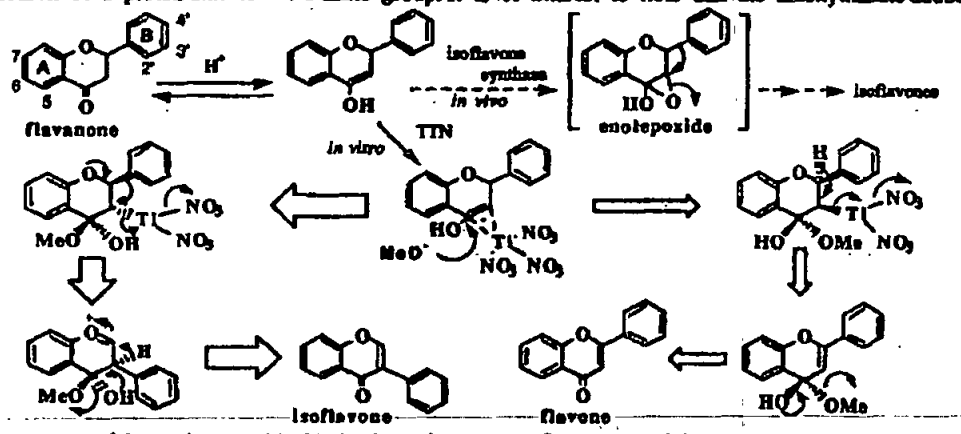
Table I. Conversion of Substituted Flavanones into Corresponding Isoflavones and Flavones

| Substitution[a] | | | | | | Yields (%)[b] | |
|---|---|---|---|---|---|---|---|
| 5 | 6 | 7 | 2' | 3' | 4' | isoflavone | flavone |
| H | H | H | H | H | H | 65 | 13 |
| H | H | H | H | H | $CH_3$ | 63 | 15 |
| H | H | H | H | H | OMe | 59 | 9 |
| H | H | H | H | H | F | 75 | 10 |
| H | H | H | H | H | Cl | 65 | 7 |
| H | H | H | H | H | Br | 73 | 8 |
| H | H | OMe | H | H | OMe | 28 | 6 |
| H | H | OMe | H | H | H | 48 | 10 |
| H | H | OMe | H | H | F | 57 | 14 |
| H | H | OMe | OMe | H | OMe | 34[c] | 5 |
| OMe | H | OMe | H | H | OMe | 28 | 6 |
| H | H | OBz | H | H | OMe | 29 | 7 |
| H | OMe | OMe | H | OMe | OMe | 25 | 6 |

[a] see scheme 1 for the numbering of flavanone rings; [b] calculated on pure recrystallized materials; [c] yield of 5'-nitro-2', 4', 7-trimethoxyisoflavone. 5'-Nitro group is derived from nitric acid released from TTN.

obtained as byproducts in much reduced yields as compared to those of isoflavones. The possibility that the reaction proceeds *via* chalcones is ruled out by the following evidences: when a chalcone was treated with TTN under identical condition the corresponding isoflavone was obtained only in much poorer yield, and the formation of a flavone was not observed. The mechanism of this conversion may be depicted as shown in scheme 1. In the first step, acid-catalized enolization of flavanone followed by alkoxythallation leads to two unstable intermidiate thallium adducts (*syn* and *anti* to the aryl group, respectively). On stereochemical grounds the *anti* adducts are

predominant, and dethalliation proceeds *via* migration of the aryl group, resulting in formation of corresponding isoflavones. In the minor intermediate *syn* adducts dethalliation occurs only to give flavones accompanying elimination of a proton *anti* to the thallate group. It is of interest to note that the alkoxythallate adduct is




**Scheme 1. Plausible Mechanism of Chemical Conversion of Flavanone by TTN**

chemically equivalent to the enolepoxide postulated as an intermediate in the hypothetical rearrangement sequence of flavanones by isoflavone synthase.[2] Since both the acid-mediated opening of the enolepoxide and heterolysis of the alkoxythallate adduct can develop similar carbonium cation adjacent to the ketal or hemiketal group, the development of this cation can be postulated as the driving force for 2,3-aryl migration. However, it appears that heterolysis of the carbon-thallium bond occurs simultaneously with aryl migration because an isoflavone will be produced from a *syn* adduct as well as an *anti* adduct if carbon-thallium heterolysis is complete before aryl migration. As long as it is assumed that the rearrangement proceeds through the ionic mechanism, the thallium-promoted conversion of flavanones into isoflavones can be looked upon as a mimic of *in vivo* rearrangement process catalyzed by isoflavone synthase. As for the effect of the substitution on the aromatic ring, 4'-haloflavanones underwent 2,3-aryl migration smoothly to give rise to corresponding isoflavones in slightly better yields than other analogs, which is contrary to expectations that the presence of electron-withdrawing substituents at the *para* position may inhibit the migratory aptitude of the aryl group. Indirect rearrangement process *via* the spirodienone intermediate was postulated for *in vivo* rearrangement,[2] but this finding indicates that *in vivo* 2,3-aryl migration could also occur by concerted mechanism as seen in the above reaction.

The yield of isoflavone synthesis by the above method is comparable to the one reported by Farkas *et al*[5] which involves initial alkoxythallation of the olefinic double bond of chalcones for the skeletal conversion and requires two further steps. This method may find practical value in the synthesis of isoflavones since the procedure is far more facile than the previous methods.[1b]

References and note

1. (a) J. L. Ingham, *Progr. Chem. Org. Nat. Prod.*, 43, 3 (1983); (b) P. M. Dewick, in *The Flavonoid: Advances in Research* (J. B. Harborne and T. J. Mabry eds), Chapter X, Chapman and Hall, New York, 1982; (c) J. L. Ingham, in *Phytoalexins* (J. A. Bailly and J. W. Mansfield eds), p. 21, Blackie, Glasgow and London, 1982.
2. (a) M. Hagmann and H. Grisebach, *FEBS Lett.*, 175, 199 (1984); (b) G. Kochs and H. Grisebach, *Eur. J. Biochemistry*, 155, 311 (1986).
3. T. Hakamatsuka, H. Noguchi, Y. Ebizuka and U. Sankawa, *Chem. Pharm. Bull.*, 37, 249 (1989).
4. The reaction did not proceed without $HClO_4$ in the reaction medium. The addition of such strong acid is presumed to be indispensable to induce the enol form of flavanone as described later.
5. L. Farkas, A. Gottsegen and M. Nogradi, *J. Chem. Soc. Perkin I*, 1974, 305.

(Received in Japan 6 September 1990)

# United States Patent [19]

**Feuer et al.**

[11] **4,166,862**

[45] **Sep. 4, 1979**


[54] **ANIMAL FEED CONTAINING ANABOLIC ISOFLAVONES**


[75] Inventors: **Laszlo Feuer; Mihály Nógrádi; Agnes Géttsegen; Borbala Vermes; János Strelisky; Andras Wolfner; Lorant Farkas; Sandor Antus; Maria K. Tóth,** all of Budapest, Hungary

[73] Assignee: **Chinoin Gyogyszer es Vegyeszeti Termekek Gyara Rt.,** Budapest, Hungary



[21] Appl. No.: **470,444**

[22] Filed: **May 16, 1974**

**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 146,773, May 25, 1971, Pat. No. 3,833,730.

[51] **Int. Cl.**[2] ..... **A61K 31/35**

[52] **U.S. Cl.** ..... **424/283**

[58] **Field of Search** ..... 424/263, 283; 260/345.2



[56] **References Cited**

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*Primary Examiner*—Anna P. Fagelson
*Attorney, Agent, or Firm*—Karl F. Ross



[57] **ABSTRACT**

An animal feed containing 0.00002 to 0.1% by weight of:
2-methyl-7-methoxy-4'-nitroisoflavone,
7-[hydroxyethoxy]-2-methylisoflavone,
7-carbethoxy-2-methylisoflavone,
7-ethoxyisoflavone,
7-n-benzyloxy-2-methylisoflavone,
7-p-nitrobenzyloxyisoflavone,
7-p-nitrobenzyloxy-2-methylisoflavone,
7-p-chlorobenzyloxyisoflavone,
7-cetyloxy-2-methylisoflavone,
7-(2-octyloxy)-isoflavone,
7-benzyloxy-3',4'-dimethoxyisoflavone, and
7-hexadecyloxyisoflavone

as a weight-gain promoting anabolic ingredient.


**18 Claims, No Drawings**

# ANIMAL FEED CONTAINING ANABOLIC ISOFLAVONES

This application is a continuation-in-part of application Ser. No. 146,773, filed May 25, 1971 (Now U.S. Pat. No. 3,833,730) and is related to applications Ser. No. 371,560 filed June 19, 1973, now U.S. Pat. No. 3,907,830, and Ser. No. 374,056 filed June 27, 1973, now U.S. Pat. No. 3,864,362, both as continuations in part of application Ser. No. 146,773.

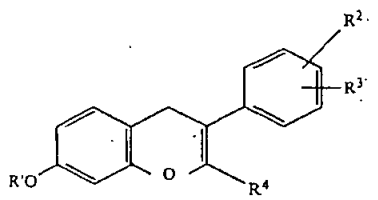
In order to reduce protein deficiency in nutrition or to terminate it thorough and significant research is being carried out throughout the world. One of the most obvious ways to achieve this objective is the use of additives to nutrients and feed, which improve the utilization of the nutrients introduced into the organism. In animal husbandry, these additives result in higher bodyweight increase for an identical feed consumption and breeding period.

However, it has been rather difficult to develop an appropriate substance for increasing animal body weight since the use of substances with hormonal effects and of antibiotics is not permitted in most of the countries.

Isoflavone compounds have been subjected to a detailed investigation from the aspect of their body weight increasing effect. These compounds are rather widespread in plants, and a great number of them show oestrogenic properties. (cf. Virtanen, A. J.: Angew. Chem. 70, 544, (1958); Virtanen, A. J., Hietala, P. K.: Acta Chem. Scand. 12, 579 (1958). Grazing animals become infertile on consuming certain varieties of clover; research into this problem has shown that genistein and daidzein present in the plants consumed by pasturing animals are responsible for this effect because of their marked oestrogenic action (Chang, E. W. et al.: Ann. N.Y. Acad. Sci. 61, 625, (1955).

For the determination of the oestrogenic effect of isoflavones a reliable method has been evolved by East, J. (J. Endocrin. 13, 94, (1955). Since that time a number of authors have dealt very thoroughly with this problem (Matrone, G. et al.: Nutrition 59, 235, (1956); Gabor, M.: Naturwiss. 46, 650, (1959); Crabbe P. et al.: J. Am. Chem. Soc. 85, 5258, (1958).

The present invention relates to an animal feed containing as an active ingredient at least one compound of the formula



(I)

or a salt thereof, in which formula $R^1$ represents a substituted or unsubstituted, saturated or unsaturated alkyl, group, $R^2$ and $R^3$ represent hydrogen, an alkoxy, nitro, halogen, sulpho or hydroxy group, and $R^4$ is hydrogen, an alkyl or—COOH group. More specifically R' is isopropyl, benzyl or halobenzyl, R and $R^3$ are hydrogen or nitro and $R^4$ is hydrogen, methyl or carboxy.

Preferably the ingredient is a compound selected from the group which consists of

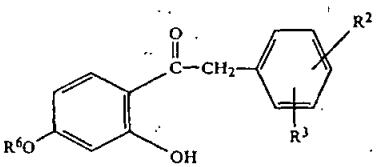
2-methyl-7-methoxy-4'-nitroisoflavone,
7-[hydroxyethoxy]-2-methylisoflavone,
7-carbethoxy-2-methylisoflavone,
7-(3-N-pyridyl)-propoxyisoflavone,
7-ethoxyisoflavone,
7-benzyloxy-2-methylisoflavone,
7-p-nitrobenzyloxyisoflavone,
7-p-nitrobenzyloxy-2-methylisoflavone,
7-p-chlorobenzyloxyisoflavone,
7-cetyloxy-2-methylisoflavone,
7-(2-octyloxy)-isoflavone,
7-benzyloxy-3', 4'-dimethoxyisoflavone, and
7-hexadecyloxyisoflavone

The composition according to the present invention can be favorably applied as a feed additive. In that case one of the compounds of formula I, or a salt thereof, is added to the feed in amounts from 0.00002 to 0.1%, prior to or after the admixture of further additives.

The active ingredient according to the present invention may bear, if desired, also substituents on the alkyl group $R^1$. Substituents in this position may be a heterocycle, a dialkylamino group, a carbethoxy, hydroxyalkyl, alkoxy-alkyl or aryl or a substituted aryl group. The $R^1$ alkyl group may be substituted with a nitrogen containing heterocyclic group.

If desired, the compound is mixed with further additives. Substances with biological activity such as vitamins, aminoacids, choline chloride, salts of mineral acids, trace elements and other known substances of biological importance are suitable. The feed additive can be applied in premixes, in admixture with other components possessing biological effects. As further additives various diluents, solvents, sliding and molding substances, and carriers may be used. The feed additive can be mixed with the feed as a powder, granulate, powder mixture, emulsion or suspension. It is also possible to use the feed composition in mixtures added to the drinking water of the animals.

The new compounds can be made by a process wherein ketones of the formula



(II)

(a) are reacted with an alkyl orthoformate in the presence of a basic catalyst, or

(b) are reacted with hydrogen cyanide and/or cyanides in the presence of hydrogen halide, or

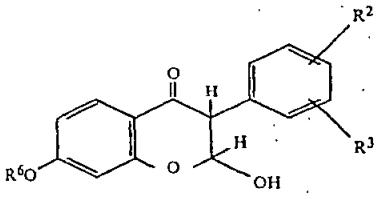
(c) are reacted with an alkyl formate in the presence of an alkali metal, or

(d) are reacted with an alkyl oxalyl halogenide, followed, if desired, by saponification and/or decarboxylation of the obtained isoflavone ester, or

(e) are reacted with an organic anhydride, or

(f) are reacted with a N, N-dialkyl acid amide in the presence of phosphorus oxychloride, or whereby

(g) 2-hydroxy-isoflavanone derivatives of the formula



(III)

are dehydrated, and finally, if necessary, the $R^6$ group is converted into an $R^5$ group and/or the compounds are converted into salts or respectively, liberated from their salts, in the formulae

$R^2$ and $R^3$ represent hydrogen, a halogen, alkoxy, nitro, sulpho or hydroxy group,

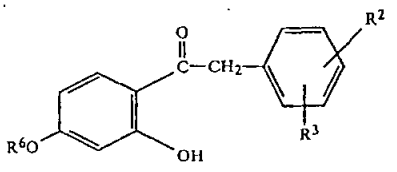
$R^4$ is hydrogen, an alkyl or —COOH group,

$R^5$ is an optionally substituted and/or unsaturated alkyl group with a carbon chain longer than two carbon atoms or carrying a substituent, and which alkyl group may also be unsaturated, $R^6$ is hydrogen, or, if desired, a substituted alkyl group or acyl group.

In carrying out variant (a) of the process according to the present invention the preferred method is to react an appropriately substituted ketone with an orthoformic ester in an aprotic solvent of higher boiling point. Pyridine, dimethyl formamide or diethyleneglycol dimethylether may be used as solvents, while preferably piperidine, morpholine, pyrrolidine and other secondary amines may serve as basic catalysts.

In carrying out variant (b), the preferred method is to react the ketones with hydrogen cyanide, in an aprotic solvent, in the presence of dry gaseous hydrochloric acid or of other hydrogen halides and Lewis acids, respectively. In this reaction, also aprotic solvents of non-basic nature, preferably diethylether or other dialkylethers can be applied. Zinc chloride or other Lewis acids may be used as catalysts. The reaction is carried out with hydrogen cyanide or with one of its appropriate salts, preferably with zinc cyanide. The mixture may be saturated with dry gaseous hydrochloric acid, and lastly, the formed substituted $\alpha$-formimino-2-hydroxy-phenyl-benzyl-ketone hydrochlorides are decomposed by treatment with water.

In carrying out variant (c) of the process according to the invention, ketones of the formula



are reacted with alkyl formates in the presence of an alkali metal. A preferred method is to dissolve an appropriately substituted 2-hydroxy-phenyl-benzyl-ketone in ethyl formate, and adding the solution dropwise to powdered sodium metal, then decomposing the reaction mixture with water, and separating the formed isoflavone.

According to variant (d) of the process according to the present invention appropriately substituted 2-hydroxy-phenyl-benzyl ketones are reacted with alkyl oxalyl halides. The formed 2-carbalkoxy-isoflavone derivative can be converted, if desired, into an isoflavone derivative unsubstituted in position 2, by hydrolysis of the ester group followed by decarboxylation. This process can be carried out preferably with methyl or ethyl oxalyl chloride in the presence of a basic acid-binding agent in an appropriate aprotic solvent (such as preferably pyridine or another tertiary amine capable of binding acids).

According to variant (e) of the process according to the invention, the appropriately substituted 2-hydroxy-phenyl-benzyl ketone is reacted with organic acid anhydrides in the presence of a basic catalyst. The anhydrides of acetic, propionic or benzoic acids can be used as organic acid anhydrides. The anhydride is heated in the presence of a basic catalyst, preferably of the alkali metal salt of the acid component of the anhydride or of a tertiary amine, in the absence of solvents or in an aprotic solvent of higher boiling point such as pyridine or dimethyl formamide.

On carrying out variant (f) of the process according to the invention, the ketone is reacted with N, N-dialkyl acid amides in the presence of phosphorus oxychloride, preferably in a way that the appropriately substituted 2-hydroxy-phenyl-benzyl ketone is heated with the N,N-dialkyl acid amide (dimethyl formamide, dimethyl acetamide) and phosphorus oxychloride, and using the N,N-dialkyl acid amide itself as a solvent.

In carrying out variant (g) of the process according to the invention, 2-hydroxy-isoflavanones of the formula IV are dehydrated by heating alone or in an acidic medium in a polar solvent.

In the course of the process according to the present invention, in the first step, from the compounds of the formula III or IV, derivatives can be obtained in which the substituent $R^6$ is not the $R^5$ group desired to be present in the end product. In these cases the $R^6$ group may be converted into an $R^5$ group. This operation can be carried out by the partial or complete alkylation of mono- and poly-hydroxy-isoflavones, respectively. The alkylation can be preferably performed with alkyl or substituted alkyl halides, alkyl sulphates, olefins and epoxides, preferably in a way that the mentioned alkylating agents are heated with the isoflavones to be alkylated, in appropriate solvents, ketones, dimethyl formamide or ethers having a longer carbon chain, in the case of haloid compounds preferably in the presence of an acid-binding agent such as an alkali carbonate, and in the case of alkyl bromides and alkyl chlorides preferably in the presence of an alkali iodide as additive.

The mentioned operation can also be carried out by the partial or complete deacylation and, respectively, the partial or complete dealkylation of acyloxy- and polyacyloxy-, alkyloxy- and polyalkyloxy-isoflavones. Acyloxy- and polyacyloxy isoflavones are formed when the procedure according to variant (e) is carried out with di- and polyhydroxy-phenyl-benzyl ketones, respectively, carrying a 2-positioned hydroxy group. Deacylation is preferably performed in an acidic or alkaline medium in the presence of a polar solvent.

The operation can also be performed by the decarboxylation of isoflavone-2-carboxylic acids. Isoflavone-2-carboxylic acids are formed in variant (d) of the process according to the invention and their decarboxylation may be preferably carried out by heating them in the presence of catalysts such as powdered copper or in the absence of catalysts.

On the basis of the abundant literature on studies concerning the oestrogenic properties of isoflavones, and of the data of our research in this field, we were

able to produce isoflavone compounds as described which are devoid of any oestrogenic effects. According to the present invention, the compounds
2-methyl-7-methoxy-4'-nitroisoflavone,
7-[hydroxyethoxy]-2-methylisoflavone,
7-carbethoxy-2-methylisoflavone,
7-(3-N-pyridyl)-propoxyisoflavone,
7-ethoxyisoflavone,
7-n-benzyloxy-2-methylisoflavone,
7-p-nitrobenzyloxyisoflavone,
7-p-nitrobenzyloxy-2-methylisoflavone,
7-p-chlorobenzyloxyisoflavone,
7-cetyloxy-2-methylisoflavone,
7-(2-octyloxy)-isoflavone,
7-benzyloxy-3', 4'-dimethoxyisoflavone, and
7-hexadecyloxyisoflavone
show a marked weight-gain increasing effect which essentially exceeds that of isoflavone compounds possessing definitely oestrogenic effects, and this weight-gain increasing effect is associated with a reduction or complete absence of oestrogenic effects.

Thus, in the course of our observations in discovering in a field still not described in literature, i.e. in the group non-oestrogenic isoflavones, a very interesting novel biological effect, that of increasing the weight yield has been found. Beside this action, the compound possesses an anatoxic effect free of androgen effects, as well (proved by N-retention and by the musculus levator test or by measuring the skeletal muscle increasing effect).

Certain compounds described in this disclosure are also suitable for other applications in animal husbandry such as for increasing the yield of milk, egg and wool, for the improvement of the economy of fish production in fisheries, as well as in the breeding of fur-farm animals, in game economy etc.

Concerning the effect of the compounds the pharmacological tests given below are of special interest.

Test of anabolic effect: The investigation was carried out with castrated rats by means of the musculus levator ani test and vesicula seminalis test. The preparations were administered orally for a period of three weeks. The tests were performed by the method of Eisenberg and Gordan (Eisenberg, E., Gordan, G. S. J.: J. Pharmacol. 99, 38 (1950). In addition, also the weight of the prepared diaphragm of the animals was established. According to these tests, the weight of musculus levator ani rose by a Student significance of p 0.01, the weight of vesicula seminalis did not increase while the weight of the prepared diaphragm of the animals increased by a Student significance of p 0.05. On the basis of these results the preparations proved to be anabolic and free of androgen effect.

During the tests, a total of 30 mg/kg of active ingredient was administered to the animals.

The investigation of nitrogen retention was carried out also with rats. Under a systematic treatment, the nitrogen excretion of the treated animals decreased on the 20th and 30th day, respectively, by a significance of p 0.05.

The results of these investigations similarly points to the anabolic effect.

Exeminations with S-35 labelled methionine showed that with treatment, increased methionine incorporation takes place in the muscle tissues of the treated animals.

The muscle-activity-increase was investigated by the forced swimming test of rats. The animals were forced to swim in water of 29° C. with a load of 3 g/100 g body weight. The calorie content and quantity of feed administered was the same as with control animals.

The difference between the periods of forced swimming until exhaustion of the control animals and the animals treated for 45 days and forced to swim daily was 33 minutes (the control animals were subjected to identical treatment with the exception of the active ingredient), i.e. the swimming period (performance) of the control animals increased from 166 to 196 minutes while that of the treated animals from 162 to 225 minutes.

These experiments were carried out by administering daily doses of 5 mg/kg of body weight.

In further experiments we succeeded in partially suppressing the anticatabolic effect of cortisone with the compound.

On examining the data of the analysis of body weight it was found that the weight increase of the muscle tissue was specifically greater than that of the fat tissue, and that fat content of the muscle tissue decreased, while that of proteins rose.

The acute toxicity tests proved the full inocuity of the preparation. During a 48 hour period of observation no mice died on administering orally 4000 mg/kg of body weight doses or subcutaneously 3500 mg/kg doses. In rat tests, no perceptible alterations were observed during a 48 hour period, after administering orally or subcutaneously 3500 mg/kg of body weight doses.

On dogs, no alterations were observed during a week of administering 3500 mg/kg body weight doses.

The subacute toxicity tests were performed on rats. When administering daily doses of 200 mg/kg of body weight and 500 mg/kg of body weight orally, no alterations could be observed after one month of test period. Similar results were obtained in the subacute tests carried out with mice.

As regards to chronic toxicity up to the present, the three-month chronic toxicity tests have been completed. After administering daily 100 mg/kg of body weight and 10 mg/kg of body weight doses for three months to male and female rats, no perceptable alterations were observed (full blood investigation, histological and other clinical tests).

Similarly negative results were obtained in the toxicity test with dogs after the first 3 months of observation (here the applied doses were 20 mg/kg of body weight and 50 mg/kg of body weight).

The oestrogenic effect of the compounds was investigated by the uterus test on infantile mice, after oral and subcutaneous administration. No oestrogenic effects were observed.

After administering daily 5 mg/kg of body weight of preparations to chickens for 30 days, the endocrine glands of the experimental animals were subjected to a detailed histological investigation. No perceivable alterations were observed.

The weight-gain increasing effect induced by doses of 2 g/100 kg of feed was in the various animal species as follows:
8 to 15% in calves
7 to 10% in cattle
7 to 10% in hogs
8 to 20% in poultry
10 to 20% in rabbits
8 to 12% in guinea pigs

The periods of administration varied from one to four months, depending on the animal species and conditions of breeding. The treated animals did not obtain greater

amounts of feed than the control tests during the treatment period. Moreover, in several cases some saving of feed was attained in spite of the weight-gain increasing effect.

It was observed during the treatment period that the experimentally treated animals showed an increased vitality, and that the weight increase was mainly due to an increase of the muscle mass. This was particularly evident in pig fattening trials when in case of bacon pigs the ratio of pigs of class A, low in fat, was significantly higher.

In rats, also the effect exerted on the reproductive organs was separately examined. The capability of reproduction and the number of brood were in case of males and females pretreated with the active ingredient the same as that on untreated controls.

In an investigation on the uptake and excretion of C-14 labelled isoflavones it was found that the uptake is rather quick both in case of oral and of intramuscular administration, half of the ingredient introduced was excreted with urine while the other half with faeces.

In a number of organs, activity detectable by radiography was present 48 hours after completion of the treatment.

## EXAMPLE 1

27 g of 2-hydroxy-4-isopropyloxy-phenyl-benzyl ketone, 22 g of ethyl orthoformate and 5 g of morpholine are boiled for 8 hours in 200 ml of dimethyl formamide. The ethanol formed during the reaction is removed through a fractionation head. Then the major part of solvent is distilled off in a vacuum and the residue is diluted with dilute aqueous hydrochloric acid. The crude product is filtered and recrystallized from acetone, yielding 24 g of 7-isopropyloxy-isoflavone of m.p. 115°–117° C.

On applying a similar method, 7-n-butyloxy-isoflavone, m.p. 152°–153° C., can be prepared from 4-n-butyloxy-2-hydroxy-phenyl-benzyl-ketone (m.p. 71°–73° C.), and 7-n-amyloxy-isoflavone, m.p. 142°–143° C. from 4-n-amyloxy-2-hydroxy-phenyl-benzyl ketone (m.p. 72°–75° C.).

## EXAMPLE 2

28.6 g of 2-hydroxy-4-n-butyloxy-phenyl-benzyl ketone are dissolved in 50 ml of anhydrous ether, 25 g of zinc cyanide are added, and the solution is saturated, under cooling, with dry hydrogen chloride gas. After allowing the mixture to stand for 24 hours, the solvent is decanted from the separated oil, the oil triturated with ether, the ether is decanted, and the residue is heated with 1000 ml of water for 30 minutes on a water bath. The product which precipitates on cooling is filtered, and recrystallized from a mixture of methanol and acetone, thus 15 g of 7-n-butyloxy-isoflavone are obtained.

7-Isopropyloxy-isoflavone and 7-n-amyloxy-isoflavone already described in Example 1 can be produced in a similar way.

## EXAMPLE 3

A solution of 18 g of 2-hydroxy-4-isopropyloxy-phenyl-benzyl ketone in 150 g of ethyl formate is added in small portions under cooling to 9 g of powdered sodium. After allowing the reaction mixture to stand for some hours, it is treated with ice water containing hydrochloric acid, the ethyl formate is distilled off, the residual aqueous mixture is boiled for an hour, and the product precipitated on cooling is recrystallized from acetone, thus 11 g of 7-isopropyloxy-isoflavone are obtained, m.p. 115°–117° C. In a similar way, 7-n-butyloxy-isoflavone and 7-n-amyloxy-isoflavone already described in Example 1 can also be produced.

## EXAMPLE 4

To a solution of 13.5 g of 2-hydroxy-4-isopropyloxy-phenyl-benzyl ketone in 120 ml of pyridine, 11 ml of ethyloxalyl chloride are added under cooling. After allowing the reaction mixture to stand for a day, it is diluted with water, extracted with chloroform and repeatedly shaken with a 10% aqueous hydrochloric acid solution. On evaporating the solution, the residue is treated for 5 hours with a mixture of 100 ml of methanol and 50 ml of a 10% aqueous solution of sodium hydroxide, the methanol is distilled off, and the aqueous solution is acidified. The product is filtered, thoroughly dried and, after addition of 5 g of powdered copper, heated to 250° C. On completion of the evolution of gas, the residue is crystallized from methanol, yielding 5 g of 7-isopropyloxy-isoflavone, m.p. 116°–117° C.

## EXAMPLE 5

28.6 g of 2-hydroxy-4-n-butyloxy-phenyl-benzyl ketone and 25 g of anhydrous sodium acetate are boiled for 14 hours with 120 ml of acetic anhydride under a reflux condenser. The reaction mixture is poured into water, allowed to stand and the precipitated substance is recrystallized from a mixture of methanol and acetone, yielding 25 g of colorless crystals of 7-n-butyloxy-2-methyl-isoflavone, m.p. 91°–93° C. In a similar way, 7-isopropyloxy-2-methyl-isoflavone, m.p. 152°–154° C.; 7-n-amyloxy-2-methyl-isoflavone, m.p. 87°–89° C., and 2-methyl-3',4',5,7-isoflavone, m.p. 214°–215° C. can also be produced.

## EXAMPLE 6

16 g of phosphorus oxychloride are mixed with 50 ml of dimethyl formamide with cooling. After 15 minutes, 27 g of 2-hydroxy-4-isopropyloxy-phenyl-benzyl ketone are added, and the mixture is boiled for 18 hours under reflux condenser. On dilution with water, the precipitate is filtered, dried, boiled with 200 ml of methanol, and the methanolic extract is evaporated to a small volume. On recrystallizing the separated crude product from acetone, 10 g of 7-isopropyloxy-isoflavone described in Example 1 are obtained.

## EXAMPLE 7

23.8 g of 7-hydroxy-isoflavone in 200 ml of anhydrous acetone are boiled, under stirring, with 18 g of n-hexyl bromide, 18 g of potassium carbonate and 1 g of potassium iodide for 72 hours under reflux condenser. The inorganic salts are removed by filtration, the filtrate is subjected to steam distillation in order to remove acetone and excess reagent, the precipitate is filtered and recrystallized from acetone, yielding 20 g of 7-n-hexyloxy-isoflavone, m.p. 120°–122° C. In a similar way, also 7-isopropyloxy-isoflavone, 7-n-butyloxy-isoflavone, and 7-n-amyloxy-isoflavone already characterized in Example 1, 7-isopropyloxy-2-methyl-isoflavone, 7-n-butyloxy-isoflavone and 7-n-amyloxy-isoflavone already characterized in Example 5, and

7-n-propyloxy-isoflavone, m.p. 162°–164° C.,
7-n-propyloxy-2-methyl-isoflavone, m.p. 120°–122° C.,
7-n-hexyloxy-2-methyl-isoflavone, m.p. 62°–64° C.,
7-benzyloxy-2-methyl-isoflavone, m.p. 139°–141° C.,

7-(4-chlorobenzyloxy)-isoflavone, m.p. 182°–184° C.,
7-(4-chlorobenzyloxy)-2-methyl-isoflavone, m.p. 154°–156° C.,
7-(4-nitrobenzyloxy)-2-methyl-isoflavone, m.p. 201°–203° C.,
7-carbethoxy-methoxy-3',4'-dimethoxy-isoflavone, m.p. 149°–152° C.,
7-carbethoxy-methoxy-3',4'-dimethoxy-2-methyl-isoflavone, m.p. 132°–134° C.,
7-(2-ethoxy-ethoxy)-isoflavone, m.p. 139°–140° C.,
7-(2ethoxy-ethoxy)-2-methyl-isoflavone, m.p. 104°–105° C.,
7-(2-ethoxy-ethoxy)-3',4'-dimethoxy-2-methyl-isoflavone, m.p. 132°–133° C.,
7-(2-hydroxy-ethyoxy)-isoflavone, m.p. 144°–146° C.,
7-(2-hydroxy-ethoxy)-2-methyl-isoflavone, m.p. 151°–153° C.,
7-(2-hydroxy-ethoxy)-3',4'-dimethoxy-isoflavone, m.p. 145°–156° C., and
7-(3-chloropropyloxy)-isoflavone, m.p. 137°–138° C. can be prepared.

## EXAMPLE 8

12 g of 7-hydroxy-isoflavone are boiled for 2 hours under reflux condenser with 10 g of potassium carbonate and 9 g of sec-butyl bromide in 40 ml of dimethyl formamide. On pouring the reaction mixture on water, the separated product is recrystallized from acetone, yielding 12 g of 7-sec-butyloxy-isoflavone, m.p. 87°–89° C. In a similar way, also 7-sec-butyloxy-2-methyl-isoflavone, m.p. 107°–109° C., and all the other isoflavone derivatives described in Example 7 can be prepared.

## EXAMPLE 9

10 g of 5,7-diacetoxy-2-methyl-isoflavone of m.p. 177°–178° C. prepared by the method described in Example 5 are boiled for 10 minutes with 50 ml of methanol and with a solution of 5 g of sodium hydroxide in 30 ml of water. On acidifying the solution with a 10% aqueous sulphuric acid, the precipitated product is filtered, affording 6 g of 5,7-dihydroxy-2-methyl-isoflavone, m.p. 228°–229° C.

## EXAMPLE 10

Formulation of poultry raising feed:

| | |
|---|---|
| Maize | 40.0 kg |
| Feed wheat | 20.0 kg |
| Bran | 6.0 kg |
| Extracted soybeans | 13.0 kg |
| Extracted groundnut | 11.5 kg |
| Powdered alfalfa | 1.4 kg |
| Extracted sunflower seed | 4.0 kg |
| Potassium-phosphorus composite "Foszkal" | 0.5 kg |
| Feed lime | 2.3 kg |
| Feed sodium chloride | 0.3 |
| Vitamin premix 2 | 0.5 kg |
| Mineral premix II | 0.5 kg |
| Total: | 100.0 kg |

+2 g of 7-isopropyloxy-isoflavone/100 kg of feed.

## EXAMPLE 11

Formulation of pig feed:

| | |
|---|---|
| Bran | 22.0 kg |
| Extracted soybeans | 15.0 kg |
| Extracted groundnut | 6.0 kg |
| Powdered linseed | 14.0 kg |
| Powdered alfalfa | 4.0 kg |
| Powdered milk | 15.0 kg |
| Fish meal | 10.0 kg |
| Yeast | 2.0 kg |
| Feed lime | 6.0 kg |
| Feed soidum chloride | 1.5 kg |
| Vitamin premix | 3.0 kg |
| Mineral premix | 1.5 kg |
| Total: | 100.0 kg |

+2 g of 7-isopropyloxy-isoflavone/100 kg of feed.

## EXAMPLE 12

10 g of 7-hydroxy-2-methyl-isoflavone, 10 g of anhydrous potassium carbonate, 1 g of potassium iodide and 12.5 g of benzyl chloride are boiled in 200 ml of anhydrous acetone for 2 hours with stirring, under a reflux condenser. On subjecting the mixture to steam distillation, the crude product precipitating from the water is filtered, dried, and recrystallized from a mixture of 100 ml of methanol and 40 ml of acetone, affording white needle crystals of 7-benzyloxy-2-methyl-isoflavone, m.p. 139°–141° C.

## EXAMPLE 13

(a) To a solution of 12 g of 2-hydroxy-4-methoxyphenyl-benzyl ketone in 120 ml of anhydrous pyridine, 11 ml of ethoxalyl chloride are added dropwise, with stirring and cooling. After standing overnight, the mixture is poured onto water, extracted with chloroform, washed with 10% hydrochloric acid until free of pyridine, then with water until it is free of acid. After drying and evaporation an oil, 7-methoxy-2,3-dihydro-isoflavone-2-ol-2-carboxylic ethylester is obtained which is subsequently heated with 120 ml of glacial acetic acid and 12 ml of concentrated hydrochloric acid for half an hour on a water bath. On cooling and pouring onto water, 2-carbethoxy-7-methoxy-isoflavone precipitates. Recrystallized from methanol, m.p. 130°–132° C.

(b) 13 ml of 2 N sodium hydroxide are added to a solution of 8.25 g of the ester obtained in paragraph (a) in 240 ml of acetone, and the mixture is stirred for 5 hours. After removing the major part of acetone, the residue is acidified with 10% hydrochloric acid. Addition of some water precipitated snow-white 7-methoxy-isoflavone-2-carboxylic acid which was separated. M.p. 241°–243° C. (with decomposition).

## EXAMPLE 14

10.5 g of 7-hydroxy-isoflavone in 200 ml of anhydrous acetone are boiled for 2 hours with 11.8 g of p-nitrobenzyl iodide in the presence of 5.7 g of anhydrous potassium carbonate under reflux condenser. On distilling off about half of the volume of acetone, the residue is poured onto 1000 ml of water. The precipitating crude product is subjected to suction and recrystallized from glacial acetic acid, affording light yellow plates of 7-p-nitrobenzyloxy-isoflavone, m.p. 225°–226° C.

Test results for anabolic isoflavone derivative

Test animals: chicks

Composition of the poultry starter feed

Corn = 60%; Soy (45%) 20%; Corn meal 2%; Fishmeal (65%) 10%; Yeast 3.3%; Calcium phosphate 0.6%; Calcium 2.3%; Salt 0.3%; Vitamin mixture I 1.0%; Mineral mixture I 0.5%.

Content:

Dry 86%; Starch (kg/100 kg) 69.5; Raw eggwhite 19.5%; Digestible raw eggwhite 17.1%.

Composition of the poultry maintenance feed:

Corn 50%; Wheat 14.9%; Soy (45%) 12.5%; Hazel nut bits 9%; Corn meal 2.0%; Fish meal (65%) 4.5%; Meat meal (45%) 3.0%; Calcium phosphate 1.0%; Calcium 1.8%; Salt 0.3%; Vitamin mixture II 0.5%; Mineral mixture I 0.5%.

Content:

Dry 86%; Starch (kg/100 kg) 69.5%; Raw egg white 19.5%; Digestible raw egg white 17.1%.

Composition of the vitamin mixtures

| | Vitamin mixture I. 0.5% | Vitamin mixture II. 0.5% |
|---|---|---|
| A-Vitamin | 2,000,000 IU | 1,200,000 IU |
| D-3 Vitamin | 400,000 IU | 300,000 IU |
| E-Vitamin | 4,000 IU | 2,000 IU |
| K-3 Vitamin | 400 mg | 400 mg |
| B-1 Vitamin | 400 mg | 200 mg |
| B-2 Vitamin | 800 mg | 700 mg |
| B-3 Vitamin | 1,200 mg | 2,000 mg |
| B-6 Vitamin | 400 mg | 500 mg |
| B-12 Vitamin | 10 mg | 4 mg |
| Niacin | 4,000 mg | 5,000 mg |
| Cholinchloride | 100,000 mg | 100,000 mg |
| Bacitracin | 6,000 mg | 4,000 mg |
| Ethoxy-methyl-quinoline | 25,000 mg | 25,000 mg |
| Furazolidon | 20,000 mg | —mg |
| Ardinon | — | 25,000 mg |

Composition of mineral mixture I

| | |
|---|---|
| Manganese | 20,000 mg |
| Iron | 2,000 mg |
| Zinc | 8,000 mg |
| Copper | 400 mg |
| Iodine | 150 mg |
| Ethoxy-methyl-quinoline in 100,000 g bran. | 100 mg |

The material is used as a starter feed and as a maintenance feed in a concentration of 2 g/100 kg feed. The mixing is carried out in two steps: a first mixing to obtain a concentration of 1000 ppm and a second mixing to 20 ppm.

After the mixing the concentration was tested qualitatively as a control.

During the tests the temperature and humidity of the atmosphere was held constant. The length of the tests was 4–5 weeks and the weight of the chicks was checked once a week. In the first week the animals were given the starter feed, in the second week starter feed and maintenance feed in equal portions, and from the third week pure maintenance feed.

The results of the tests with the chicks are assembled in the following Table:

| | No. of animals | Age of the animals at the start of the tests | Length of the treatment | Starting weight averages | Final weight averages | Weight change % over control | Feed rating |
|---|---|---|---|---|---|---|---|
| | Number | Day | Weeks | g | g | + | % |
| 2-Methyl-7-methoxy-4'-nitro-isoflavone | 20 | 7 | 4 | 95 | 469 | +6.3 | 94.5 |
| 7-[2-Hydroxyethoxy-]-2-methyl-isoflavone | 20 | 7 | 4 | 95 | 470 | +6.4 | 94.1 |
| 7-Carbethoxy-2-methyl-isoflavone | 20 | 7 | 4 | 95 | 450 | +1.8 | 98.6 |
| Control group | 20 | 7 | 4 | 95 | 442 | — | — |
| 7-3-(N-Pyridyl)-propoxy-isoflavone | 20 | 3 | 4 | 41 | 353 | +3 | 98 |
| 7-Ethoxy-isoflavone | 20 | 3 | 4 | 41 | 353 | +6 | 95 |
| 7-Isopropoxy-isoflavone | 20 | 3 | 4 | 42 | 374 | +9 | 93 |
| Control group | 20 | 3 | 4 | 41 | 343 | — | — |
| 7-n-Benzyloxy-2-methyl-isoflavone | 20 | 5 | 4 | 49 | 390 | +8 | 92 |
| 7-p-Nitrobenzyloxy-isoflavone | 20 | 5 | 4 | 49 | 386 | +7 | 94 |
| 7-p-Nitrobenzyloxy-2-methyl-isoflavone | 20 | 5 | 4 | 49 | 372 | +3 | 98 |
| 7-p-Chlorobenzyloxy-isoflavone | 20 | 5 | 4 | 49 | 372 | — | — |
| Control group | 20 | 5 | 4 | 49 | 361 | — | — |
| 7-Cetyloxy-2-methyl-isoflavone | 20 | 7 | 4 | 100 509 | +5.1 | 97 | |
| Control group | 20 | 7 | 4 | 99 | 485 | — | — |
| 7-Benzyloxy-3', 4'-dimethoxy-isoflavone | 20 | 7 | 5 | 96 | 1087 | +9.4 | 95.5 |
| Control group | 20 | 7 | 5 | 98 | 993 | — | — |
| 7-(2-Octyloxy)-iso- | | | | | | | |

-continued

| | No. of animals | Age of the animals at the start of the tests | Length of the treatment | Starting weight averages | Final weight averages | Weight change % over control | Feed rating |
|---|---|---|---|---|---|---|---|
| | Number | Days | Weeks | g | g | + | % |
| flavone | 30 | 10 | 5 | 112 | 1023 | +2.2 | 98.5 |
| 7-Hexadecyloxy-isoflavone | 30 | 10 | 5 | 112 | 1020 | +2.2 | 98.6 |
| Control group | 30 | 10 | 5 | 113 | 1001 | — | — |

We claim:

1. An animal feed containing 0.00002 to 0.1% by weight of 2-methyl-7-methoxy-4'-nitroisoflavone as a weight-gain-promoting anabolic ingredient.

2. An animal feed containing 0.00002 to 0.1% by weight of 7-(hydroxyethoxy)-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

3. An animal feed containing 0.00002 to 0.1% by weight of 7-carbethoxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

4. An animal feed containing 0.00002 to 0.1% by weight of 7-benzyloxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

5. An animal feed containing 0.00002 to 0.1% by weight of 7-p-nitrobenzyloxyisoflavone as a weight-gain-promoting anabolic ingredient.

6. An animal feed containing 0.00002 to 0.1% by weight of 7-p-nitrobenzyloxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

7. An animal feed containing 0.00002 to 0.1% by weight of 7-p-chlorobenzyloxyisoflavone as a weight-gain-promoting anabolic ingredient.

8. An animal feed containing 0.00002 to 0.1% by weight of 7-cetyloxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

9. An animal feed containing 0.00002 to 0.1% by weight of 7-benzyloxy-3',4'-dimethoxyisoflavone as a weight-gain-promoting anabolic ingredient.

10. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 2-methyl-7-methoxy-4'-nitroisoflavone as a weight-gain-promoting anabolic ingredient.

11. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-hydroxy-ethoxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

12. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-carbethoxy-2-methyl-isoflavone as a weight-gain-promoting anabolic ingredient.

13. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-benzyloxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

14. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-p-nitrobenzyloxy-isoflavone as a weight-gain-promoting anabolic ingredient.

15. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-p-nitrobenzyloxy-2-methyl-isoflavone as a weight-gain-promoting anabolic ingredient.

16. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-p-chlorobenzyloxyisoflavone as a weight-gain-promoting anabolic ingredient.

17. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-cetyloxy-2-methylisoflavone as a weight-gain-promoting anabolic ingredient.

18. In a method of feeding animals the improvement which comprises adding to the animal feed 0.00002 to 0.1% by weight of 7-benzyloxy-3',4'-dimethoxyisoflavone as a weight-gain-promoting anabolic ingredient.

* * * * *

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(54) **Basische Äther, diese enthaltende pharmazeutische Zubereitungen und Verfahren zu ihrer Herstellung.**

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**JOURNAL FÜR PRAKTISCHE CHEMIE, Band 311, 1969 Leipzig J. KLOSA "Synthesen neuer Aminoalkyläther von 7-Hydroxy-chromonen" Seiten 183 bis 186**

**Die Akte enthält technische Angaben, die nach dem Eingang der Anmeldung eingereicht wurden und die nicht in dieser Patentschrift enthalten sind.**

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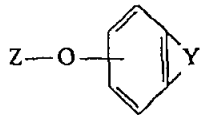
**EP 0 031 885 B1**

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BUNDESDRUCKEREI BERLIN

**Beschreibung**

Die Erfindung betrifft neue basische Äther der allgemeinen Formel I



(I)

worin

| | | |
|---|---|---|
| Z | $CH_3-NR^2-CH_2CH_2-CHR^1-$, | |
| | (1-$R^2$-3-piperidyl)-$CHR^3-$, | |
| | (1-$R^2$-2-piperidyl)-$CH_2-CHR^3-$ oder | |
| | 1-$R^2$-3-$R^4$-4-hexahydroazepinyl, | |
| $R^1$ | Cyclopropyl oder Ar, | |
| $R^2$ | H, Alkyl mit 1–4 C-Atomen, Allyl, Cycloalkylalkyl mit 4–8 C-Atomen oder Benzyl, | |
| $R^3$ | H oder Ar, | |
| $R^4$ | H oder Alkyl mit 1–4 C-Atomen, | |
| Y | $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ oder $-CH_2-CHQ^1-CHQ^2-CO-$, | |
| $Q^1$ und $Q^2$ | jeweils H, Alkyl mit 1–4 C-Atomen, Cycloalkyl mit 3–6 C-Atomen oder Ar und | |
| Ar | Phenyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl | |

bedeuten, worin jedoch

$R^1$ Cyclopropyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl

bedeutet, wenn gleichzeitig

Z eine $(CH_3)_2N-CH_2CH_2-CHR^1$-Gruppe in 7-Stellung und
Y $-O-C(C_6H_5)=CH-CO-$ oder $-O-C(C_6H_5)=C(CH_3)-CO-$

bedeuten, sowie ihre physiologisch unbedenklichen Säureadditionssalze.

Der Erfindung lag die Aufgabe zugrunde, neue Verbindungen aufzufinden, die zur Herstellung von Arzneimitteln verwendet werden können. Diese Aufgabe wurde durch die Bereitstellung der Verbindungen der Formel I gelöst.

Es wurde gefunden, daß die Verbindungen der Formel I und ihre physiologisch unbedenklichen Säureadditionssalze wertvolle pharmakologische Eigenschaften besitzen. So zeigen sie insbesondere Wirkungen auf das Zentralnervensystem, vor allem antidepressive Wirkungen. Im einzelnen kann eine reserpinantagonistische Wirkung (feststellbar z. B. an Mäusen gegenüber Reserpin in Anlehnung an die Methode von Askew, Life Science, Bd. 10 [1963], Seiten 725–730), eine antikataleptische Wirkung (feststellbar z. B. an Ratten gegenüber Tetrabenazin in Anlehnung an die Methode von Giurgea et al., Medicina Experimentalis, Bd. 9, [1963], Seiten 249–262) und eine antiptotische Wirkung (feststellbar z. B. gegenüber Reserpin in Anlehnung an die Methodik von Domenjoz und Theobald, Arch. int. pharmacodyn., Bd. 120 [1959], Seite 450 ff., mit der Bewertung nach Rubin et al., J. Pharmacol. Exp. Therap., Bd. 120 [1957], Seiten 125–136) nachgewiesen werden. Weiterhin kann die Wirkung des 5-Hydroxytryptophans bei Mäusen (Methode: ähnlich wie Ross et al., Acta pharmacol. et toxicol., Bd. 39 [1976], Seiten 152–166) potenziert werden und die zentralen Auswirkungen einer Erregung und Temperatursteigerung, die D-Amphetaminsulfat (z. B. 1,5 mg/kg s.c., 1 Stunde nach der Prüfsubstanz verabfolgt, die ebenfalls s.c. appliziert wird) und Aggregation (Zusammensetzung von 5 Ratten in einem Glas) auslösen (Methodik nach Müller-Calgan et al. in Zippel, H. P. (Ed.): Memory and Transfer of Information, Plenum Press, New York–London, 1973, Seiten 87–125) können, erhöht und/oder verlängert werden. Die Substanzen haben einen Einfluß auf die biogenen Amine des ZNS. So führen sie z. B. in vitro zur Aufnahmehemmung von Noradrenalin, 5-Hydroxytryptamin und Dopamin (Methodik: Kannengießer et al., Biochem. Pharmacol., Bd. 22 (1973), Seiten 73–84) in Synaptosomen und hemmen in vivo (Methodik: Carlsson et al., Europ. J. Pharmacol., Bd. 5 [1969], Seiten 357–366; 367–373) die durch Tyraminderivate induzierte Katecholamin- und Serotonin-Freisetzung im Gehirn. Weiterhin treten blutdrucksenkende und spasmolytische Wirkungen auf, die nach hierfür geläufigen Methoden ermittelt werden können.

Verbindungen der Formel I und ihre physiologisch unbedenklichen Säureadditionssalze können daher als Arzneimittelwirkstoffe und auch als Zwischenprodukte zur Herstellung anderer Arzneimittelwirkstoffe verwendet werden.

Aus Journal für praktische Chemie, Band 311, Seiten 183 bis 186 (1969) sind ähnliche Verbindun-

gen bekannt, insbesondere das 7-(1-Phenyl-3-dimethylamino-propoxy)-flavon und das 3-Methyl-7-(1-phenyl-3-dimethylamino-propoxy)-flavon. Diese Substanzen wurden auf Analgesie, Spasmolyse, Coronaraktivität und Permeabilitätswirkung getestet und sie zeigten dabei keine pharmakodynamisch brauchbaren Eigenschaften. Im Hinblick auf diese Feststellung waren die wertvollen pharmakologischen Eigenschaften der vorliegenden neuen Verbindungen daher besonders überraschend.

Gegenstand der Erfindung sind die basischen Äther der Formel I sowie ihre physiologisch unbedenklichen Säureadditionssalze.

In den Resten $R^2$, $R^4$, $Q^1$ und $Q^2$ bedeutet Alkyl vorzugsweise Methyl, ferner auch Äthyl, n-Propyl, Isopropyl, n-Butyl, Isobutyl, sek.-Butyl oder tert.-Butyl. Cycloalkyl (in den Resten $Q^1$ und $Q^2$) bedeutet vorzugsweise Cyclopropyl, Cyclobutyl, Cyclopentyl oder Cyclohexyl, ferner auch 1- oder 2-Methylcyclopropyl, 1-, 2- oder 3-Methylcyclobutyl, 1-, 2- oder 3-Methylcyclopentyl, 1- oder 2-Äthylcyclopropyl usw. Alkoxy (im Rest Ar) ist vorzugsweise Methoxy, ferner auch Äthoxy, n-Propoxy, Isopropoxy, n-Butoxy, Isobutoxy, sek.-Butoxy oder tert.-Butoxy. Cycloalkylalkyl (im Rest $R^2$) ist vorzugsweise Cyclopropylmethyl, aber auch z. B. 1- oder 2-Cyclopropyläthyl, 1-, 2- oder 3-Cyclopropylpropyl, 1-Cyclopropyl-1-methyl-äthyl, 1-, 2-, 3- oder 4-Cyclopropylbutyl, 1-Cyclopropyl-1-methylpropyl, 1-, 2-, 3-, 4- oder 5-Cyclopropylpentyl, Cyclobutylmethyl, 1- oder 2-Cyclobutyläthyl, 1-, 2- oder 3-Cyclobutylpropyl, 1-, 2-, 3- oder 4-Cyclobutylbutyl, Cyclopentylmethyl, 1- oder 2-Cyclopentyläthyl, 1-, 2- oder 3-Cyclopentylpropyl, Cyclohexylmethyl, 1- oder 2-Cyclohexyläthyl oder Cycloheptylmethyl. Alkylthio (im Rest Ar) ist vorzugsweise Methylthio, aber auch Äthylthio, n-Propylthio, Isopropylthio, n-Butylthio, Isobutylthio, sek.-Butylthio oder tert.-Butylthio.

Der Rest Ar ist vorzugsweise Phenyl, in zweiter Linie p-Fluorphenyl, p-Chlorphenyl, p-Methoxyphenyl, 3,4-Methylendioxyphenyl oder m-Trifluormethylphenyl, weiterhin o- oder m-Fluorphenyl, o- oder m-Chlorphenyl, o- oder m-Methoxyphenyl, o-, m- oder p-Methylthiophenyl, 2,3-Methylendioxyphenyl, o- oder p-Trifluormethylphenyl. Falls mehrere Reste Ar im gleichen Molekül vorhanden sind, so können sie gleich oder voneinander verschieden sein.

Im einzelnen steht $R^1$ vorzugsweise für Phenyl, p-Fluorphenyl, p-Chlorphenyl oder p-Methoxyphenyl; $R^2$ vorzugsweise für H oder Methyl, in zweiter Linie vorzugsweise für Äthyl, Allyl, Cyclopropylmethyl oder Benzyl; $R^3$ vorzugsweise für H oder Phenyl; $R^4$ vorzugsweise für H oder Methyl.

Dementsprechend bedeutet der Rest Z vorzugsweise 1-Phenyl-3-dimethylaminopropyl, in zweiter Linie vorzugsweise

1-Phenyl-3-methylaminopropyl, 1-Phenyl-3-N-benzyl-N-methylamino-propyl,
(3-Piperidyl)-methyl, α-(3-Piperidyl)-benzyl,
(1-Methyl-3-piperidyl)-methyl, α-(1-Methyl-3-piperidyl)-benzyl,
2-(2-Piperidyl)-äthyl, 1-Phenyl-2-(2-piperidyl)-äthyl,
2-(1-Methyl-2-piperidyl)-äthyl, 1-Phenyl-2-(1-methyl-2-piperidyl)-äthyl,
4-Hexahydro-azepinyl, 1-Methyl-4-hexahydroazepinyl,
3-Methyl-4-hexahydroazepinyl, 1,3-Dimethyl-4-hexahydroazepinyl,

ferner z. B.

1-Phenyl-3-methyläthyl-amino-propyl, 1-Phenyl-3-(N-methyl-N-allylamino)-propyl,
1-Phenyl-3-(N-methyl-N-cyclopropylmethylamino)-propyl,
1-Äthyl-3-piperidyl)-methyl, α-(1-Äthyl-3-piperidyl)-benzyl,
(1-Allyl-3-piperidyl)-methyl, α-(1-Allyl-3-piperidyl)-benzyl,
(1-Cyclopropylmethyl-3-piperidyl)-methyl, α-(1-Cyclopropylmethyl-3-piperidyl)-benzyl,
(1-Benzyl-3-piperidyl)-methyl, α-(1-Benzyl-3-piperidyl)-benzyl,
2-(1-Äthyl-2-piperidyl)-äthyl, 1-Phenyl-2-(1-äthyl-2-piperidyl)-äthyl,
2-(1-Allyl-2-piperidyl)-äthyl, 1-Phenyl-2-(1-allyl-2-piperidyl)-äthyl,
2-(1-Cyclopropylmethyl-2-piperidyl)-äthyl,
1-Phenyl-2-(1-cyclopropylmethyl-2-piperidyl)-äthyl,
2-(1-Benzyl-2-piperidyl)-äthyl, 1-Phenyl-2-(1-benzyl-2-piperidyl)-äthyl,
3-Äthyl-4-hexahydroazepinyl, 1-Methyl-3-äthyl-4-hexahydroazepinyl,
1-Äthyl-, 1-Allyl-, 1-Cyclopropylmethyl- oder 1-Benzyl-4-hexahydroazepinyl,
1-Cyclopropyl-3-methylamino-propyl, 1-p-Fluorphenyl-3-methylamino-propyl,
1-p-Chlorphenyl-3-methylamino-propyl, 1-m-Trifluormethyl-phenyl-3-methylamino-propyl,
1-Cyclopropyl-3-dimethylamino-propyl, 1-p-Fluorphenyl-3-dimethylamino-propyl,
1-p-Chlorphenyl-3-dimethylamino-propyl, 1-m-Trifluormethylphenyl-3-dimethylamino-propyl.

Die Reste $Q^1$ und $Q^2$ können gleich oder verschieden sein. Vorzugsweise steht der eine dieser Reste für H oder Alkyl mit 1—3 C-Atomen, der andere für H oder Ar, wobei Ar insbesondere Phenyl, p-Fluorphenyl, p-Chlorphenyl, p-Methoxyphenyl, 3,4-Methylendioxyphenyl oder m-Trifluormethylphenyl bedeutet.

Der Rest Y bedeutet dementsprechend vorzugsweise $-O-CHQ^3-CHQ^4-CH_2-$ (worin die Reste $Q^3$ und $Q^4$ jeweils H oder Alkyl mit 1—3 C-Atomen, der eine dieser Reste jedoch vorzugsweise H bedeutet;

Chromane), $-O-CHAr-CHQ^4-CH_2-$ (Flavane), $-O-CHQ^3-CHAr-CH_2-$ (Isoflavane), $-O-CHQ^3-CHQ^4-CO-$ (Chromanone), $-O-CHAr-CHQ^4-CO-$ (Flavanone), $-O-CHQ^3-CHAr-CO-$ (Isoflavanone), $-O-CQ^3=CQ^4-CO-$ (Chromone), $-O-CAr=CQ^4-CO-$ (Flavone), $-O-CQ^3=CAr-CO-$ (Isoflavone) oder $-CH_2-CHQ^3-CHQ^4-CO-$ (Tetralone).

Unter diesen sind die Isoflavone, in zweiter Linie die Tetralone und die Flavone besonders bevorzugt.

Dementsprechend sind Gegenstand der Erfindung insbesondere diejenigen Verbindungen der Formel I, in denen mindestens einer der genannten Reste eine der vorstehend angegebenen, insbesondere der vorstehend angegebenen bevorzugten Bedeutungen hat. Einige bevorzugte Gruppen von Verbindungen können durch die folgenden Teilformeln Ia bis Ic ausgedrückt werden, die der Formel I entsprechen und worin die nicht bezeichneten Reste die bei Formel I angegebene Bedeutung haben, worin jedoch in Ia

Z $CH_3-NR^2-CH_2CH_2-CHR^1-$,
$R^1$ Phenyl,
$R^2$ H, Methyl oder Benzyl,
Y $-O-CH_2CH_2CH_2-$, $-O-CHAr-CH_2CH_2-$, $-O-CH_2-CH(C_6H_5)-CH_2-$, $-O-CH_2CH_2-CO-$, $-O-CHAr-CHQ^4-CO-$, $-O-CAr=CQ^4-CO-$, $-O-CH=CAr-CO-$ oder $-CH_2CH_2CH_2-CO-$,
Ar Phenyl, p-Fluorphenyl, p-Chlorphenyl, p-Methoxyphenyl, 3,4-Methylendioxyphenyl oder m-Trifluormethylphenyl und
$Q^4$ H oder Alkyl mit 1–3 C-Atomen bedeuten;

in Ib

Z $CH_3-NR^2-CH_2CH_2-CHR^1$,
$R^1$ Phenyl,
$R^2$ H, Methyl oder Benzyl und
Y $-O-C(C_6H_5)=CH-CO-$, $-O-CH=C(C_6H_5)-CO-$ oder $-CH_2CH_2CH_2-CO-$ bedeuten;

in Ic

Z $CH_3-NR^2-CH_2CH_2-CHR^1-$,
$R^1$ Phenyl,
$R^2$ H, Methyl oder Benzyl und
Y $-O-CH=C(C_6H_5)-CO-$ bedeuten.

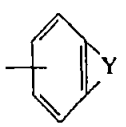
Die Verbindungen der Formel I können ein oder mehrere asymmetrische Kohlenstoffatome besitzen. Sie können daher als Racemate, falls mehrere asymmetrische Kohlenstoffatome vorhanden sind, auch als Gemische mehrerer Racemate sowie in verschiedenen optisch-aktiven Formen vorliegen.

Gegenstand der Erfindung ist ferner ein Verfahren zur Herstellung der Verbindungen der Formel I sowie ihrer physiologisch unbedenklichen Säureadditionssalze, dadurch gekennzeichnet, daß man ein Phenol der allgemeinen Formel II

$$HO-G \qquad (II)$$

worin

G die Gruppe



bedeutet, oder eines seiner Salze mit einem Amin der allgemeinen Formel III

$$Z-X \qquad (III)$$

worin

X Cl, Br, J oder OH bedeutet und
Z die angegebene Bedeutung hat

oder mit einem seiner reaktionsfähigen Derivate umsetzt und daß man gegebenenfalls in einer erhaltenen Verbindung der Formel I eine sekundäre Aminogruppe durch Behandeln mit einem alkylierenden, allylierenden, cycloalkylierenden oder benzylierenden Mittel in die entsprechende tertiäre Aminogruppe oder eine N-Benzylgruppe durch Behandeln mit einem Reduktionsmittel in eine NH-Gruppe und/oder eine erhaltene Base der Formel I durch Behandeln mit einer Säure in eines ihrer physiologisch unbedenklichen Säureadditionssalze umwandelt.

Die Herstellung der Verbindungen der Formel I erfolgt im übrigen nach an sich bekannten Methoden, wie sie in der Literatur (z. B. in den Standardwerken wie Houben-Weyl, Methoden der Organischen Chemie, Georg-Thieme-Verlag, Stuttgart; Organic Reactions, John Wiley & Sons, Inc., New York) beschrieben sind, und zwar unter Reaktionsbedingungen, wie sie für die genannten Umsetzungen bekannt und geeignet sind. Dabei kann man auch von an sich bekannten, hier nicht näher erwähnten Varianten Gebrauch machen.

Die Ausgangsstoffe der Formeln II und III können gewünschtenfalls auch in situ gebildet werden, derart, daß man sie aus dem Reaktionsgemisch nicht isoliert, sondern sofort weiter zu den Verbindungen der Formel I umsetzt.

Die Phenole der Formel II sind in der Regel bekannt. Falls sie neu sind, können sie nach an sich bekannten Methoden hergestellt werden, z. B. durch Spaltung entsprechender Benzyl- oder Methyläther.

In den Basen der Formel III bedeutet der Rest X vorzugsweise Cl oder Br. Reaktionsfähige Derivate der Basen der Formel III sind insbesondere die reaktionsfähigen Ester der Alkohole der Formel III (X = OH), vorzugsweise die entsprechenden Alkylsulfonate (worin die Alkylgruppe 1–6 C-Atome besitzt) und die entsprechenden Arylsulfonate (worin die Arylgruppe 6–10 C-Atome besitzt), z. B. die entsprechenden Methan-, Benzol-, p-Toluol- oder Naphthalin-1- oder -2-sulfonate.

Die Basen der Formel III sind teilweise bekannt. Die bisher noch nicht bekannten Basen der Formel III können nach an sich bekannten Methoden in Analogie zu bekannten Verbindungen hergestellt werden. So sind die Verbindungen der Formel III (X = OH) z. B. durch Reduktion entsprechender Ester oder Ketone vom Typ

$CH_3-NR^2-CH_2CH_2-COR^1$, (1-$R^2$-3-piperidyl)–COOAlkyl,
(1-$R^2$-3-piperidyl)–CO–$R^3$, (1-$R^2$-2-piperidyl)–$CH_2$–COOAlkyl,
(1-$R^2$-2-piperidyl)–$CH_2$–CO–$R^3$ oder 1-$R^2$-3-$R^4$-hexahydroazepin-4-on

erhältlich, die Verbindungen der Formel III (X = Cl, Br oder J) aus den Alkoholen mit anorganischen Halogeniden wie $SOCl^2$, $PBr^3$ oder HJ, die Sulfonate durch Veresterung der Alkohole mit den entsprechenden Sulfonylchloriden. Die tertiären unter den Aminen der Formel III (in denen $R^2$ nicht H bedeutet) sind auch aus den sekundären Aminen (III, $R^2$ = H) durch Alkylierung, Allylierung, Cycloalkylalkylierung oder Benzylierung zugänglich. Umgekehrt können die sekundären Amine (III, $R^2$ = H) aus den entsprechenden N-Alkylderivaten (III, $R^2$ = Alkyl mit 1–4 C-Atomen) durch Desalkylierung mit Chlorameisensäureäthylester erhalten werden. Weiterhin können die Amine der Formel III durch Reduktion entsprechender Pyridine oder Azepine hergestellt werden. So kann man z. B. 2-Methylpyridin mit $C_6H_5Li$ metallieren, anschließend mit Benzaldehyd zu 1-Phenyl-2-(2-pyridyl)-äthanol umsetzen und dieses zu 1-Phenyl-2-(2-piperidyl)-äthanol reduzieren. 1-Methyl-4-hydroxy-hexahydroazepin ist zugänglich durch Ringerweiterung von 1-Methyl-4-piperidon mit $CH_2N_2$ zu 1-Methyl-hexahydroazepin-4-on und Reduktion mit $NaBH_4$.

Vor der Umsetzung mit III wird das Phenol II zweckmäßig zunächst in ein Salz übergeführt, insbesondere in ein Metallsalz, z. B. ein Alkalimetallsalz (Li-, Na- oder K-Salz) oder Thalliumsalz. Man kann das Phenol mit einem metallsalz-bildenden Reagenz umsetzen, z. B. einem Alkalimetall (z. B. Na), einem Alkalimetallhydrid oder -amid (z. B. LiH, NaH, $NaNH_2$ oder $KNH_2$), einem Metallalkoholat (worin der Alkohol-Teil vorzugsweise 1–4 C-Atome besitzt, z. B. Lithium-, Natrium-, Kalium- oder Thalliummethylat, -äthylat oder -tert.-butylat), einer Organometallverbindung (z. B. Butyllithium, Phenyllithium oder Phenylnatrium), einem Metallhydroxid, -carbonat oder -bicarbonat (z. B. des Li, Na, K oder Ca). Die Herstellung des Phenolats wird vorteilhafterweise in Gegenwart eines Lösungsmittels oder Lösungsmittelgemischs vorgenommen. Geeignete Lösungsmittel sind z. B. Kohlenwasserstoffe (wie Hexan, Benzol, Toluol oder Xylol), halogenierte Kohlenwasserstoffe (wie $CH_2Cl_2$, $CHCl_3$ oder $CCl_4$), Äther (wie Diäthyläther, Diisopropyläther, Tetrahydrofuran (THF), Dioxan oder Diäthylenglykoldimethyläther), Amide wie Dimethylformamid (DMF), Alkohole (wie Methanol oder Aethanol), Ketone (wie Aceton oder Butanon).

Das Phenol II oder dessen Salz wird mit dem Amin III vorzugsweise in Gegenwart eines Verdünnungsmittels umgesetzt, z. B. desjenigen Lösungsmittels, das für die Herstellung des Salzes verwendet worden ist, das jedoch durch ein anderes Lösungsmittel ersetzt oder mit einem solchen verdünnt sein kann. Eine besondere Variante besteht darin, zweiphasig (z. B. in $CH_2Cl_2$/wässeriger Natronlauge) zu arbeiten, wobei auch ein Katalysator (z. B. ein Kronenäther, ein Ammoniumsalz wie ein Trialkylbenzylammoniumhalogenid oder ein Phosphoniumsalz) zugesetzt werden kann. Die Umsetzung wird in der Regel bei Temperaturen zwischen −20° und 180° durchgeführt, vorzugsweise zwischen 20 und 160°.

Das Phenolat kann auch in situ gebildet werden. In diesem Falle läßt man das Phenol II und das Amin III miteinander in Gegenwart einer Base reagieren.

Eine Variante der Reaktion besteht in der Umsetzung eines Phenols der Formel II mit einem Hydroxya-



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min der Formel III (X = OH) in Gegenwart eines Dehydratisierungsmittels, z. B. eines Azodicarbonsäuredialkylesters in Anwesenheit von Triphenylphosphin in einem inerten Lösungsmittel wie THF bei −10 bis +30°.

Weiterhin kann man gewünschtenfalls ein erhaltenes sekundäres Amin der Formel I ($R^2$ = H) am Stickstoffatom alkylieren, wobei man tertiäre Amine der Formel I ($R^2$ = Alkyl mit 1−4 C-Atomen) erhält. Als N-Alkylierungsmittel eignen sich z. B. die entsprechenden Alkylhalogenide, z. B. Methylchlorid, Methylbromid, Methyljodid, Äthylchlorid, Äthylbromid, Äthyljodid, n-Propylchlorid, -bromid oder -jodid usw., ferner die entsprechenden Dialkylsulfate wie Dimethylsulfat und die entsprechenden Sulfonsäurealkylester wie p-Toluolsulfonsäure-methylester. Eine Methylgruppe kann ferner beispielsweise durch Behandeln mit Ameisensäure und wässeriger Formaldehydlösung eingeführt werden, zweckmäßig durch mehrstündiges Erhitzen auf Temperaturen zwischen 50 und 100°. Allgemein wird die N-Alkylierung zweckmäßig in Gegenwart oder Abwesenheit eines inerten Lösungsmittels bei Temperaturen zwischen 0 und 120°, vorzugsweise zwischen 40 und 100° vorgenommen, wobei auch ein Katalysator zugegen sein kann, vorzugsweise eine Base wie Kalium-tert.-butylat.

Eine Alkylierung gelingt auch durch Behandeln einer sekundären Base I ($R^2$ = H) mit einem Aldehyd oder Keton in Gegenwart von Wasserstoff und einem Hydrierungskatalysator (z. B. Raney-Nickel) bei Temperaturen zwischen 50 und 100° und Drucken zwischen 1 und 200 at; so erhält man mit Aceton die entsprechende Isopropylverbindung I ($R^2$ = Isopropyl).

Eine Alkylierung ist auch mehrstufig möglich. Man kann beispielsweise eine Verbindung der Formel I ($R^2$ = H) zunächst in an sich bekannter Weise acylieren (z. B. durch Behandeln mit Acetanhydrid/Pyridin acetylieren) und das erhaltene N-Acylierungsprodukt (z. B. N-Acetylierungsprodukt) anschließend zum gewünschten tertiären Amin reduzieren, beispielsweise mit einem komplexen Metallhydrid wie $LiAlH_4$ in einem inerten Lösungsmittel wie Diäthyläther oder THF, vorzugsweise bei Temperaturen zwischen 20 und 60°.

In ganz analoger Weise kann ein sekundäres Amin der Formel I ($R^2$ = H) mit allylierenden, cycloalkylalkylierenden oder benzylierenden Mitteln (z. B. Allylchlorid oder -bromid, Cyclopropylmethylhalogeniden wie Cyclopropylmethylchlorid oder -bromid, Benzylhalogeniden wie Benzylchlorid oder -bromid) behandelt werden, wobei Verbindungen der Formel I ($R^2$ = Allyl, Cycloalkylalkyl mit 4−8 C-Atomen oder Benzyl) gebildet werden.

Weiterhin kann in einer Verbindung der Formel I ($R^2$ = Benzyl) die Benzylgruppe nach einer in der Literatur angegebenen Methode reduktiv entfernt werden, vorzugsweise durch Hydrogenolyse in Gegenwart eines Edelmetallkatalysators oder mit Hilfe eines desalkylierenden Agens, z. B. $ClCOC_2H_5$/NaOH.

Eine erhaltene Base der Formel I kann mit einer Säure in das zugehörige Säureadditionssalz übergeführt werden. Für diese Umsetzung eignen sich Säuren, die physiologisch unbedenkliche Salze liefern. So können anorganische Säuren verwendet werden, z. B. Schwefelsäure, Halogenwasserstoffsäuren wie Chlorwasserstoffsäure oder Bromwasserstoffsäure, Phosphorsäuren wie Orthophosphorsäure, Salpetersäure, Sulfaminsäure, ferner organische Säuren, im einzelnen aliphatische, alicyclische, araliphatische, aromatische oder heterocyclische ein- oder mehrbasige Carbon-, Sulfon- oder Schwefelsäuren, wie Ameisensäure, Essigsäure, Propionsäure, Pivalinsäure, Diäthylessigsäure, Malonsäure, Bernsteinsäure, Pimelinsäure, Fumarsäure, Maleinsäure, Milchsäure, Weinsäure, Äpfelsäure, Benzoesäure, Salicylsäure, 2-Phenylpropionsäure, Citronensäure, Gluconsäure, Ascorbinsäure, Nicotinsäure, Isonicotinsäure, Methan- oder Äthansulfonsäure, Äthandisulfonsäure, 2-Hydroxyäthansulfonsäure, Benzolsulfonsäure, p-Toluolsulfonsäure, Naphthalin-mono- und -disulfonsäuren, Laurylschwefelsäure.

Die freien Basen der Formel I können, falls gewünscht, aus ihren Salzen durch Behandlung mit starken Basen wie Natrium- oder Kaliumhydroxid, Natrium- oder Kaliumcarbonat in Freiheit gesetzt werden.

Gegenstand der Erfindung ist ferner die Verwendung der Verbindungen der Formel I und ihrer physiologisch unbedenklichen Salze zur Herstellung pharmazeutischer Zubereitungen, insbesondere auf nicht-chemischem Wege. Hierbei können sie zusammen mit mindestens einem Träger- oder Hilfsstoff und gegebenenfalls in Kombination mit einem oder mehreren weiteren Wirkstoff(en) in eine geeignete Dosierungsform gebracht werden.

Gegenstand der Erfindung sind ferner Mittel, insbesondere pharmazeutische Zubereitungen, enthaltend eine Verbindung der Formel I und/oder eines ihrer physiologisch unbedenklichen Säureadditionssalze. Diese Zubereitungen können als Arzneimittel in der Human- oder Veterinärmedizin eingesetzt werden. Als Trägersubstanzen kommen organische oder anorganische Stoffe in Frage, die sich für die enterale (z. B. orale), parenterale oder topikale Applikation eignen und mit den neuen Verbindungen nicht reagieren, beispielsweise Wasser, pflanzliche Öle, Benzylalkohole, Polyäthylenglykole, Gelatine, Kohlehydrate wie Lactose oder Stärke, Magnesiumstearat, Talk, Vaseline. Zur enteralen Applikation dienen insbesondere Tabletten, Dragees, Kapseln, Sirupe, Säfte, Tropfen oder Suppositorien, zur parenteralen Applikation Lösungen, vorzugsweise ölige oder wässerige Lösungen, ferner Suspensionen, Emulsionen oder Implantate, für die topikale Anwendung Salben, Cremes oder Puder. Die neuen Verbindungen können auch lyophilisiert und die erhaltenen Lyophilisate z. B. zur Herstellung von Injektionspräparaten verwendet werden. Die angegebenen Zubereitungen können sterilisiert sein und/oder Hilfsstoffe wie Gleit-, Konservierungs-, Stabilisierungs- und/oder Netzmittel, Emulgatoren, Salze zur Beeinflussung des osmotischen Druckes, Puffersubstanzen, Farb-, Geschmacks- und/oder Aromastoffe enthalten. Sie können, falls erwünscht, auch einen oder mehrere weitere Wirkstoffe enthalten, z. B. ein oder

mehrere Vitamine.

Gegenstand der Erfindung sind ferner die Verbindungen der Formel I und ihrer physiologisch unbedenklichen Säureadditionssalze zur Verwendung bei der Bekämpfung von Krankheiten, insbesondere von Depressionen verschiedener Ätiologie und Symptomatologie, sowie bei der therapeutischen Behandlung des menschlichen oder tierischen Körpers.

Dabei werden die erfindungsgemäßen Substanzen in der Regel in Analogie zu bekannten, im Handel befindlichen Psychopharmaka (z. B. Imipramin) verabreicht, vorzugsweise in Dosierungen zwischen 2 und 500 mg, insbesondere zwischen 10 und 50 mg pro Dosierungseinheit. Die tägliche Dosierung liegt vorzugsweise zwischen 0,05 und 10 mg/kg Körpergewicht. Die spezielle Dosis für jeden bestimmten Patienten hängt jedoch von den verschiedensten Faktoren ab, beispielsweise von der Wirksamkeit der eingesetzten speziellen Verbindung, vom Alter, Körpergewicht, allgemeinen Gesundheitszustand, Geschlecht, von der Kost, vom Verabfolgungszeitpunkt und -weg, von der Ausscheidungsgeschwindigkeit, Arzneistoffkombination und Schwere der jeweiligen Erkrankung, welcher die Therapie gilt. Die orale Applikation ist bevorzugt.

Jede der in den folgenden Beispielen genannten Verbindungen der Formel I ist zur Herstellung von pharmazeutischen Zubereitungen besonders geeignet.

In den nachfolgenden Beispielen bedeutet »übliche Aufarbeitung«:

Man gibt, falls erforderlich, Wasser hinzu, extrahiert mit einem organischen Lösungsmittel wie Benzol, Chloroform oder Dichlormethan, trennt ab, trocknet die organische Phase über Natriumsulfat, filtriert, dampft ein und reinigt durch Chromatographie und/oder Kristallisation.

Die Rf-Werte wurden an Kieselgel mit Toluol : Triäthylamin (8 : 2) erhalten, sofern nicht anders angegeben.

## Beispiel 1

Eine Lösung von 23,8 g 7-Hydroxy-isoflavon in 250 ml 0,5 n äthanolischer KOH wird eingedampft, der Rückstand in 200 ml DMF gelöst, auf 150° erhitzt und unter Rühren mit einer Lösung von 20 g 1-Chlor-1-phenyl-3-dimethylamino-propan in 50 ml DMF versetzt. Man rührt 1,5 Stunden bei 150° und fällt durch Zugabe von Wasser 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon. F. 128–130° (aus Aceton). Hydrochlorid, F. 259–260°.

## Beispiele 2 bis 220

Analog Beispiel 1 erhält man durch Verätherung der Na-Salze von

6- oder 7-Hydroxy-chroman, 6- oder 7-Hydroxyflavan, 6- oder 7-Hydroxyisoflavan,
6- oder 7-Hydroxychromanon, 6- oder 7-Hydroxyflavanon, 6- oder 7-Hydroxyisoflavanon,
6- oder 7-Hydroxychromon, 5-, 6-, 7- oder 8-Hydroxyflavon, 5-, 6-, 7- oder 8-Hydroxyisoflavon,
5-, 6- oder 7-Hydroxytetralon mit 1-Chlor-1-phenyl-3-methylaminopropan,
1-Chlor-1-phenyl-3-dimethylaminopropan, 1-Chlor-1-phenyl-3-N-benzyl-N-methylaminopropan,
3-Chlormethyl-piperidin, 3-(α-Chlorbenzyl)-piperidin, 1-Methyl-3-chlormethyl-piperidin,
1-Methyl-3-(α-chlorbenzyl)-piperidin, 2-(2-Chloräthyl)-piperidin,
2-(2-Chlor-2-phenyläthyl)-piperidin, 1-Methyl-2-(2-chloräthyl)-piperidin,
1-Methyl-2-(2-chlor-2-phenyläthyl)-piperidin, 1-Benzyl-2-(2-chloräthyl)-piperidin,
4-Chlorhexahydroazepin, 1-Methyl-4-chlor-hexahydroazepin,
3-Methyl-4-chlor-hexahydroazepin, 1,3-Dimethyl-4-chlorhexahydroazepin

oder den entsprechenden Bromverbindungen:

2. 6-(1-Phenyl-3-methylamino-propoxy)-chroman, Hydrochlorid, F. 164°.
3. 7-(1-Phenyl-3-methylamino-propoxy)-chroman.
4. 6-(1-Phenyl-3-dimethylamino-propoxy)-chroman, Hydrochlorid, F. 137–139°.
5. 7-(1-Phenyl-3-dimethylamino-propoxy)-chroman, Hydrochlorid, F. 182–183°.
6. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chroman, Hydrochlorid, F. 139–140°.
7. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chroman.
8. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chroman.
9. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chroman.
10. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-chroman, Hydrochlorid, F. 100–103°.
11. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-chroman.
12. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chroman.
13. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chroman.
14. 6-(1-Phenyl-3-methylamino-propoxy)-flavan.
15. 7-(1-Phenyl-3-methylamino-propoxy)-flavan.

16. 6-(1-Phenyl-3-dimethylamino-propoxy)-flavan.
17. 7-(1-Phenyl-3-dimethylamino-propoxy)-flavan.
18. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavan.
19. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavan.
20. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-flavan.
21. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-flavan.
22. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavan.
23. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavan.
24. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavan.
25. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavan.
26. 6-(1-Phenyl-3-methylamino-propoxy)-isoflavan.
27. 7-(1-Phenyl-3-methylamino-propoxy)-isoflavan.
28. 6-(1-Phenyl-3-dimethylamino-propoxy)-isoflavan.
29. 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavan, F. 98–100°.
30. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavan.
31. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavan.
32. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-isoflavan.
33. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-isoflavan.
34. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavan.
35. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavan.
36. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavan.
37. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavan.
38. 6-(1-Phenyl-3-methylamino-propoxy)-chromanon.
39. 7-(1-Phenyl-3-methylamino-propoxy)-chromanon.
40. 6-(1-Phenyl-3-dimethylamino-propoxy)-chromanon.
41. 7-(1-Phenyl-3-dimethylamino-propoxy)-chromanon, Hydrochlorid, F. 188–191°.
42. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chromanon.
43. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chromanon.
44. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chromanon.
45. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chromanon.
46. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-chromanon.
47. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-chromanon, Hydrochlorid, F. 182–184°.
48. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chromanon.
49. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chromanon.
50. 6-(1-Phenyl-3-methylamino-propoxy)-flavanon.
51. 7-(1-Phenyl-3-methylamino-propoxy)-flavanon.
52. 6-(1-Phenyl-3-dimethylamino-propoxy)-flavanon.
53. 7-(1-Phenyl-3-dimethylamino-propoxy)-flavanon.
54. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavanon.
55. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavanon.
56. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-flavanon.
57. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-flavanon.
58. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavanon.
59. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavanon.
60. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavanon.
61. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavanon.
62. 6-(1-Phenyl-3-methylamino-propoxy)-isoflavanon.
63. 7-(1-Phenyl-3-methylamino-propoxy)-isoflavanon.
64. 6-(1-Phenyl-3-dimethylamino-propoxy)-isoflavanon.
65. 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavanon.
66. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavanon.
67. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavanon.
68. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-isoflavanon.
69. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-isoflavanon.
70. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavanon.
71. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavanon.
72. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavanon.
73. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavanon.
74. 6-(1-Phenyl-3-methylamino-propoxy)-chromon.
75. 7-(1-Phenyl-3-methylamino-propoxy)-chromon.
76. 6-(1-Phenyl-3-dimethylamino-propoxy)-chromon.
77. 7-(1-Phenyl-3-dimethylamino-propoxy)-chromon, Hydrochlorid, F. 199°.
78. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
79. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
80. 6-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chromon.

81. 7-[α-(1-Methyl-3-piperidyl)-benzyloxy]-chromon.
82. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-chromon.
83. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-chromon.
84. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chromon.
85. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-chromon.
86. 5-(1-Phenyl-3-methylamino-propoxy)-flavon.
87. 6-(1-Phenyl-3-methylamino-propoxy)-flavon, Rf 0,43 (Methylenchlorid/Triäthylamin 8 : 2).
88. 7-(1-Phenyl-3-methylamino-propoxy)-flavon.
89. 5-(1-Phenyl-3-dimethylamino-propoxy)-flavon, F. 140–141°.
90. 6-(1-Phenyl-3-dimethylamino-propoxy)-flavon, F. 142–142,5°.
91. 8-(1-Phenyl-3-dimethylamino-propoxy)-flavon.
92. 5-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavon.
93. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavon. F. 93–94°.
94. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-flavon.
95. 5-(3-Piperidyl-methoxy)-flavon.
96. 6-(3-Piperidyl-methoxy)-flavon.
97. 7-(3-Piperidyl-methoxy)-flavon.
98. 5-(α-3-Piperidyl-benzyloxy)-flavon.
99. 6-(α-3-Piperidyl-benzyloxy)-flavon.
100. 7-(α-3-Piperidyl-benzyloxy-flavon.
101. 5-(1-Methyl-3-piperidyl-methoxy)-flavon.
102. 6-(1-Methyl-3-piperidyl-methoxy)-flavon.
103 7-(1-Methyl-3-piperidyl-methoxy)-flavon.
104. 5-(α-1-Methyl-3-piperidyl-benzyloxy)-flavon.
105. 6-(α-1-Methyl-3-piperidyl-benzyloxy)-flavon.
106. 7-(α-1-Methyl-3-piperidyl-benzyloxy)-flavon.
107. 5-[2-(2-Piperidyl)-äthoxy]-flavon.
108. 6-[2-(2-Piperidyl)-äthoxy]-flavon.
109. 7-[2-(2-Piperidyl)-äthoxy]-flavon.
110. 5-[1-Phenyl-2-(2-piperidyl)-äthoxy]-flavon.
111. 6-[1-Phenyl-2-(2-piperidyl)-äthoxy]-flavon.
112. 7-[1-Phenyl-2-(2-piperidyl)-äthoxy]-flavon.
113. 5-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavon.
114. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavon.
115. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-flavon.
116. 5-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-flavon.
117. 6-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-flavon.
118. 7-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-flavon.
119. 5-(4-Hexahydroazepinyloxy)-flavon.
120. 6-(4-Hexahydroazepinyloxy)-flavon.
121. 7-(4-Hexahydroazepinyloxy)-flavon.
121. 7-(4-Hexahydroazepinyloxy)-flavon.
122. 5-(1-Methyl-4-hexahydroazepinyloxy)-flavon.
123. 6-(1-Methyl-4-hexahydroazepinyloxy)-flavon.
124. 7-(1-Methyl-4-hexahydroazepinyloxy)-flavon.
125. 5-(3-Methyl-4-hexahydroazepinyloxy)-flavon.
126. 6-(3-Methyl-4-hexahydroazepinyloxy)-flavon.
127. 7-(3-Methyl-4-hexahydroazepinyloxy)-flavon.
128. 5-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavon.
129. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavon.
130. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-flavon.
131. 5-(1-Phenyl-3-methylamino-propoxy)-isoflavon.
132. 6-(1-Phenyl-3-methylamino-propoxy)-isoflavon.
133. 7-(1-Phenyl-3-methylamino-propoxy)-isoflavon, Hydrochlorid, F. 210–211°.
134. 5-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon.
135. 6-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon.
136. 8-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon.
137. 5-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon.
138. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon.
139. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon, F. 115–118°, Hydrochlorid, F. 210–212°.
140. 5-(3-Piperidyl-methoxy)-isoflavon.
141. 6-(3-Piperidyl-methoxy)-isoflavon.
142. 7-(3-Piperidyl-methoxy)-isoflavon.
143. 5-(α-3-Piperidyl-benzyloxy)-isoflavon.

144. 6-(α-3-Piperidyl-benzyloxy)-isoflavon.
145. 7-(α-3-Piperidyl-benzyloxy)-isoflavon.
146. 5-(1-Methyl-3-piperidyl-methoxy)-isoflavon.
147. 6-(1-Methyl-3-piperidyl-methoxy)-isoflavon.
148. 7-(1-Methyl-3-piperidyl-methoxy)-isoflavon, Hydrochlorid, F. 257–260°.
149. 5-(α-1-Methyl-3-piperidyl-benzyloxy)-isoflavon.
150. 6-(α-1-Methyl-3-piperidyl-benzyloxy)-isoflavon.
151. 7-(α-1-Methyl-3-piperidyl-benzyloxy)-isoflavon, Hydrochlorid, F. 143–145°.
152. 5-[2-(2-Piperidyl)-äthoxy]-isoflavon.
153. 6-[2-(2-Piperidyl)-äthoxy]-isoflavon.
154. 7-[2-(2-Piperidyl)-äthoxy]-isoflavon.
155. 5-[1-Phenyl-2-(2-piperidyl)-äthoxy]-isoflavon.
156. 6-[1-Phenyl-2-(2-piperidyl)-äthoxy]-isoflavon.
157. 7-[1-Phenyl-2-(2-piperidyl)-äthoxy]-isoflavon.
158. 5-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavon.
159. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavon.
160. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-isoflavon, Hydrochlorid, F. 206–207°.
161. 5-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-isoflavon.
162. 6-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-isoflavon.
163. 7-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-isoflavon.
164. 5-(4-Hexahydroazepinyloxy)-isoflavon.
165. 6-(4-Hexahydroazepinyloxy)-isoflavon.
166. 7-(4-Hexahydroazepinyloxy)-isoflavon.
167. 5-(1-Methyl-4-hexahydroazepinyloxy)-isoflavon.
168. 6-(1-Methyl-4-hexahydroazepinyloxy)-isoflavon.
169. 7-(1-Methyl-4-hexahydroazepinyloxy)-isoflavon.
170. 5-(3-Methyl-4-hexahydroazepinyloxy)-isoflavon.
171. 6-(3-Methyl-4-hexahydroazepinyloxy)-isoflavon.
172. 7-(3-Methyl-4-hexahydroazepinyloxy)-isoflavon.
173. 5-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavon.
174. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavon.
175. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-isoflavon.
176. 5-(1-Phenyl-3-methylamino-propoxy)-1-tetralon.
177. 6-(1-Phenyl-3-methylamino-propoxy)-1-tetralon.
178. 7-(1-Phenyl-3-methylamino-propoxy)-1-tetralon.
179. 5-(1-Phenyl-3-dimethylamino-propoxy)-1-tetralon.
180. 6-(1-Phenyl-3-dimethylamino-propoxy)-1-tetralon.
181. 7-(1-Phenyl-3-dimethylamino-propoxy)-1-tetralon, Hydrochlorid, F. 167–169°.
182. 5-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-1-tetralon.
183. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-1-tetralon, Rf 0,4 (Äthylacetat).
184. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-1-tetralon, Hydrochlorid, F. 178–180°.
185. 5-(3-Piperidyl-methoxy)-1-tetralon.
186. 6-(3-Piperidyl-methoxy)-1-tetralon.
187. 7-(3-Piperidyl-methoxy)-1-tetralon.
188. 5-(α-3-Piperidyl-benzyloxy)-1-tetralon.
189. 6-(α-3-Piperidyl-benzyloxy)-1-tetralon.
190. 7-(α-3-Piperidyl-benzyloxy)-1-tetralon.
191. 5-(1-Methyl-3-piperidyl-methoxy)-1-tetralon.
192. 6-(1-Methyl-3-piperidyl-methoxy)-1-tetralon.
193. 7-(1-Methyl-3-piperidyl-methoxy)-1-tetralon.
194. 5-(α-1-Methyl-3-piperidyl-benzyloxy)-1-tetralon.
195. 6-(α-1-Methyl-3-piperidyl-benzyloxy)-1-tetralon.
196. 7-(α-1-Methyl-3-piperidyl-benzyloxy)-1-tetralon.
197. 5-[2-(2-Piperidyl)-äthoxy]-1-tetralon.
198. 6-[2-(2-Piperidyl)-äthoxy]-1-tetralon.
199. 7-[2-(2-Piperidyl)-äthoxy]-1-tetralon.
200. 5-[1-Phenyl-2-(2-piperidyl)-äthoxy]-1-tetralon.
201. 6-[1-Phenyl-2-(2-piperidyl)-äthoxy]-1-tetralon.
202. 7-[1-Phenyl-2-(2-piperidyl)-äthoxy]-1-tetralon.
203. 5-[2-(1-Methyl-2-piperidyl)-äthoxy]-1-tetralon.
204. 6-[2-(1-Methyl-2-piperidyl)-äthoxy]-1-tetralon.
205. 7-[2-(1-Methyl-2-piperidyl)-äthoxy]-1-tetralon, Hydrochlorid, F. 188–190°.
206. 5-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-1-tetralon.
207. 6-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-1-tetralon.
208. 7-[1-Phenyl-2-(1-methyl-2-piperidyl)-äthoxy]-1-tetralon.

209. 5-(4-Hexahydroazepinyloxy)-1-tetralon.
210. 6-(4-Hexahydroazepinyloxy)-1-tetralon.
211. 7-(4-Hexahydroazepinyloxy)-1-tetralon.
212. 5-(1-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
213. 6-(1-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
214. 7-(1-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
215. 5-(3-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
216. 6-(3-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
217. 7-(3-Methyl-4-hexahydroazepinyloxy)-1-tetralon.
218. 5-(1,3-Dimethyl-4-hexahydroazepinyloxy)-1-tetralon.
219. 6-(1,3-Dimethyl-4-hexahydroazepinyloxy)-1-tetralon.
220. 7-(1,3-Dimethyl-4-hexahydroazepinyloxy)-1-tetralon.

Beispiele 221 bis 313

Analog Beispiel 1 erhält man aus den entsprechenden Na-Phenolaten und den entsprechenden Chlor- oder Brom-aminen:

221. 6-(1-Cyclopropyl-3-dimethylamino-propoxy)-isoflavon.
222. 7-(1-Cyclopropyl-3-dimethylamino-propoxy)-isoflavon.
223. 6-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-isoflavon.
224. 7-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-isoflavon, Hydrochlorid, F. 217°.
225. 6-(1-p-Chlorphenyl-3-dimethylamino-propoxy)-isoflavon.
226. 7-(1-p-Chlorphenyl-3-dimethylamino-propoxy)-isoflavon, Hydrochlorid, F. 247°.
227. 6-(1-p-Methoxyphenyl-3-dimethylamino-propoxy)-isoflavon.
228. 7-(1-p-Methoxyphenyl-3-dimethylamino-propoxy)-isoflavon, F. 111—113°.
229. 6-(1-p-Methylthiophenyl-3-dimethylamino-propoxy)-isoflavon.
230. 7-(1-p-Methylthiophenyl-3-dimethylamino-propoxy)-isoflavon.
231. 6-(1-Phenyl-3-dimethylamino-propoxy)-4'-methoxy-flavan, Hydrochlorid, F. 166—168°.
232. 7-(1-Phenyl-3-dimethylamino-propoxy)-4'-methoxy-flavan.
233. 3-Methyl-6-(1-phenyl-3-dimethylamino-propoxy)-3',4'-methylendioxy-flavanon, Hydrochlorid, F. 80—120°.
234. 3-Methyl-7-(1-phenyl-3-dimethylamino-propoxy)-3',4'-methylendioxy-flavanon.
235. cis-3-Äthyl-6-(1-phenyl-3-dimethylamino-propoxy)-4'-methoxy-flavanon, Rf 0,55.
236. trans-3-Äthyl-6-(1-phenyl-3-dimethylamino-propoxy)-4'-methoxy-flavanon, Rf 0,57.
237. cis-3-n-Propyl-6-(1-phenyl-3-dimethylamino-propoxy)-flavanon, Rf 0,44.
238. 3-tert.-Butyl-6-(1-phenyl-3-methylamino-propoxy)-chromon.
239. 3-tert.-Butyl-7-(1-phenyl-3-methylamino-propoxy)-chromon.
240. 3-tert.-Butyl-6-(1-phenyl-3-dimethylamino-propoxy)-chromon.
241. 3-tert.-Butyl-7-(1-phenyl-3-dimethylamino-propoxy)-chromon.
242. 3-tert.-Butyl-6-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
243. 3-tert.-Butyl-7-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
244. 3-tert.-Butyl-6-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
245. 3-tert.-Butyl-7-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
246. 3-tert.-Butyl-6-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
247. 3-tert.-Butyl-7-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
248. 3-tert.-Butyl-6-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
249. 3-tert.-Butyl-7-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
250. 2-Cyclopentyl-6-(1-phenyl-3-methylamino-propoxy)-chromon.
251. 2-Cyclopentyl-7-(1-phenyl-3-methylamino-propoxy)-chromon.
252. 2-Cyclopentyl-6-(1-phenyl-3-dimethylamino-propoxy)-chromon.
253. 2-Cyclopentyl-7-(1-phenyl-3-dimethylamino-propoxy)-chromon.
254. 2-Cyclopentyl-6-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
255. 2-Cyclopentyl-7-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
256. 2-Cyclopentyl-6-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
257. 2-Cyclopentyl-7-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
258. 2-Cyclopentyl-6-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
259. 2-Cyclopentyl-7-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
260. 2-Cyclopentyl-6-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
261. 2-Cyclopentyl-7-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
262. 3-Cyclopentyl-6-(1-phenyl-3-methylamino-propoxy)-chromon.
263. 3-Cyclopentyl-7-(1-phenyl-3-methylamino-propoxy)-chromon.
264. 3-Cyclopentyl-6-(1-phenyl-3-dimethylamino-propoxy)-chromon.
265. 3-Cyclopentyl-7-(1-phenyl-3-dimethylamino-propoxy)-chromon.



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266. 3-Cyclopentyl-6-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
267. 3-Cyclopentyl-7-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
268. 3-Cyclopentyl-6-[α-1-(methyl-3-piperidyl)-benzyloxy]-chromon.
269. 3-Cyclopentyl-7-[α-1-(methyl-3-piperidyl)-benzyloxy]-chromon.
270. 3-Cyclopentyl-6-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
271. 3-Cyclopentyl-7-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
272. 3-Cyclopentyl-6-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
273. 3-Cyclopentyl-7-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
274. 2-Cyclohexyl-6-(1-phenyl-3-methylamino-propoxy)-chromon.
275. 2-Cyclohexyl-7-(1-phenyl-3-methylamino-propoxy)-chromon.
276. 2-Cyclohexyl-6-(1-phenyl-3-dimethylamino-propoxy)-chromon.
277. 2-Cyclohexyl-7-(1-phenyl-3-dimethylamino-propoxy)-chromon.
278. 2-Cyclohexyl-6-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
279. 2-Cyclohexyl-7-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
280. 2-Cyclohexyl-6-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
281. 2-Cyclohexyl-7-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
282. 2-Cyclohexyl-6-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
283. 2-Cyclohexyl-7-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
284. 2-Cyclohexyl-6-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
285. 2-Cyclohexyl-7-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
286. 3-Cyclohexyl-6-(1-phenyl-3-methylamino-propoxy)-chromon.
287. 3-Cyclohexyl-7-(1-phenyl-3-methylamino-propoxy)-chromon.
288. 3-Cyclohexyl-6-(1-phenyl-3-dimethylamino-propoxy)-chromon.
289. 3-Cyclohexyl-7-(1-phenyl-3-dimethylamino-propoxy)-chromon.
290. 3-Cyclohexyl-6-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
291. 3-Cyclohexyl-7-(1-phenyl-3-N-benzyl-N-methylamino-propoxy)-chromon.
292. 3-Cyclohexyl-6-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
293. 3-Cyclohexyl-7-[α-(1-methyl-3-piperidyl)-benzyloxy]-chromon.
294. 3-Cyclohexyl-6-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
295. 3-Cyclohexyl-7-[2-(1-methyl-2-piperidyl)-äthoxy]-chromon.
296. 3-Cyclohexyl-6-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
297. 3-Cyclohexyl-7-(1,3-dimethyl-4-hexahydroazepinyloxy)-chromon.
298. 3-Methyl-6-(1-phenyl-3-dimethylamino-propoxy)-3',4'-methylendioxy-flavon, F. 74—76°.
299. 3-Methyl-7-(1-phenyl-3-dimethylamino-propoxy)-3',4'-methylendioxy-flavon.
300. 2-Äthyl-6-(1-phenyl-3-dimethylamino-propoxy)-isoflavon
301. 2-Äthyl-7-(1-phenyl-3-dimethylamino-propoxy)-isoflavon, Hydrochlorid, F. 226—228°.
302. 6-(1-Phenyl-3-dimethylamino-propoxy)-4'-fluor-isoflavon.
303. 7-(1-Phenyl-3-dimethylamino-propoxy)-4'-fluor-isoflavon, F. 112—113°; Hydrochlorid, F. 234—235°.
304. 6-(1-Phenyl-3-dimethylamino-propoxy)-4'-chlor-isoflavon.
305. 7-(1-Phenyl-3-dimethylamino-propoxy)-4'-chlor-isoflavon, Hydrochlorid, F. 259—260°.
306. 6-(1-Phenyl-3-dimethylamino-propoxy)-3'-trifluormethyl-isoflavon.
307. 7-(1-Phenyl-3-dimethylamino-propoxy)-3'-trifluormethyl-isoflavon, Hydrochlorid, F. 174—176°.
308. 6-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-4'-fluor-isoflavon.
309. 7-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-4'-fluor-isoflavon, F. 121—123°, Hydrochlorid, F. 229—230°.
310. 6-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-4'-chlor-isoflavon.
311. 7-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-4'-chlor-isoflavon, Hydrochlorid, F. 213—215°.
312. 6-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-3'-trifluormethyl-isoflavon.
313. 7-(1-p-Fluorphenyl-3-dimethylamino-propoxy)-3'-trifluormethyl-isoflavon, Hydrochlorid, F. 174—176°.

## Beispiel 314

Man löst 23,8 g 7-Hydroxyisoflavon in 500 ml absolutem Toluol, versetzt mit 7,1 ml Thallium-(I)-äthylat und rührt 1 Stunde bei 20°. Nach dem Eindampfen wird der Rückstand in 100 ml absolutem Acetonitril gelöst, mit 20 g 1-Chlor-1-phenyl-3-dimethylaminopropan versetzt, 3 Stunden unter Rühren gekocht und eingedampft. Nach üblicher Aufarbeitung erhält man 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon. F. 128—130°.

Beispiel 315

Man löst 2,7 g Azodicarbonsäureäthylester in 25 ml THF, gibt unter Kühlung und Rühren 4 g Triphenylphosphin hinzu, tropft anschließend eine Lösung von 2 g 1-Chlor-1-phenyl-3-dimethylamino-propan in 15 ml THF und dann eine Lösung von 2,38 g 7-Hydroxyisoflavon in 10 ml THF hinzu und rührt 2 Stunden bei 0°. Nach Stehenlassen über Nacht bei 20° wird wie üblich aufgearbeitet. Man erhält 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon. F. 128–130°.

Beispiel 316

Zu einer Lösung von 23,8 g 7-Hydroxyisoflavon in 100 ml $CH_2Cl_2$ gibt man 200 ml 50%ige wässerige Natronlauge und 1 g Triäthylbenzylammoniumchlorid, tropft unter Rühren 20 g 1-Chlor-1-phenyl-3-dimethylamino-propan hinzu und rührt noch eine Stunde nach. Nach üblicher Aufarbeitung erhält man 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon. F. 128–130°.

Beispiel 317

Eine Lösung von 20 g 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon in 400 ml Methanol wird an 3 g 5%igem Pd–C bei 20° und 1 at bis zum Stillstand hydriert. Man filtriert, dampft ein und erhält 7-(1-Phenyl-3-methylamino-propoxy)-isoflavon, Hydrochlorid, F. 210–211°.

Beispiel 318

Zu 38,5 g 7-(1-Phenyl-3-methylamino-propoxy)-isoflavon tropft man unter Rühren und Kühlen 30 g Ameisensäure und anschließend bei 20° 7 g 25%ige Formaldehydlösung. Man erhitzt bis zum Ende der Gasentwicklung auf dem Wasserbad, kühlt ab, gießt auf Eis, arbeitet wie üblich auf und erhält 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon. F. 128–130°.

Beispiel 319

Man gibt 12 g Allylbromid und 26 g wasserfreies Kaliumcarbonat zu einer Lösung von 38,5 g 7-(1-Phenyl-3-methyl-amino-propoxy)-isoflavon in 1 l absolutem Toluol, kocht 20 Stunden, kühlt ab, gießt in Wasser, arbeitet wie üblich auf und erhält 7-(1-Phenyl-3-N-allyl-N-methylaminopropoxy)-isoflavon. Hydrochlorid, F. 192–194°.

Beispiele 320 bis 326

Analog Beispiel 319 erhält man mit Allylbromid, n-Butyljodid, Cyclopropylmethylchlorid oder Benzylbromid:

320. 6-(1-Phenyl-3-N-allyl-N-methylamino-propoxy)-isoflavon.
321. 6-(1-Phenyl-3-N-n-butyl-N-methylamino-propoxy)-isoflavon.
322. 7-(1-Phenyl-3-N-n-butyl-N-methylamino-propoxy)-isoflavon.
323. 6-(1-Phenyl-3-N-cyclopropylmethyl-N-methylamino-propoxy)-isoflavon.
324. 7-(1-Phenyl-3-N-cyclopropylmethyl-N-methylamino-propoxy)-isoflavon, Hydrochlorid, F. 225–227°.
325. 6-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon.
326. 7-(1-Phenyl-3-N-benzyl-N-methylamino-propoxy)-isoflavon.

Beispiele 327 bis 331

Analog Beispiel 1 erhält man aus den entsprechenden Na-phenolaten und den entsprechenden basischen Chloriden:

327. 6-(1-Cyclopropyl-3-methylamino-propoxy)-flavon, Rf 0,45 (Chloroform/Triäthylamin 8 : 2).
328. 6-(1-Phenyl-3-dimethylamino-propoxy)-4'-methoxy-flavon, F. 125–127°.
329. 6-(1-Phenyl-3-dimethylamino-propoxy)-3',4'-methylendioxy-flavon, F. 159–160°.
330. 7-(1-Cyclopropyl-3-methylamino-propoxy)-chroman, Rf 0,24 (Methanol).
331. 7-(1-Cyclopropyl-3-dimethylamino-propoxy)-chroman, Rf 0,74 (Chloroform/Triäthylamin 8 : 2).



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Die nachstehenden Beispiele betreffen pharmazeutische Zubereitungen, die Amine der Formel I oder ihre Säureadditionssalze enthalten:

Beispiel A

Tabletten

Ein Gemisch von 1 kg 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon-hydrochlorid, 4 kg Lactose, 1,2 kg Kartoffelstärke, 0,2 kg Talk und 0,1 kg Magnesiumstearat wird in üblicher Weise zu Tabletten gepreßt, derart, daß jede Tablette 10 mg Wirkstoff enthält.

Beispiel B

Dragees

Analog Beispiel A werden Tabletten gepreßt, die anschließend in üblicher Weise mit einem Überzug aus Saccharose, Kartoffelstärke, Talk, Tragant und Farbstoff überzogen werden.

Beispiel C

Kapseln

2 kg 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon-hydrochlorid werden in üblicher Weise in Hartgelatinekapseln gefüllt, so daß jede Kapsel 20 mg des Wirkstoffs enthält.

Beispiel D

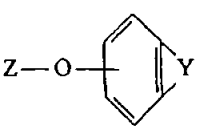
Ampullen

Eine Lösung von 1 kg 7-(1-Phenyl-3-dimethylamino-propoxy)-isoflavon-hydrochorid in 30 l zweifach destilliertem Wasser wird steril filtriert, in Ampullen abgefüllt, unter sterilen Bedingungen lyophilisiert und steril verschlossen. Jede Ampulle enthält 10 mg Wirkstoff.

Analog sind Tabletten, Dragees, Kapseln und Ampullen erhältlich, die einen oder mehrere der übrigen Wirkstoffen der Formel I und/oder ihre physiologisch unbedenklichen Säureadditionssalze enthalten.

**Patentansprüche**

1. Basische Äther der allgemeinen Formel I



(I)

worin

| | |
|---|---|
| Z | $CH_3-NR^2-CH_2CH_2-CHR^1-$, (1-$R^2$-3-piperidyl)-$CHR^3-$, (1-$R^2$-2-piperidyl)-$CH_2-CHR^3-$ oder 1-$R^2$-3-$R^4$-4-hexahydroazepinyl, |
| $R^1$ | Cyclopropyl oder Ar, |
| $R^2$ | H, Alkyl mit 1–4 C-Atomen, Allyl, Cycloalkylalkyl mit 4–8 C-Atomen oder Benzyl, |
| $R^3$ | H oder Ar, |
| $R^4$ | H oder Alkyl mit 1–4 C-Atomen, |
| Y | $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ oder $-CH_2-CHQ^1-CHQ^2-CO-$, |
| $Q^1$ und $Q^2$ | jeweils H, Alkyl mit 1–4 C-Atomen, Cycloalkyl mit 3–6 C-Atomen oder Ar und |
| Ar | Phenyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl |

bedeuten, worin jedoch

$R^1$ Cyclopropyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl

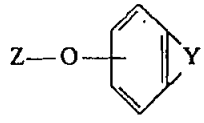
bedeutet, wenn gleichzeitig

Z eine $(CH_3)_2N–CH_2CH_2–CHR^1$-Gruppe in 7-Stellung und
Y $–O–C(C_6H_5)=CH–CO–$ oder $–O–C(C_6H_5)=C(CH_3)–CO–$

bedeuten, sowie ihre physiologisch unbedenklichen Säureadditionssalze.

2. 6-(1-Phenyl-3-dimethylamino-propoxy)-flavon.

3. Verfahren zur Herstellung von basischen Äthern der allgemeinen Formel I



(I)

worin

| | |
|---|---|
| Z | $CH_3–NR^2–CH_2CH_2–CHR^1–$, (1-$R^2$-3-piperidyl)–$CHR^3–$, (1-$R^2$-2-piperidyl)–$CH_2–CHR^3–$ oder 1-$R^2$-3-$R^4$-4-hexahydroazepinyl, |
| $R^1$ | Cyclopropyl oder Ar, |
| $R^2$ | H, Alkyl mit 1–4 C-Atomen, Allyl, Cycloalkylalkyl mit 4–8 C-Atomen oder Benzyl, |
| $R^3$ | H oder Ar, |
| $R^4$ | H oder Alkyl mit 1–4 C-Atomen, |
| Y | $–O–CHQ^1–CHQ^2–CH_2–$, $–O–CHQ^1–CHQ^2–CO–$, $–O–CQ^1=CQ^2–CO–$ oder $–CH_2–CHQ^1–CHQ^2–CO–$, |
| $Q^1$ und $Q^2$ | jeweils H, Alkyl mit 1–4 C-Atomen, Cycloalkyl mit 3–6 C-Atomen oder Ar und |
| Ar | Phenyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl |

bedeuten, worin jedoch

$R^1$ Cyclopropyl oder durch F, Cl, Alkoxy oder Alkylthio mit jeweils 1–4 C-Atomen, Methylendioxy oder $CF_3$ substituiertes Phenyl

bedeutet, wenn gleichzeitig

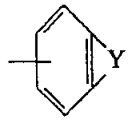
Z eine $(CH_3)_2N–CH_2CH_2–CHR^1$-Gruppe in 7-Stellung und
Y $–O–C(C_6H_5)=CH–CO–$ oder $–O–C(C_6H_5)=C(CH_3)–CO–$

bedeuten, sowie von deren physiologisch unbedenklichen Säureadditionssalzen, dadurch gekennzeichnet, daß man ein Phenol der allgemeinen Formel II

$$HO—G \qquad (II)$$

worin

G die Gruppe



bedeutet, oder eines seiner Salze mit einem Amin der allgemeinen Formel III

$$Z—X \qquad (III)$$

worin

X Cl, Br, J oder OH bedeutet und
Z die angegebene Bedeutung hat



X 263

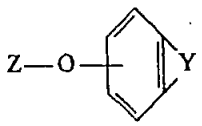
oder mit einem seiner reaktionsfähigen Derivate umsetzt und daß man gegebenenfalls in einer erhaltenen Verbindung der Formel I eine sekundäre Aminogruppe durch Behandeln mit einem alkylierenden, allylierenden, cycloalkylalkylierenden oder benzylierenden Mittel in die entsprechende tertiäre Aminogruppe oder eine N-Benzylgruppe durch Behandeln mit einem Reduktionsmittel in eine NH-Gruppe und/oder eine erhaltene Base der Formel I durch Behandeln mit einer Säure in eines ihrer physiologisch unbedenklichen Säureadditionssalze umwandelt.

4. Verfahren zur Herstellung pharmazeutischer Zubereitungen, dadurch gekennzeichnet, daß man eine Verbindung der Formel I und/oder eines ihrer physiologisch unbedenklichen Säureadditionssalze gemäß Anspruch 1 zusammen mit mindestens einem festen, flüssigen oder halbflüssigen Träger- oder Hilfsstoff und gegebenenfalls in Kombination mit einem oder mehreren weiteren Wirkstoffen in eine geeignete Dosierungsform bringt.

5. Pharmazeutische Zubereitung, gekennzeichnet durch einen Gehalt an mindestens einer Verbindung der allgemeinen Formel I und/oder einem ihrer physiologisch unbedenklichen Säureadditionssalze gemäß Anspruch 1.

**Claims**

1. Basic ethers of the general formula I



(I)

wherein

| | |
|---|---|
| Z | is $CH_3-NR^2-CH_2CH_2-CHR^1-$, (1-$R^2$-3-piperidyl)-$CHR^3-$, (1-$R^2$-2-piperidyl)-$CH_2CHR^3-$ or 1-$R^2$-3-$R^4$-4-hexahydroazepinyl, |
| $R^1$ | is cyclopropyl or Ar, |
| $R^2$ | is H, alkyl having 1–4 C atoms, alkenyl having 2–4 C atoms, cycloalkylalkyl having 4–8 C atoms or benzyl, |
| $R^3$ | is H or Ar, |
| $R^4$ | is H or alkyl having 1–4 c atoms, |
| Y | is $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ or $-CH_2-CHQ^1-CHQ^2-CO-$, |
| $Q^1$ and $Q^2$ | are each H, alkyl having 1–4 C atoms, cycloalkyl having 3–6 C atoms or Ar and |
| Ar | is phenyl or phenyl which is substituted by F, Cl, alkoxy or alkylthio, each of which has 1–4 C atoms, methylenedioxy or $CF_3$, |

but wherein

$R^1$ is cyclopropyl or phenyl which is substituted by F, Cl, alkoxy or alkylthio, each of which has 1–4 C atoms, methylenedioxy or $CF_3$,

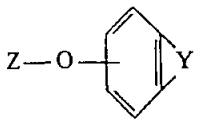
if, at the same time,

Z is a $(CH_3)_2N-CH_2CH_2-CHR^1$ group in 7-position and
Y is a $-O-C(C_6H_5)=CH-CO-$ or $-O-C(C_6H_6)=C(CH_3)-CO$-group

and also their physiologically acceptable acid addition salts.

2. 6-(1-Phenyl-3-dimethylaminopropoxy)-flavone.

3. Process for the preparation of basic ethers of the general formula I



(I)

wherein

| | |
|---|---|
| Z | is $CH_3-NR^2-CH_2CH_2-CHR^1-$, (1-$R^2$-3-piperidyl)-$CHR^3-$, (1-$R^2$-2-piperidyl)-$CH_2CHR^3-$ or 1-$R^2$-3-$R^4$-4-hexahydroazepinyl, |

| | |
|---|---|
| $R^1$ | is cyclopropyl or Ar, |
| $R^2$ | is H, alkyl having 1–4 C atoms, alkenyl having 2–4 C atoms, cycloalkylalkyl having 4–8 C atoms or benzyl, |
| $R^3$ | is H or Ar, |
| $R^4$ | is H or alkyl having 1–4 c atoms, |
| Y | is $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ or $-CH_2-CHQ^1-CHQ^2-CO-$, |
| $Q^1$ and $Q^2$ | are each H, alkyl having 1–4 C atoms, cycloalkyl having 3–6 C atoms or Ar and |
| Ar | is phenyl or phenyl which is substituted by F, Cl, alkoxy or alkylthio, each of which has 1–4 C atoms, methylenedioxy or $CF_3$, |

but wherein

$R^1$ is cyclopropyl or phenyl which is substituted by F, Cl, alkoxy or alkylthio, each of which has 1–4 C atoms, methylenedioxy or $CF_3$,

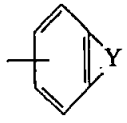
if, at the same time,

Z is a $(CH_3)_2N-CH_2CH_2-CHR^1$ group in 7-position and
Y is a $-O-C(C_6H_5)=CH-CO-$ or $-O-C(C_6H_5)=C(CH_3)-CO$-group,

and of their physiologically acceptable acid addition salts charakterised in that a phenol of the general formula II

$$HO-G \qquad (II)$$

wherein
G is the group



or one of its salts, is reacted with an amine of the general formula III

$$Z-X \qquad (III)$$

wherein

X is Cl, Br, I or OH and
Z has the meaning indicated,

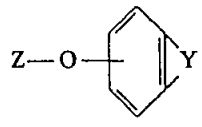
or with one of its reactive derivatives, and, if appropriate, in a resulting compound of the formula I, a secondary amino group is converted by treatment with an alkylating, alkenylating, cycloalkylalkylating or benzylating agent into the corresponding tertiary amino group or an N-benzyl group is converted by treatment with a reducing agent into an NH group and/or a resulting base of the formula I is converted by treatment with an acid into one of its physiologically acceptable acid addition salts.

4. Process for the preparation of pharmaceutical formulations, characterised in that a compound of the formula I and/or one of its physiologically acceptable acid addition salts is brought into a suitable dosage form together with at least one solid, liquid or semi-liquid excipient or adjuvant, and, if appropriate, in combination with one or more further active compounds.

5. Pharmaceutical formulation characterised in that it contains at least one compound of the general formula I and/or one of its physiologically acceptable acid additions salts.

**Revendications**

1. Ethers basiques de formule générale I:



(I)

dans laquelle

| | |
|---|---|
| Z | représente $CH_3-NR^2-CH_2CH_2-CHR^1$, $(1-R^2\text{-}3\text{-pipéridyl})-CHR^3-$, $(1-R^2\text{-}2\text{-pipéridyl})-CH_2-CHR^3-$ ou $1-R^2\text{-}3-R^4\text{-}4$-hexahydroazépinyle, |
| $R^1$ | représente un groupe cyclopropyle ou Ar, |
| $R^2$ | représente H, un groupe alkyle contenant 1 à 4 atomes de carbone, un groupe allyle, un groupe cycloalkylalkyle contenant 4 à 8 atomes de carbone ou un groupe benzyle, |
| $R^3$ | représente H ou Ar, |
| $R^4$ | représente H ou un groupe alkyle contenant 1 à 4 atomes de carbone, |
| Y | représente $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ ou $-CH_2-CHQ^1-CHQ^2-CO-$, |
| $Q^1$ et $Q^2$ | représentent chacun H, un groupe alkyle contenant 1 à 4 atomes de carbone, un groupe cycloalkyle contenant 3 à 6 atomes de carbone ou Ar, et |
| Ar | représente un groupe phényle ou un groupe phényle substitué par F, Cl, un groupe alcoxy ou un groupe alkylthio contenant chacun 1 à 4 atomes de carbone, un groupe méthylène-dioxy ou par $CF_3$, |

mais dans laquelle

$R^1$ représente un groupe cyclopropyle ou un groupe phényle substitué par F, Cl, un groupe alcoxy ou un groupe alkylthio contenant chacun 1 à 4 atomes de carbone, un groupe méthylène-dioxy ou par $CF_3$,

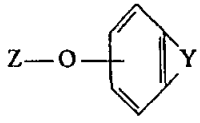
lorsque simultanément

Z représente un groupe $(CH_3)_2N-CH_2CH_2-CHR^1$ en position 7 et
Y représente $-O-C(C_6H_5)=CH-CO$ ou $-O-C(C_6H_5)=C(CH_3)-CO-$,

ainsi que leurs sels d'addition d'acide physiologiquement sans inconvénient.

2. La 6-(1-phényl-3-diméthylamino-propoxy)-flavone.

3. Procédé de préparation d'éthers basiques de formule générale I:



(I)

dans laquelle

| | |
|---|---|
| Z | représente $CH_3-NR^2-CH_2CH_2-CHR^1$, $(1-R^2\text{-}3\text{-pipéridyl})-CHR^3-$, $(1-R^2\text{-}2\text{-pipéridyl})-CH_2-CHR^3-$ ou $1-R^2\text{-}3-R^4\text{-}4$-hexahydroazépinyle, |
| $R^1$ | représente un groupe cyclopropyle ou Ar, |
| $R^2$ | représente H, un groupe alkyle contenant 1 à 4 atomes de carbone, un groupe allyle, un groupe cycloalkylalkyle contenant 4 à 8 atomes de carbone ou un groupe benzyle, |
| $R^3$ | représente H ou Ar, |
| $R^4$ | représente H ou un groupe alkyle contenant 1 à 4 atomes de carbone, |
| Y | représente $-O-CHQ^1-CHQ^2-CH_2-$, $-O-CHQ^1-CHQ^2-CO-$, $-O-CQ^1=CQ^2-CO-$ ou $-CH_2-CHQ^1-CHQ^2-CO-$, |
| $Q^1$ et $Q^2$ | représentent chacun H, un groupe alkyle contenant 1 à 4 atomes de carbone, un groupe cycloalkyle contenant 3 à 6 atomes de carbone ou Ar, et |
| Ar | représente un groupe phényle ou un groupe phényle substitué par F, Cl, un groupe alcoxy ou un groupe alkylthio contenant chacun 1 à 4 atomes de carbone, un groupe méthylène-dioxy ou par $CF_3$, |

mais dans laquelle

$R^1$ représente un groupe cyclopropyle ou un groupe phényle substitué par F, Cl, un groupe alcoxy ou un groupe alkylthio contenant chacun 1 à 4 atomes de carbone, un groupe méthylène-dioxy ou par $CF_3$,

lorsque simultanément

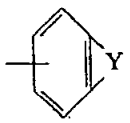
Z représente un groupe $(CH_3)_2N-CH_2CH_2-CHR^1$ en position 7, et
Y représente $-O-C(C_6H_5)=CH-CO$ ou $-O-C(C_6H_5)=C(CH_3)-CO-$,

ainsi que de leurs sels d'addition d'acide physiologiquement sans inconvénient, caractérisé en ce qu'on fait réagir un phénol de formule générale II:

$$HO—G \qquad (II)$$

dans laquelle

G représente le groupe



ou un de ses sels avec une amine de formulé générale III

$$Z—X \qquad (III)$$

dans laquelle

X représente Cl, Br, I ou OH, et
Z a la signification indiquée,

ou avec un de des dérivés réactifs et, éventuellement, dans un composé obtenu de formule I, par traitement avec un agent d'alkylation, d'allylation, de cycloalkylalkylation ou de benzylation, on transforme un groupe amino secondaire en un groupe amino tertiaire correspondant ou, par traitement avec un agent réducteur, on transforme un groupe N-benzyle en un groupe NH et/ou, par traitement avec un acide, on transforme une base obtenue de formule I en un de ses sels d'addition d'acide physiologiquement sans inconvénient.

4. Procédé en vue d'obtenir des préparations pharmaceutiques, caractérisé en ce qu'on met, sous une forme de dosage appropriée, un composé de formule I et/ou un de ses sels d'addition d'acide physiologiquement sans inconvénient suivant la revendication 1 conjointement avec au moins une substance auxiliaire ou une substance support solide, liquide ou semi-liquide et éventuellement en combinaison avec une ou plusieurs autres substances actives.

5. Préparation pharmaceutique, caractérisée en ce qu'elle contient au moins un composé de formule générale I et/ou un de ses sels d'addition d'acide physiologiquement sans inconvénient suivant la revendication 1.

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# BIOSYNTHESIS OF PTEROCARPAN PHYTOALEXINS IN *TRIFOLIUM PRATENSE*

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(*Received* 21 *July* 1976)

Key Word Index—*Trifolium pratense*; Leguminosae; red clover; biosynthesis; phytoalexin; pterocarpan; demethylhomopterocarpin; maackiain; isoflavonoid; coumestan.

Abstract—Feeding experiments have demonstrated that 7,2'-dihydroxy-4'-methoxy-isoflavone-[$^{14}$C-Me] and -isoflavanone-[$^{14}$C-Me] are extremely efficient precursors of the phytoalexin demethylhomopterocarpin in $Cu^{2+}$-treated red clover seedlings. Neither of these compounds, nor demethylhomopterocarpin-[$^{14}$C-Me], was incorporated into a second pterocarpan phytoalexin, maackiain. 3-Hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me] was a poor precursor of both pterocarpans. A biosynthetic pathway to demethylhomopterocarpin via 2'-hydroxylation of formononetin (7-hydroxy-4'-methoxyisoflavone) and subsequent reduction to the isoflavanone is proposed. The conversion of this isoflavanone into the pterocarpan may involve the corresponding isoflavanol and a carbonium ion intermediate. The branch-point to maackiain is probably at the formononetin stage. The presence of two coumestans, 9-*O*-methylcoumestrol and medicagol, previously unreported in red clover, is demonstrated. Biosynthetic implications are discussed.

## INTRODUCTION

In response to fungal infection [1], or on treatment with heavy metal ions [2], seedlings of red clover (*Trifolium pratense* L.) synthesise two pterocarpan phytoalexins, (6aR,11aR)-demethylhomopterocarpin (medicarpin) (1) and (6aR,11aR)-maackiain (2). Feeding experiments utilising $Cu^{2+}$-treated seedlings have demonstrated [2] that labelled 2',4',4-trihydroxychalcone (3) and 7-hydroxy-4'-methoxyisoflavone (formononetin) (6) were readily incorporated into both pterocarpans, and that the 2,4- and 2,4,5-oxygenation patterns in the D-ring of demethylhomopterocarpin and maackiain respectively are presumably built up from the simple 4'-*O*-methyl of formononetin. However, 2',4'-dihydroxy-4-methoxychalcone (4) and 7,4'-dihydroxyisoflavone (daidzein) (5) were inefficient biosynthetic precursors, results which may be rationalised if methylation is an integral part of the aryl migration process involved in the biosynthesis of isoflavones [3,4].

Further labelled isoflavonoids have been tested as precursors of demethylhomopterocarpin and maackiain in $Cu^{2+}$-treated red clover seedlings, to investigate the biosynthetic route to these pterocarpans, compounds which may well play an important role in the disease resistance of this and other plants [5]. Some of the results have been published in a preliminary communication [6].

## RESULTS

Compounds tested as biosynthetic precursors were 7,2'-dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] (7), (±)-7,2'-dihydroxy-4'-methoxyisoflavanone-[$^{14}$C-Me] (8), 3-hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me] (9) and (±)-3-hydroxy-9-methoxypterocarpan-[$^{14}$C-Me] (demethylhomopterocarpin) (1).

Base condensation of 4'-benzyloxy-2'-hydroxyacetophenone with 4-benzyloxy-2-methoxybenzaldehyde yielded 4,4'-dibenzyloxy-2'-hydroxy-2-methoxychalcone which was converted via thallium nitrate oxidation and treatment with acid [7] into 7,4'-dibenzyloxy-2'-methoxyisoflavone. Debenzylation followed by selective monobenzylation produced 7-benzyloxy-4'-hydroxy-2'-methoxyisoflavone, which was methylated using methyl iodide-[$^{14}$C]. The product, 7-benzyloxy-2',4'-dimethoxyisoflavone-[4'-$^{14}$C-Me] was debenzylated/selectively demethylated by treating with $AlCl_3$ in MeCN[8] to give 7,2'-dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] (7). Catalytic hydrogenation of this isoflavone (via the acetate) produced the corresponding (±)-isoflavanone-[$^{14}$C-Me] (8), and the latter compound was converted into 3-hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me] (9) by heating with mineral acid. $NaBH_4$ reduction of 7,2'-dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] followed by treatment with acid gave (±)-demethylhomopterocarpin-[$^{14}$C-Me] (1).

Small quantities (*ca* 0.5 mg) of these labelled compounds were separately administered as their Na salts in phosphate buffer to batches of 4-day-old dark-grown red clover seedlings [2], which had been treated with aqueous $CuCl_2$ for 8 hr. After a feeding period of 16 hr in the dark, the seedlings were worked up, and the pterocarpan phytoalexins were isolated from the extract. As described previously [2], the pterocarpans were isolated together, individual concentrations were assayed by UV, then half of the fraction was diluted with inactive (±)-demethylhomopterocarpin carrier, and the other half with (±)-maackiain carrier. The individual ptero-

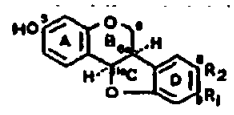
Table 1. Incorporation of [*methyl*-$^{14}$C]-labelled isoflavonoids into pterocarpans in *Trifolium pratense* seedings*

| Compound fed | Unabsorbed precursor (%) | Demethylhomopterocarpin sp. act. (dpm/mM) | Dilution | Incorp† (%) | Maackiain sp. act. (dpm/mM) | Dilution | Incorp† (%) |
|---|---|---|---|---|---|---|---|
| For mononetin | 30 | $3.45 \times 10^7$ | 34 | 0.62 | $2.00 \times 10^7$ | 59 | 0.51 |
| 7,2'- Dihydroxy-4'-methoxyisoflavone | 22 | $4.35 \times 10^6$ | 2.7 | 12.8 | $1.45 \times 10^3$ | 7900 | 0.0035 |
| (±)-7,2'-Dihydroxy-4'-methoxyisoflavanone | 27 | $3.80 \times 10^6$ | 3.2 | 7.6 | $1.02 \times 10^3$ | 11900 | 0.0019 |
| 3-Hydroxy-9-methoxypterocarp-6a-ene | 19 | $3.02 \times 10^6$ | 420 | 0.040 | $2.28 \times 10^3$ | 5600 | 0.0044 |
| (±)-Demethylhomopterocarpin | 20 | $7.92 \times 10^6$ | 1.5 | 35.6 | $1.86 \times 10^3$ | 6100 | 0.0035 |

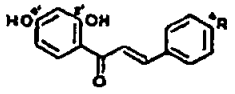
* Four-day-old, $CuCl_2$ inducer applied for 8 hr, feeding period 16 hr. † Incorporation figures are not corrected for unabsorbed precursor, or possible utilisation of only one enantiomer from racemic mixtures.

carpans were methylated and purified to constant specific activity. The results of these comparative feeding experiments are summarised in the Table 1, which also includes incorporation data for 7-hydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] (formononetin) (6), administered in the same set of experiments. Since this compound is a good precursor of both pterocarpans in red clover [2], these data act as a standard.

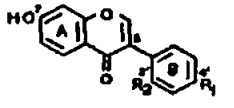
The incorporation figures indicate that isoflavone (7) and (±)-isoflavanone (8) are both excellent precursors of



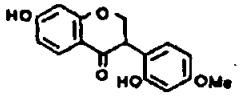
(1) $R_1$=OMe, $R_2$=H
(2) $R_1R_2$ = O-$CH_2$-O



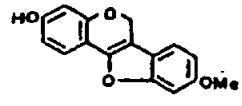
(3) R=OH
(4) R=OMe



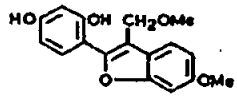
(5) $R_1$=OH, $R_2$=H
(6) $R_1$=OMe, $R_2$=H
(7) $R_1$=OMe, $R_2$=OH



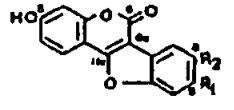
(8)



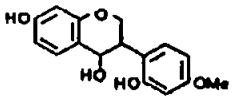
(9)



(10)



(11) $R_1$=OH, $R_2$=H
(12) $R_1$=OMe, $R_2$=H
(13) $R_1R_2$ = O-$CH_2$-O



(14)

demethylhomopterocarpin, considerably better than formononetin (6). Neither of these compounds was significantly incorporated into maackiain. The incorporation of pterocarp-6a-ene (9) into demethylhomopterocarpin was very small by comparison, and into maackiain was negligible. (±)-Demethylhomopterocarpin was not incorporated into maackiain.

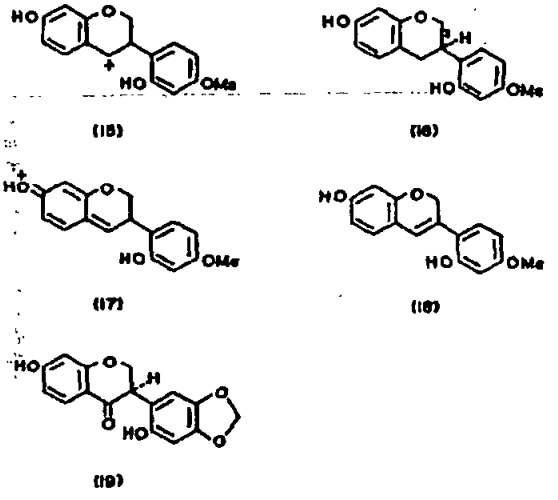
Autooxidation of pterocarp-6a-enes to the corresponding 6-oxo derivatives (coumestans) in the presence of air has frequently been reported [9–11]). To ascertain whether this process was occurring during the feeding period, thus resulting in unusually low incorporation figures for 3-hydroxy-9-methoxypterocarp-6a-ene, the stability of this pterocarp-6a-ene was investigated. An acetone solution of (9), after being stirred overnight in the presence of air showed little reaction, only very faint traces of the coumestan oxidation product being observed. In contrast, a methanolic solution treated similarly contained hardly any of the original pterocarp-6a-ene. Traces of coumestan were present, but the major product was found to be 2-(2,4-dihydroxyphenyl)-6-methoxy-3-methoxymethyl-benzofuran (10). A similar reaction has been noted [12] for anhydropisatin (3-methoxy-8,9-methylenedioxypterocarp-6a-ene), the conversion being the result of photolytic ring fission and then alcoholysis. Samples of (9) dissolved in the feeding mixture of NaOH/phosphate buffer also disappeared over 24 hr to give faint traces of the coumestan, plus other unidentified products. Maximum stability of (9) in an aqueous medium was attained by using a mixture of 2-methoxyethanol–deoxygenated water containing either NaOH or Tween-20, and keeping the solution in an $N_2$ atmosphere. Such solutions after a period of 24 hr in the light contained about half of the original pterocarp-6a-ene concentration, together with a product which was presumably the 2-methoxyethanol analogue of (10). Further feeding experiments were carried out with 3-hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me] employing these solvent systems, and conducting the first 3.5 hr of the feeding period in an $N_2$ atmosphere (all feedings performed in the dark). Although such feeding conditions were abnormal for the plant, uptake of precursor and production of the pterocarpans were not noticeably different from those observed in the other experiments carried out under "normal" conditions. Incorporation data, however, were virtually identical to those shown in the Table, and demonstrate that 3-hydroxy-9-methoxypterocarp-6a-ene is not significantly utilised for pterocarpan production in red clover.

Chromatography of the seedling extracts during these experiments consistently revealed the presence of a zone exhibiting a strong bright-blue fluorescence under UV light. Such fluorescence is characteristic of coumestans,

but the only reported coumestan in *T. pratense* is coumestrol (11) [13], and the observed zone ran at a considerably higher $R_f$ than (11). UV data ($\lambda_{max}^{EtOH}$ 343, 309, 302 nm) indicated the presence of two coumestans. Comparison with authentic specimens by TLC and UV spectroscopy (shifts in the presence of NaOAc, NaOEt, NaOAc–$H_3BO_3$) suggested these were 3-hydroxy-9-methoxycoumestan (9-*O*-methylcoumestrol) (12) and 3-hydroxy-8,9-methylenedioxy-coumestan (medicagol) (13). This was confirmed by the mass spectrum of the acetylated mixture. The acetates could be partially separated by TLC [14]. Reference material was synthesised by DDQ oxidation [11] of the corresponding (±)-pterocarpans [2].

## DISCUSSION

Preliminary feeding experiments using DL-phenylalanine-[1-$^{14}$C] [2] had suggested that the red clover phytoalexins demethylhomopterocarpin (1) and maackiain (2) were synthesised *de novo* on treatment of the plant tissue with $Cu^{2+}$ ions, and the similar incorporation



(15) (16) (17) (18) (19)

profiles observed over a period of time indicated that it was unlikely that (1) was a precursor of (2). The latter conclusion is confirmed by the negligible incorporation of labelled (1) into (2).

7,2'-Dihydroxy-4'-methoxy-isoflavone (7) and -isoflavanone (8) are extremely efficient precursors of demethylhomopterocarpin, and may well be natural intermediates on its biosynthetic pathway. Although isoflavone (7) has not been reported in nature, 7,2'-dihydroxy-4'-methoxyisoflavanone has recently been isolated [15] from *Onobrychis viciifolia* (Leguminosae). It appears likely therefore that formononetin (6) may be 2'-hydroxylated to (7) which is then reduced to the isoflavanone (8). 2'-Hydroxylation of isoflavones has been previously demonstrated [16] in *Phaseolus aureus*, and this, or the corresponding hydroxylation of isoflavanones is regarded [17] as a probable step in the biosynthetic pathway to coumestans in this and other plants. The conversion of (8) into (1) however clearly does not proceed via the pterocarp-6a-ene (9). Pterocarp-6a-enes, formed by dehydration of 2'-hydroxyisoflavanones, or oxidative cyclisation of 2'-deoxyisoflavanones, have been postulated [9, 16–18] as key intermediates in the biosynthesis of other isoflavonoid compounds. Thus, reduction might give pterocarpans, addition of water could produce 6a-hydroxypterocarpans, whilst allylic oxidation would serve as a route to coumestans. The incorporation of (9) into (1) (0.040%) is insignificant by comparison with other precursors, and even this small amount may be due to prior hydrolysis to (8), a good precursor. Incorporation into maackiain was negligible. Thus, a hypothesis for pterocarpan biosynthesis not involving pterocarp-6a-ene intermediates is clearly necessary. From these results, the most likely route from (8) to (1) would be by further reduction to the isoflavanol (14) which could lose water and cyclise to the pterocarpan [19]. Such a pathway would be analogous to laboratory syntheses [20] of pterocarpans by $NaBH_4$ reduction of 2'-hydroxyisoflavanones, followed by treatment with acid. This synthetic route probably involves a carbonium ion (15) [21], and the same entity could occupy a place in the biosynthetic pathway. Strong support comes from the results of feeding experiments [22] in lucerne (*Medicago sativa*) where demethylhomopterocarpin and the isoflavan vestitol (16), phytoalexins of lucerne, have been shown to be interconvertible, although they appear to be synthesised simultaneously from a common intermediate. Such an intermediate could be the carbonium ion (15), or its mesomeric counterpart (17), presumably enzyme-bound. Cyclisation and loss of a proton would lead to the pterocarpan (1), whilst addition of hydride ion would produce the isoflavan (16). Reversal of these pathways would explain the interconversion. Structure (17) represents the protonated form of the quinonemethide intermediate postulated [23] in the chemical conversion of (16) into (1), a reaction proposed as a chemical analogy for pterocarpan biosynthesis.

We have tested (±)-vestitol-[$^{14}$C-Me] as a precursor of the pterocarpans in red clover [22]. It was incorporated into demethylhomopterocarpin (0.90‰ dilution 19) but not into maackiain (0.0018‰ dilution 8400). Comparative figures for (±)-7,2'-dihydroxy-4'-methoxyisoflavanone-[$^{14}$C-Me] were: demethylhomopterocarpin (2.7‰ dilution 4·5), maackiain (0.0047‰ dilution 3200). These figures also suggest that vestitol is not a normal biosynthetic precursor of demethylhomopterocarpin, but may be converted into that compound by some common intermediate, e.g. carbonium ion (15). An alternative, uncharged intermediate, not yet excluded by feeding experiments, might be the isoflav-3-ene (18). However, if isoflav-3-enes are intermediates in the biosynthesis of pterocarpans and isoflavans, it is surprising that no naturally occurring example of this class of isoflavonoid has yet been reported.

Although the results strongly suggest the biosynthetic route to demethylhomopterocarpin involves 2'-hydroxyisoflavone (7) and 2'-hydroxyisoflavanone (8), isoflavanol (14) may be produced from formononetin via a 'metabolic grid' [24] of isoflavones, isoflavanones and isoflavanols. The further elaboration of the B ring of formononetin to produce the 2,4,5 substitution pattern in maackiain proceeds by a route, yet to be determined, which does not involve (7), (8), (9) or (1). Formononetin probably represents the branch-point in the pathways to (1) or (2).

The two pterocarpan phytoalexins produced by red

clover have large negative specific optical rotations (see Experimental) and can thus be assigned the 6aR,11aR configuration [25]. Thus, the enzymic reduction processes leading from isoflavone (7) to demethylhomopterocarpin via (8) and (14) are presumably stereospecific. Incorporation data for racemic substrates in the Table are, however, uncorrected for possible utilisation of only one enantiomer. In particular, if only one enantiomer of isoflavanone (8) is involved in the biosynthesis of demethylhomopterocarpin, and epimerisation does not occur, the percentage incorporation figures may be doubled. Relatively few naturally occurring isoflavanones are known, and of these, only two, (3S)-7,4'-dihydroxyisoflavanone [26] and (3R)-sophorol (19) [27] have been isolated in optically active form. (3R)-Sophorol may well occupy an important position in the biosynthetic pathway to (6aR,11aR)-maackiain.

The co-occurrence in red clover of the coumestans 9-*O*-methylcoumestrol (12) and medicagol (13), together with the corresponding pterocarpans demethylhomopterocarpin and maackiain is of biosynthetic significance, and suggests common intermediates in their biosynthetic pathways. These could well be 7,2'-dihydroxy-4'-methoxyisoflavanone (8) for the methoxy compounds, and sophorol (19) for the methylenedioxy series. Although pterocarp-6a-enes are apparently not involved in pterocarpan biosynthesis, they could still be precursors of coumestans, and would probably be derived by dehydration of 2'-hydroxyisoflavanones. The ready oxidation of pterocarp-6a-enes by such mild oxidants as atmospheric oxygen has so far precluded their use in feeding experiments as possible precursors of coumestans.

## EXPERIMENTAL

*General.* The growing of plant material, feeding techniques, isolation and purification of metabolites, and counting of radioactive samples were all as previously described [2]. TLC was carried out using 0.5 mm layers of Si gel (Merck Kiesel gel $GF_{254}$) in the solvent systems: A, $C_6H_6$–EtOAc–MeOH–petrol (60–80°), 6:4:1:3; B, $CHCl_3$–*iso*-PrOH, 10:1; C, $C_6H_6$–EtOH, 92:8; D, toluene–$Et_2O$, 1:1; E, $C_6H_6$–EtOAc, 32:1; F, $Et_2O$–petrol (60–80°), 4:1; G, petrol (60–80°)–EtOAc, 7:3. $Me_2CO$ (Analar) was used for elution of TLC zones. The synthesis of formononetin-[*methyl*-$^{14}$C] (sp. act. 0.535 mCi/mM) has been reported [4].

*7-Benzyloxy-4'-hydroxy-2'-methoxyisoflavone.* 4'-Benzyloxy-2'-hydroxyacetophenone (2g) and 4-benzyloxy-2-methoxybenzaldehyde (2g) in EtOH (100 ml) were stirred at R° overnight with KOH (20g) in $H_2O$ (20 ml). The mixture was poured into $H_2O$, acidified with conc HCl, then extracted with EtOAc (2x). The EtOAc extract was evaporated, and the residue crystallised from $CHCl_3$–MeOH to give *4,4'-dibenzyloxy-2'-hydroxy-2-methoxychalcone* (1.4g), mp 143–4°. (Found: C, 77.62; H, 5.75. $C_{30}H_{26}O_5$ requires: C, 77.2; H, 5.58%). The above chalcone (1.4g) was acetylated (dry Py–$Ac_2O$, R° overnight), and the reaction mixture poured into $H_2O$ and extracted with EtOAc (2x). The extract was washed with dil HCl(2x), then $H_2O$, and evaporated and dried. The acetate, without further purification, was dissolved in MeOH (500 ml) and stirred at R° overnight with $Tl(NO_3)_3$. $3H_2O$ (1.5g). Solid KOH (4g) was then added, and the mixture was stirred for a further 1 hr. After neutralisation with conc HCl, dil HCl (10%, 30 ml) was added, and the mixture heated under reflux for 2 hr, then filtered hot. Filtrate was concentrated under red pres, diluted with $H_2O$ and extracted with EtOAc(2x). The EtOAc extract, on evaporation yielded *7,4'-dibenzyloxy-2'-methoxyisoflavone* which was recrystallised from MeOH. Yield 1.0g, mp 133–5°, 149–50° (dimorphic). (Found: C, 77.36; H, 5.48. $C_{30}H_{24}O_5$ requires: C, 77.6; H, 5.17%). This isoflavone (0.5g) was debenzylated by heating at 80° for 4 hr with HOAc (40 ml) and conc HCl (20 ml). The mixture was poured into $H_2O$, extracted with EtOAc (2x), and the extracts washed with aq. $NaHCO_3$, then $H_2O$, and evaporated. The product was recrystallised from MeOH to give *7,4'-dihydroxy-2'-methoxyisoflavone* (0.17g), mp 283–5°. (Found: C, 67.08; H, 4.34. $C_{16}H_{12}O_5$ requires: C, 67.6; H, 4.23%). A further 0.10g was obtained by concentration of the mother liquors. This isoflavone (0.14 g) was stirred at 80° for 1.5 hr with BzCl (48 $\mu$l), dry $K_2CO_3$ (3 g) and KI (0.3 g) in dry DMF (20 ml). The mixture was poured into $H_2O$, extracted with EtOAc (2x), and the extract washed with $H_2O$ and evaporated. The product was dissolved in hot MeOH (150 ml), then concentrated to ca 40 ml, and allowed to crystallise, to yield *7-benzyloxy-4'-hydroxy-2'-methoxyisoflavone* (0.11g), mp 251–5°. (Found: C, 72.55; H, 5.06. $C_{23}H_{18}O_5$ requires: C, 73.8; H, 4.81%).

*7,2'-Dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me].* 7-Benzyloxy-4'-hydroxy-2'-methoxyisoflavone (30.2 mg) was stirred and heated under reflux in dry $Me_2CO$ (50 ml) with dry $K_2CO_3$ (5g), MeI (4 $\mu$l, 9.1 mg) and MeI-[$^{14}$C] (500 $\mu$Ci, sp. act. 55 mCi/mM; 1.31 mg) overnight. Excess MeI (0.1 ml) was then added, and the reaction was continued for a further 1 hr. The reaction mixture was filtered, evaporated and the product, *7-benzyloxy-2',4'-dimethoxyisoflavone-[4'-$^{14}$C-Me]* purified by TLC (solvent A), then dried under vacuum. This material was heated under reflux with $AlCl_3$ (1g) in dry MeCN (10 ml) overnight. The mixture was evaporated, treated with dil HCl (10%, 10 ml), then extracted with EtOAc (3 × 30 ml), and the evaporated extracts purified by TLC (solvent A). Inactive 7,2'-dihydroxy-4'-methoxyisoflavone (89.7 mg) prepared by a similar treatment of 7-benzyloxy-2',4'-dimethoxyisoflavone derived from 4'-benzyloxy-2'-hydroxyacetophenone and 2,4-dimethoxybenzaldehyde, was added to the product, and the material was recrystallised from aq. MeOH to yield *7,2'-dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me]* (88.8 mg). The mother liquors yielded a further 14.0 mg of product, which was purified to constant sp. act. (0.518 mCi/mM) using TLC (solvents A, B and C). UV $\lambda_{max}^{EtOH}$ nm (log $\varepsilon$): 240 (4.31), 249 (4.34), 259 *sh* (4.26), 292 (4.15), 305 inf (4.07).

*(±)-7,2'-Dihydroxy-4'-methoxyisoflavanone-[$^{14}$C-Me].* 7,2'-Dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] (60.6 mg) was acetylated in dry Py (3 ml) with $Ac_2O$ (0.3 ml) at R° overnight. The mixture was poured into $H_2O$, cooled, and *7,2'-diacetoxy-4'-methoxyisoflavone-[$^{14}$C-Me]* (68.3 mg) filtered off and dried. This isoflavone (56.0 mg) was hydrogenated overnight at R° and atmospheric pressure in EtOAc (20 ml) and HOAc (0.5 ml) over Pd–C (10%, 27 mg) catalyst. The reaction mixture was filtered, evaporated, then stirred at R° with KOH (0.5 g) in EtOH (10 ml) for 3 hr. The mixture was then concentrated to a small bulk, diluted with $H_2O$ (25 ml) and acidified to pH4 with 10% HCl. The product, *(±)-7,2'-dihydroxy-4'-methoxyisoflavanone-[$^{14}$C-Me]* (37.8 mg) was obtained by extraction with EtOAc (3 × 25 ml), then TLC (solvent A). A portion (12.6 mg) was purified to constant sp. act. (0.546 mCi/mM) by TLC (solvents B and C). Unlabelled material (recrystd. aq. MeOH) had mp 212–4°, MS (probe) 70 eV *m/e* (rel. int.): 286 $M^+$ (17), 150 (100), 137 (65), 136 (27), 121 (27), 108 (47), 107 (24); $M^+$ 286.0832, $C_{16}H_{14}O_5$ requires 286.0841. UV $\lambda_{max}^{EtOH}$ nm (log $\varepsilon$: 227 *sh* (4.26), 278 (4.23), 311 (3.93).

*3-Hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me].* A stream of $N_2$ was passed through refluxing MeOH (20 ml) for 0.5 hr. (±)-7,2'-Dihydroxy-4'-methoxyisoflavanone-[$^{14}$C-Me] (25.2 mg) and conc HCl (0.2 ml) were added, and the mixture was heated under reflux in an $N_2$ stream for 2 hr. The soln was evaporated and the product, *3-hydroxy-9-methoxypterocarp-6a-ene-[$^{14}$C-Me]* (9.9 mg) was isolated by TLC (solvent A) and purified to constant sp. act. (0.572 mCi/mM) by TLC (solvent D). Unlabelled material (recrystd aq. MeOH) had mp 171–3°. MS (probe) 70 eV *m/e* (rel. int.): 268 $M^+$ (100), 267 $M^+$-1 (65), 253 $M^+$-Me (55), 252 $M^+$-1-Me (13); $M^+$ 268.0734, $C_{16}H_{12}O_4$ requires 268.0735. UV $\lambda_{max}^{EtOH}$ mm (log $\varepsilon$): 231 (4.21), 242 (4.19), 250 sh (4.15), 295 inf (3.92), 335 (4.49), 353 (4.44).

*(±)-3-Hydroxy-9-methoxypterocarpan-[$^{14}$C-Me].* (*Demethyl-*

*homopterocarpin*-[$^{14}$*C-Me*]). 7,2'-Dihydroxy-4'-methoxyisoflavone-[$^{14}$C-Me] (19.8 mg) in dry THF (5 ml) was stirred at R° and treated slowly with $NaBH_4$ (50 mg) in dry EtOH (5 ml). After 2 hr, further solid $NaBH_4$ (50 mg) was added. The mixture was stirred overnight, $Me_2CO$ (2 ml) was added, and solvents were then evaporated. The residue was treated with HCl (10%, 20 ml), and extracted with EtOAc (3 × 20 ml). TLC (solvent A) of the evapd. extracts yielded (±)-*demethylhomopterocarpin*-[$^{14}$*C-Me*] (15.3 mg) which was purified to constant sp. act. (0.514 mCi/mM) by TLC (solvent D).

*Solvolysis of 3-hydroxy-9-methoxypterocarp-6a-ene.* The pterocarp-6a-ene (39.2 mg) in MeOH (50 ml) was stirred vigorously in a stream of air in the light for 24 hr. The soln was evaporated and purified by TLC (solvent A). The major band was rechromatographed (solvent E), to give a product, *2-(2,4-dihydroxyphenyl)-6-methoxy-3-methoxymethylbenzofuran* (10) which crystallised on cooling, mp 123–4°, MS (probe) 70 eV *m/e* (rel. int.): 300 $M^+$ (30), 268 $M^+$-MeOH (100), 267 $M^+$-MeOH-1 (88), 253 $M^+$-MeOH-Me (64); $M^+$ 300.1001, $C_{17}H_{16}O_5$ requires 300.0998. UV $\lambda_{max}^{EtOH}$ nm: 267,309. NMR (60 MHz, $CDCl_3$, TMS): $\delta$ 3.43 (3H, s, OMe), 3.80 (3H, s, OMe), 4.50 (2H, s, $CH_2OMe$), 6.07 (1H, OH), 6.40 (1H, *dd*, $J$ = 9, 3 Hz, H-5), 6.45 (1H, *d*, $J$ = 3 Hz, H-7), 6.83 (1H, *dd*, $J$ = 9, 3 Hz, H-5'), 6.95 (1H, *d*, $J$ = 3 Hz, H-3'), 7.25 (1H, *d*, $J$ = 9 Hz, H-4), 7.42 (1H, *d*, $J$ = 9 Hz, H-6'), 7.55 (1H, s, OH).

*Optical rotation of pterocarpans.* Demethylhomopterocarpin and maackiain were isolated [2] from $CuCl_2$-treated red clover seedlings (from 15g seeds), and purified further by TLC (solvent E). UV analysis [2] showed this fraction to contain demethylhomopterocarpin (1.08 mg) and maackiain (1.175 mg). The measured optical rotation, $\alpha_D^{20}$ of an EtOH (1 ml) solution was −0.041°. Lit. values of $[\alpha]_D$ for ethanolic solutions of (6aR, 11aR)-demethylhomopterocarpin and (6aR,11aR)-maackiain are −192° [28] and −214° [29] respectively. Using these values, the calculated rotation for the above pterocarpan mixture was −0.046°.

*Isolation of coumestans from* T pratense. Red clover seedlings (6-day-old, wet wt 650 g) were homogenised with $H_2O$ (1 l) and left for 5 hr at R° to permit autolysis of glycosides present. The slurry was worked up as previously [2]. The coumestan zone could not be obtained pure by TLC alone, and was thus acetylated (Py-$Ac_2O$) and the acetates purified by TLC (solvents F and G). MS (probe) 70 eV *m/e*: 338.0421, $C_{18}H_{10}O_7$ requires 338.0427; 324.0634, $C_{18}H_{12}O_6$ requires 324.0634. The coumestans were regenerated by hydrolysis (KOH/MeOH) and purified by TLC (solvent A). The coumestan content was *ca* 50 μg.

*Synthesis of coumestans.* The appropriate (±)-pterocarpan [2] (100 mg) was dissolved in dioxan (5 ml) and treated with DDQ (300 mg). The mixture was left at R° overnight. The pptd coumestan was filtered off and recrystallised from THF–MeOH. Yield *ca* 80 mg. mp's: *9-O-methylcoumestrol* 338–40° (lit. [30] 337–8°); *medicagol* 325–6° (lit. [31] 326–7°).

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(54) Title: COMPOUNDS USEFUL FOR THE INHIBITION OF ALDH

(57) Abstract: The present invention provides novel antidipsotropic compounds. The invention further provides methods of inhibiting ALDH-2 using the compounds described herein. Methods for modulating alcohol consumption, alcohol dependence and/or alcohol abuse by administering the compounds of the invention to an individual are also provided. The present invention further provides a rationale for designing additional novel antidipsotropic compounds.

# COMPOUNDS USEFUL FOR THE INHIBITION OF ALDH

## RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 60/391,907, filed on June 27, 2002, hereby incorporated by reference in its entirety for all purposes.

## BACKGROUND OF THE INVENTION

### Field of the Invention

The present invention relates to compounds and methods for modulating alcohol consumption, alcohol dependence and/or alcohol abuse.

### Description of the Related Art

Excessive alcohol consumption, alcohol dependence and alcohol abuse are among the most serious drug problems of Western societies. In the United States, it is estimated that up to 10% of the population abuse alcohol. The economic cost to the nation is estimated at more than $185 billion per year. Excessive alcohol consumption also brings with it social and psychological damages such as children born with fetal alcohol syndrome, alcohol related accidents, marital disharmony, spousal or child abuse, and the like. Accordingly, safe and effective treatments for limiting and/or preventing alcohol consumption are needed.

Success in the development of pharmacotherapies for addictive disorders, including nicotine and opioid addiction, and a more widespread recognition that alcohol dependence and abuse is a medical rather than moral problem have harnessed growing support in the search for and development of pharmaceutical agents for this medical condition. However, as alcoholism is a complex disease promoted by a wide range of factors, it has been difficult to develop effective modulators of alcohol consumption.

The compound daidzin has been shown to selectively suppress ethanol intake in ethanol-preferring Syrian golden hamsters as well as in other ethanol-drinking animal models such as rats (Keung et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:10008; Keung et al (1994) *EXS* 71:371; Overstreet et al. (1996) *Alcohol. Clin. Exp. Res.* 20:659; Lin et al. (1996)

*Alcohol. Clin. Exp. Res.* 20:1083-1087). However, daidzin administered orally does not affect hamster ethanol intake.

Daidzin inhibits the conversion of monoamines such as serotonin (5-HT) and dopamine (DA) into their respective acid metabolites, 5-hydroxyindole-3-acetic acid (5-HIAA) and 3, 4,-dihydroxyphenylacetic acid (DOPAC), in isolated hamster and rat liver mitochondria (Keung et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:2198). Recent data indicates that the antidipsotropic activity of daidzin is not mediated by the monoamines but rather by their respective metabolic aldehyde intermediates, 5-hydroxyindoleacetaldehyde (5-HIAL) and/or 3, 4-dihydroxyphenylacetaldehyde (DOPAL), which accumulate in the presence of daidzin.

Several recent patents and a pending patent application address the use of daidzin and daidzin analogs useful for the inhibition of aldehyde dehydrogenase and/or alcohol consumption. U.S. Patent Nos. 5,204,369, 5,624,910 and 5,885,028 disclose methods of inhibiting the mitochondrial aldehyde dehydrogenase (ALDH-2) or treating alcohol consumption using daidzin and specific daidzin analogs. U.S. Patent No. 6,121,010 discloses screening assays used to identify compounds for reducing alcohol consumption. U.S. Patent No. 6,255,497 provides compounds which are daidzin analogs. U.S. Utility Application No. 09/616,718 is directed to methods for inhibiting ALDH-2 and reducing alcohol consumption in a human using daidzin analogs. Each issued patent and patent application is incorporated herein by reference in its entirety.

Despite the development of compounds such as daidzin and daidzin analogs, there remains a need to develop antidipsotropic compounds having improved efficacy and enhanced pharmacological properties.

## SUMMARY OF THE INVENTION

Accordingly, the present invention provides novel compounds for inhibiting mitochondrial aldehyde dehydrogenase (ALDH-2). These compounds have antidipsotropic properties and are useful for modulating (i.e., reducing) alcohol consumption, therapeutically treating alcohol dependence (i.e., alcoholism) and/or therapeutically treating alcohol abuse. In one embodiment, the present invention provides methods of synthesizing novel compounds for inhibiting ALDH-2.

Another embodiment of the present invention is directed to methods of inhibiting ALDH-2 activity using the compounds set forth herein. Another embodiment of the present invention is directed to a method of modulating (i.e., reducing or inhibiting) alcohol consumption, alcohol dependence, and/or alcohol abuse in an individual by increasing the concentration of an aldehyde in the individual using the compounds set forth herein. In one embodiment, the aldehyde is formed during the catabolism of a neurotransmitter such as, for example 5-HT or DA. In another embodiment, the aldehyde is 5-HIAL and/or DOPAL. In still another embodiment, alcohol consumption is modulated in a mammal such as a human.

The compounds may be administered to individuals using a variety of routes. In one embodiment, the compounds are administered orally. In another embodiment, the compounds are administered intraperitoneally and/or intramuscularly.

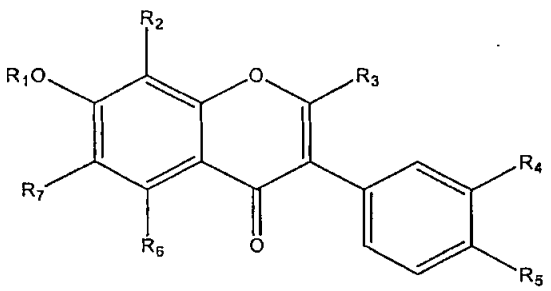
Yet another embodiment of the present invention is directed to identifying a compound that modulates (e.g., inhibits) ALDH-2 activity using the compounds set forth herein by contacting ALDH-2 with the compound and determining the ability of the compound to modulate ALDH-2 activity. In one embodiment, the compound increases the concentration of an aldehyde. In another embodiment, the aldehyde is 5-HIAL and/or DOPAL. In another embodiment, the compound does not inhibit MAO.

Compounds of the present invention are set forth herein below. Compounds of the invention may have an OH or an $NH_2$ moiety at the $R_5$ position. Certain compounds of the invention may have a straight chain alkyl at the $R_1$ position.

Combinations of features and methods described herein are also provided.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to compounds of formula I




wherein

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched $(C_1-C_6)$alkyl, $(C_1-C_6)$haloalkyl, $(C_2-C_6)$alkenyl, $(C_3-C_6)$alkadienyl, $(C_1-C_6)$alkoxy, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_2-C_6)$alkynyloxy, $(C_1-C_6)$alkoxy$(C_1-C_6)$alkyl, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_1-C_6)$alkylcarbonyl, $(C_3-C_6)$cycloalkylcarbonyl, $(C_1-C_6)$alkoxycarbonyl, $(C_4-C_6)$alkoxycarbonylalkyl, $(C_1-C_6)$hydroxyalkyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, heterocyclyloxy, and heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1-C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen, alkoxy, and sugars;

$R_3$ is selected from the group consisting of hydrogen $C_1-C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, hydroxy, halo, branched or unbranched, $(C_1-C_6)$alkyl, $(C_1-C_6)$haloalkyl, $(C_2-C_6)$alkenyl, $(C_3-C_6)$alkadienyl, $(C_1-C_6)$alkoxy, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_2-C_6)$alkynyloxy, $(C_1-C_6)$alkoxy$(C_1-C_6)$alkyl, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_1-C_6)$alkylcarbonyl, $(C_3-C_6)$cycloalkylcarbonyl, $(C_1-C_6)$alkoxycarbonyl, $(C_4-C_6)$alkoxycarbonylalkyl, $(C_1-C_6)$hydroxyalkyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, heterocyclyloxy, and heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1-C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1-C_6$ alkoxy.

Because the compounds of formula I above may contain a number of optically active carbon atoms, they may exist as enantiomers, diastereomers, stereoisomers, or their mixtures. The compounds of formula I may optionally exist as tautomers.

As used herein, the term "alkyl" includes both branched and straight chain alkyl groups. Typical alkyl groups include, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, isopentyl, n-hexyl, n-heptyl, isooctyl, nonyl, decyl and the like.

As used herein, the term "halo" refers to fluoro, chloro, bromo or iodo.

As used herein, the term "haloalkyl" refers to an alkyl group substituted with one or more halo groups such as, for example, chloromethyl, 2-bromoethyl, 3-iodopropyl, trifluoromethyl, perfluoropropyl and the like.

As used herein, the term "hydroxyalkyl" refers to an alkyl group substituted with one or more hydroxy groups such as, for example, hydroxymethyl, 2,3-dihydroxybutyl and the like.

As used herein, the term "alkenyl" refers to an ethylenically unsaturated hydrocarbon group, straight or branched chain, having 1 or 2 ethylenic bonds such as, for example, vinyl, allyl, 1-butenyl, 2-butenyl, isopropenyl, 2-pentenyl and the like.

As used herein, the term "alkylcarbonyl" refers to an alkylketo functionality, for example acetyl, n-butyryl and the like.

As used herein, the term "heterocyclyl" or "heterocycle" refers to a substituted or unsubstituted; saturated, partially unsaturated, or unsaturated 5 or 6-membered ring containing one, two or three heteroatoms, preferably one or two heteroatoms independently selected from oxygen, nitrogen and sulfur. Examples of heterocyclyls include, for example, pyridyl, thienyl, furyl, pyrimidinyl, pyrazinyl, quinolinyl, isoquinolinyl, pyrrolyl, indolyl, tetrahydrofuryl, pyrrolidinyl, piperidinyl, tetrahydropyranyl, morpholinyl, piperazinyl, dioxolanyl, dioxanyl and the like.

As used herein, the term "alkoxy" includes both branched and straight chain alkyl groups attached to a terminal oxygen atom. Typical alkoxy groups include, for example, methoxy, ethoxy, n-propoxy, isopropoxy, tert-butoxy and the like.

As used herein, the term "haloalkoxy" refers to an alkoxy group substituted with one or more halo groups such as, for example chloromethoxy, trifluoromethoxy, difluoromethoxy, perfluoroisobutoxy and the like.

As used herein, the term "alkylthio" includes both branched and straight chain alkyl groups attached to a terminal sulfur atom such as, for example methylthio.

As used herein, the term "haloalkylthio" refers to an alkylthio group substituted with one or more halo groups such as, for example trifluoromethylthio.

As used herein, the term "alkoxyalkyl" refers to an alkyl group substituted with one or more alkoxy groups such as, for example, isopropoxymethyl, or methoxymethoxymethyl.

Prophylactic Methods

In one aspect, the invention provides a method for modulating alcohol consumption and/or preventing alcohol dependence and/or abuse in a subject, by administering to the subject an agent which inhibits ALDH-2 and/or increases aldehyde concentrations (such as 5-hydroxyindoleacetaldehyde and/or 3,4-dihydroxyphenylacetaldehyde). Administration of a prophylactic agent can occur prior to the manifestation of symptoms of diseases or disorders characteristic of alcohol dependence and/or abuse, such that alcohol dependence and/or abuse is prevented or, alternatively, delayed in its progression.

Therapeutic Methods

Another aspect of the invention pertains to methods of modulating (e.g., reducing) alcohol consumption, alcohol dependence and/or alcohol abuse for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting ALDH-2 with a compound that inhibits ALDH-2. In yet another exemplary embodiment, the modulatory method involves administering a compound that increases the concentration of an aldehyde (e.g., 5-HIAL and/or DOPAL) formed during catabolism of a neurotransmitter (e.g., 5-HT and/or DA). Preferably, the compound does not inhibit MAO, or inhibits MAO only to a small degree.

Another embodiment of the present invention involves a method of modulating alcohol consumption for the treatment of alcohol abuse or dependence which includes the step of administering a therapeutically effective amount of a compound which inhibits ALDH-2, and/or increases the concentration of an aldehyde (e.g., 5-HIAL and/or DOPAL) formed during catabolism of a neurotransmitter (e.g., 5-HT and/or DA). As defined herein, a therapeutically effective amount of compound (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20

mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the compound can include a single treatment or, preferably, can include a series of treatments. It will also be appreciated that the effective dosage of in used for treatment may increase or decrease over the course of a particular treatment.

Monitoring of Effects During Clinical Trials

Monitoring the influence of compounds on the modulation of alcohol consumption, dependence and/or abuse can be applied in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to decrease alcohol consumption, dependence and/or abuse, can be monitored in clinical trials of subjects exhibiting alcohol dependence and/or abuse. In such clinical trials, decreased consumption of alcohol can be used as a "read out."

For example, and not by way of limitation, ALDH-2 activity is decreased in cells treated with a compound which inhibits ALDH-2 and as a consequence diverts part of 5-HT metabolic flux from the oxidative pathway, which leads to the formation of 5-hydroxyindoleacetic acid (5-HIAA) to the reductive pathway, further leading to the formation of 5-hydroxytryptophol (5-HTOL). Thus, to study the effect of agents on alcohol dependence and/or abuse, for example, in a clinical trial, urine samples can be collected and levels of 5-HIAA and 5-HTOL in the samples can be determined. Decreased levels of 5-HIAA and increased levels of 5-HTOL will indicate inhibition of ALDH-2 activity. In this way, the urine [5-HTOL]/[5-HIAA] ratio can serve as a marker, indicative of the physiological response of the cells to the compound. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the compound.

In one embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with a compound including the steps of (i) obtaining pre-administration urine samples from a subject before and after alcohol detoxification but prior to administration of the compound; (ii) determining the [5-HTOL]/[5-HIAA] ratios in the pre-administration samples; (iii) obtaining one or more post-administration samples from the subject;

(iv) determining the [5-HTOL]/[5-HIAA] ratio in the post-administration samples; (v) comparing the [5-HTOL]/[5-HIAA] ratios in the pre-administration samples with that in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the compound may be desirable to increase the [5-HTOL]/[5-HIAA] ratio to higher levels than detected, i.e., to increase the effectiveness of the agent. In one embodiment, the ratio of [5-HTOL]/[5-HIAA] in the urine of a detoxified subject is increased back to that of pre-detoxified state. Alternatively, decreased administration of the compound may be desirable to decrease [5-HTOL]/[5-HIAA] ratio to lower levels than detected, i.e., to decrease the effectiveness of the compound. According to such an embodiment, ALDH-2 inactivation and/or an increase in urine [5-HTOL]/[5-HIAA] ratio may be used as an indicator of the effectiveness of a compound, even in the absence of an observable phenotypic response.

Pharmaceutical Compositions

Methods of administering a compound to an individual include providing pharmaceutically acceptable compositions. In one embodiment, pharmaceutically acceptable compositions comprise a therapeutically effective amount of one or more of the compounds described herein, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. The pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment, spray or patches applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam. In another embodiment, the therapeutic compound is administered orally. The compounds of the invention can be formulated as pharmaceutical compositions for administration to a subject, e.g., a mammal, including a human.

The compounds of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a compound to be administered in which any toxic effects are outweighed by the therapeutic effects of the compound. The term subject is intended

to include living organisms such as mammals. Examples of subjects include humans, monkeys, pigs, dogs, cats, hamsters, rabbits, mice, rats, and transgenic species thereof. Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a compound of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions that may inactivate the compound.

A compound of the invention can be administered to a subject in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. The term "pharmaceutically acceptable carrier" as used herein is intended to include diluents such as saline and aqueous buffer solutions. To administer a compound of the invention other than by parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with a material to enhance absorption and/or prevent its inactivation. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neiuroimmunol.* 7:27). The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils, and in the form of nanocrystals. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of

microorganisms such as bacteria and fungi. The pharmaceutically acceptable carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, accepted methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the composition may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for

the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the therapeutic treatment of individuals.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

## EXAMPLE I

### Oral Potency of Hexzein

The oral potency of hexzein (7-*O*-ω-carboxypentyldaidzein) in reducing ethanol intake in Syrian golden hamsters *(Meso cricetus auratus)* was examined under a two-bottle free-choice protocol. Hexzein was mixed with Purina rodent meal (5755M) at 0 mg: 1 g (control), 2 mg: 1 g, 4 mg: 1 g, and 5 mg: 1 g ratios and the mixtures were pressed into 1 x 1 x 0.5 inch pellets. Food pellets, ethanol solution and water were provided continuously throughout the study and their intake were measured every day at 5 pm. Hexzein given in this manner did reduce ethanol intake. Ethanol intake in hamsters on 2 mg/g, 4 mg/g, and 5 mg/g hexzein-Chow were reduced by 18.3±3.8%, 47.8±7.4% and 56.5±5.4%, respectively. Daidzin, given in a similar manner and at equivalent dose range, failed to reduce ethanol intake. Tissue distribution studies showed that pharmacologically relevant concentrations of hexzein were found in the liver of hamsters on hexzein-Chow. However, no daidzin could be detected in the liver of hamsters on daidzin-Chow. These results indicated that hexzein is more potent than daidzin and has more desirable pharmacokinetic properties.

## EXAMPLE II

### Daidzin Mimics Effects of Ethanol Monoamine Metabolism

The effects of daidzin on monoamine metabolism were studied in hamster and rat liver and brain using tissue slices using 5-HT as the substrate. The effects of ethanol drinking on 5-HT metabolism in hamster liver and brain were examined. Freshly removed tissues were cut into small pieces (about 1 mm cubes) with a tissue-slicer, suspended in ice-cold phosphate buffered saline (PBS) (0.1g/ml), and forced through a mesh 40 stainless steel screen mounted on a Millipore Swinnex Filter Holder. The minced tissues were allowed to

sediment on ice before PBS was removed using a Pasteur pipette. The tissue slices were washed 5 more times each with 10 volumes of ice-cold PBS. After the final wash, tissues were suspended in 1 volume of PBS and dispensed using a micro-pipette with tips of i.d.= 2 mm. 5-HT metabolism catalyzed by the tissue slices was assayed in a 1 ml assay medium containing PBS, 1μM 5-HT, 100:1 tissue slices, and specified concentrations of daidzin or ethanol. Reactions were initiated by the addition of tissues and allowed to proceed for 30 min at 37°C in a shaking water bath. Reactions were terminated by addition of 0.1 ml ice-cold 1 M $HClO_4$, and 10 mM EDTA. Samples were kept on ice for 30 min and centrifuged to obtained clear supernatants. Contents of 5-HT, 5-HIAL, 5-HTOL and 5-HIAA in the supernatants were analyzed by HPLC as described before (Rooke et al. (2000) *J. Med. Chem.* 43:4169).

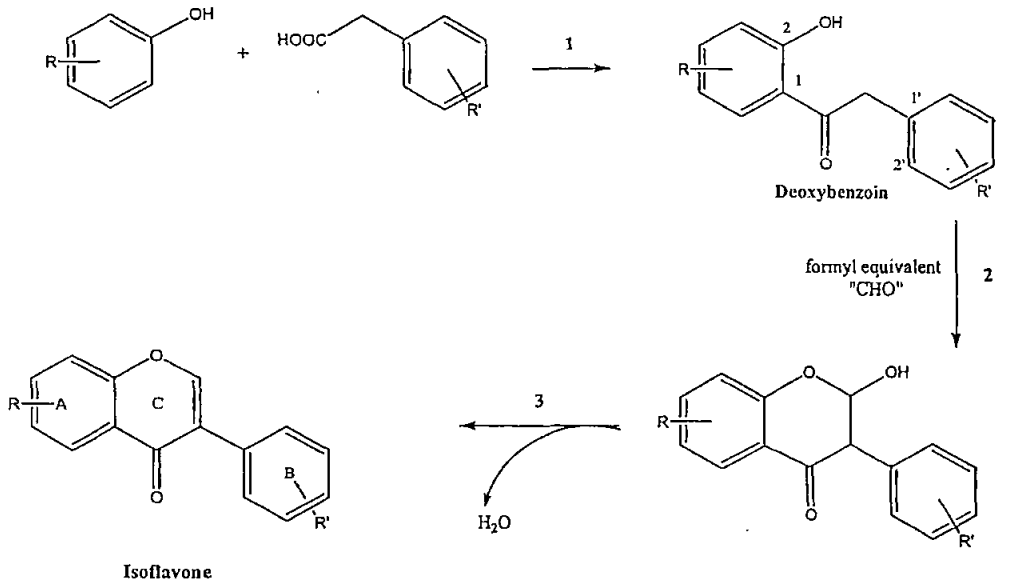
Daidzin inhibited 5-HIAA formation but not 5-HT depletion in 5-HT metabolism catalyzed by hamster and rat liver slices. Inhibitions were concentration-dependent and accompanied by concomitant increases in the formation of 5-HTOL and other unidentified metabolic products. Ethanol affected 5-HT metabolism in hamster and rat liver slices in a similar manner as daidzin. Daidzin and ethanol did not effect 5-HT metabolism in the brain slices. Daidzin, administered at a dose that would suppress hamster ethanol intake by about 50%, increased urine [5-HTOL]/[5-HIAA] ratio.

## EXAMPLE III

### Synthesis, Purification, and Structural Identification of Compounds

The 7-*O*-substituents of the derivatives of daidzein (compounds 2, 4, 6, 8, 11, 13 and 18), puerarin (compounds 17 and 36), 7-hydroxyisoflavone (compounds 3, 5, 7, 9, 10, 12 and 14), 7-hydroxy-2-ethoxycarbonylisoflavone (compounds 15 and 19), 7-hydroxy-4'-fluoro isoflavone (compound 24), 7-hydroxy-4'-bromoisoflavone (compound 26), 7-hydroxy-4-nitroisoflavone (compound 28), and 7-hydroxy-4'-methylisoflavone (compound 30) were synthesized according to the method described in Rooke et al. (2000) *J. Med. Chem.* 43:4169; and Baker and Robinson (1928) *J. Chem. Soc.* 3115. The following 4' substituted derivatives of isoflavone were synthesized: 7-hydroxy-4'- flouroisoflavone (compound 23), 7-hydroxy-4'-bromoisoflavone (compound 25), 7-hydroxy-4'nitroisoflavone (compound 27), 7-hydroxy-4'-methylisoflavone (compound 29), 4',7dimethoxy-isoflavone (compound 31), and 7,8-dimethoxyisoflavone (compound 34). The syntheses were based on the formulation of

substituted deoxybenzoins as shown in scheme 1, step 1, below, in accordance with teachings of PCT Publication WO 91/15483, and Baker, W.; Chadderton, J.; Harborne, J. B.; Ollis W.D. A new synthesis of isoflavones. Part II. *J. Chem. Soc.*, 1953, 1860-1864.




**Scheme 1**

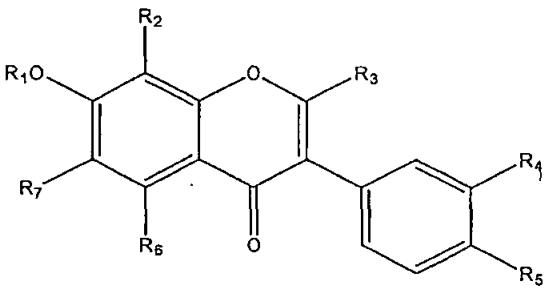
Deoxybenzoins undergo α-keto formylation, intramolecular acetal formation, and a facile dehydration to give the pyrone ring C and hence, the isoflavones as shown in Scheme 1, Step 2 and below. Substituted deoxybenzoins were synthesized by reacting appropriate substituted phenyl acetic acids with properly substituted resorcinols in the presence of $BF_3$. The starting material (7-hydroxy isoflavone, compound 1) for the synthesis of the 7-*O*-substituted isoflavones was prepared by cyclization of 2,4-dihydroxyphenyl benzyl ketone (Aldrich Chemical Co. Milwaukee, WI) as described by WO 91/15483, and shown in steps 2 and 3 of scheme 1, and further described in Gao et al. (2003) Bioorganic & Medicinal Chemistry, in press. 7-Hydroxy-2-ethoxycarbonylisoflavone (compound 20) was prepared by cyclization of the corresponding deoxybenzoin in the presence of an appropriate formyl equivalent as shown in Li, D.Y.; Gao, Z. J.; Ji, Q. E. Synthesis of isoflavones derivatives (I)-7-methoxy-3'-N, N-dialkylaminomethyl-4'-hydroxy isoflavones (*Chinese J. Med. Chem.* 1991, 1 (2), 38-42). The 2-carboxy derivatives (compounds 16, 21, and 22) were obtained by hydrolyzing their respective ethyl esters. The 4'-amino derivatives (compounds 32 and 33)

were prepared by reducing their corresponding 4'-nitro derivatives (compounds 27 and 28) under the condition set forth in WO 91/15483, and Li, D.Y.; Gao, Z. J.; Ji, Q. E. Synthesis of isoflavones derivatives (I)-7-methoxy-3'-N, N-dialkylaminomethyl-4'-hydroxy isoflavones. *Chinese J. Med. Chem.* 1991, 1 (2), 38-42. Full explanations of the syntheses are set forth below. All compounds synthesized were purified by Sephadex-LH-20 and/or silica gel columns. Products were identified by $^{1}$HNMR, $^{13}$C-NMR, MS, and elemental analyses. Purity of all compounds prepared in this study was greater than 97.8% as judged by HPLC with UV detection at 254 nm. Molecular weights, melting points, molecular formulae, and elemental analysis data of these analogs are included in EXAMPLE IV, below.

## EXAMPLE IV

## COMPOUND SYNTHESIS

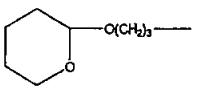
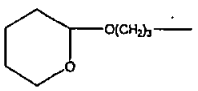
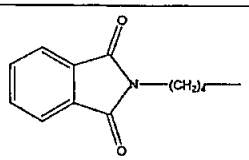
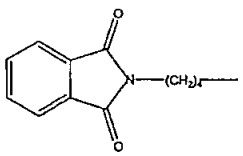
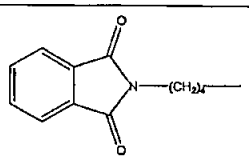
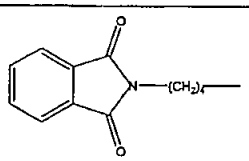
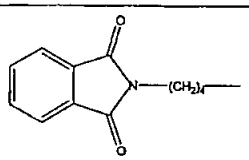
Compounds of Formula I were synthesized in accordance with the present invention.

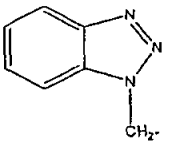
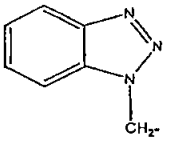



Formula I

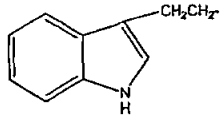
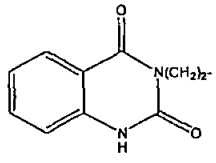
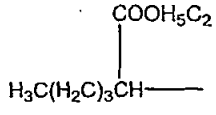
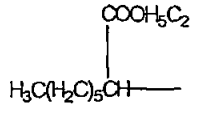
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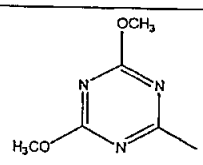
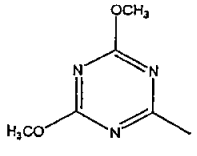
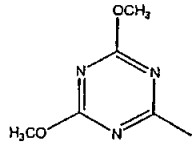
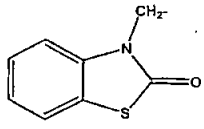
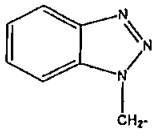
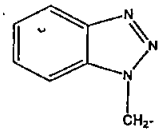
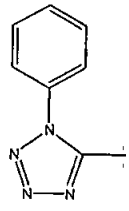
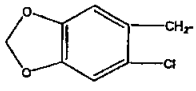
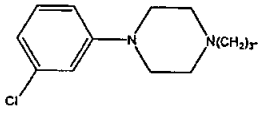
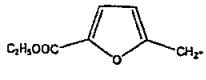
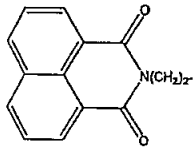
| Analog | R1 | R2 | R3 | R4 | R5 | R6 | R7 |
|---|---|---|---|---|---|---|---|
| 1 | H | H | H | H | H | H | H |
| 2 | $CH_3$ | H | H | H | OH | H | H |
| 3 | $CH_3$ | H | H | H | H | H | H |
| 4 | $(CH_3)_2CH$- | H | H | H | OH | H | H |
| 5 | $(CH_3)_2CH$- | H | H | H | H | H | H |
| 6 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | OH | H | H |

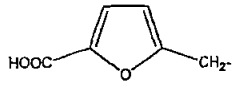
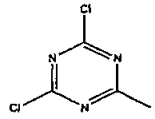
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 7 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | H | H | H |
| 8 | H-$(OCH_2CH_2)_3$ | H | H | H | OH | H | H |
| 9 | H-$(OCH_2CH_2)_3$ | H | H | H | H | H | H |
| 10 | $CH_3C(O)$- | H | H | H | H | H | H |
| 11 |  | H | H | H | OH | H | H |
| 12 |  | H | H | H | H | H | H |
| 13 |  | H | H | H | OH | H | H |
| 14 |  | H | H | H | H | H | H |
| 15 |  | H | COOEt | H | H | H | H |
| 16 |  | H | COOH | H | H | H | H |
| 17 |  | Glc | H | H | OH | H | H |

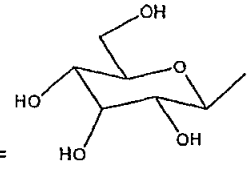
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18 |  | H | H | H | OH | H | H |
| 19 |  | H | COOEt | H | H | H | H |
| 20 | H | H | COOEt | H | H | H | H |
| 21 | H | H | COOH | H | H | H | H |
| 22 | $HOOC(CH_2)_5$ | H | COOH | H | H | H | H |
| 23 | H | H | H | H | F | H | H |
| 24 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | F | H | H |
| 25 | H | H | H | H | Br | H | H |
| 26 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | Br | H | H |
| 27 | H | H | H | H | $NO_2$ | H | H |
| 28 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | $NO_2$ | H | H |
| 29 | H | H | H | H | $CH_3$ | H | H |
| 30 | $C_2H_5OC(O)(CH_2)_5$- | H | H | H | $CH_3$ | H | H |
| 31 | $CH_3$ | H | H | H | -$OCH_3$ | H | H |
| 32 | H | H | H | H | $NH_2$ | H | H |
| 33 | $CH_3CH_2OC(O)(CH_2)_5$- | H | H | H | $NH_2$ | H | H |
| 34 | $CH_3$- | -$OCH_3$ | H | H | H | H | H |
| 35 | $CH_3CH_2$ | H | H | H | H | H | H |
| 36 | $C_2H_5OC(O)(CH_2)_5$- | Glc | H | H | OH | H | H |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 37 | $CH_3$ | H | H | H | H | H | Cl |
| 38 | H | H | H | OH | OH | H | H |
| 39 | $CH_3C(O)$- | H | $CH_3$ | H | H | H | H |
| 40 | H | H | H | H | OH | H | H |
| 41 | H | H | H | H | -$OCH_3$ | H | H |
| 42 | Glc | H | H | H | OH | H | H |
| 43 | H | H | H | H | OH | OH | H |
| 44 | Glc | H | H | H | OH | OH | H |
| 45 | $CH_3$- | H | H | H | OH | OH | H |
| 46 | H | H | H | H | -$OCH_3$ | OH | H |
| 47 | Glc | H | H | H | OH | H | -$OCH_3$ |
| 48 | H | Glc | H | H | OH | H | H |
| 49 | $CH_3(CH_2)_5$- | H | H | H | OH | H | H |
| 50 | HO-$(CH_2)_6$- | H | H | H | OH | H | H |
| 51 | $HOOC(CH_2)_{11}$- | H | H | H | OH | H | H |
| 52 | $C_2H_5OOCCH_2$- | H | H | H | OH | H | H |
| 53 | $C_2H_5OOC(CH_2)_4$- | H | H | H | OH | H | H |
| 54 | $HOOC(CH_2)_7$- | H | H | H | OH | H | H |
| 55 | HO-$(CH_2)_9$- | H | H | H | HO-$(CH_2)_9$- | H | H |
| 56 | $HOOC(CH_2)_{15}$- | H | H | H | OH | H | H |
| 57 | HO-(CH2)$_9$- | H | H | H | OH | H | H |
| 58 | $(CH_3)_2CH$- | H | H | H | $(CH_3)_2CH$- | H | H |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 60 | $C_2H_5OOC(CH_2)_4$- | H | H | H | $C_2H_5OOC(CH_2)_4$- | H | H |
| 61 | $CH_3(CH_2)_5$- | H | H | H | $CH_3(CH_2)_5$- | H | H |
| 62 | $HOOC(CH_2)_4$- | H | H | H | OH | H | H |
| 63 | HO-$(CH_2)_{12}$- | H | H | H | OH | H | H |
| 65 | $C_2H_5OOC(CH_2)_5$- | H | H | H | $C_2H_5OOC(CH_2)_5$- | H | H |
| 67 | $CH_2$=$CH(CH_2)_4$- | H | H | H | OH | H | H |
| 68 | $CH_2$=$CH(CH_2)_4$- | H | H | H | $CH_2$=$CH(CH_2)_4$- | H | H |
| 69 | $CH_3(CH_2)_{11}$- | H | H | H | OH | H | H |
| 70 | $CH_3(CH_2)_{11}$- | H | H | H | $CH_3(CH_2)_{11}$- | H | H |
| 71 | $C_2H_5OC(O)CH(CH_3)$- | H | H | H | OH | H | H |
| 72 | $C_2H_5OC(O)CH(CH_3)$- | H | H | H | $C_2H_5OC(O)CH(CH_3)$- | H | H |
| 73 |  | H | H | H | OH | H | H |
| 74 | $HOC(O)CH(CH_3)$- | H | H | H | OH | H | H |
| 75 | HO-$(CH_2)_2$- | H | H | H | OH | H | H |
| 78 |  | H | H | H | OH | H | H |
| 79 |  | H | H | H | OH | H | H |
| 80 |  | H | H | H | OH | H | H |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 81 |  | H | H | H | OH | H | H |
| 82 |  | H | H | H |  | H | H |
| 83 |  | H | H | H | OH | H | H |
| 85 |  | H | H | H |  | H | H |
| 86 | H | H | H | H |  | H | H |
| 87 |  | H | H | H | OH | H | H |
| 88 |  | H | H | H | OH | H | H |
| 89 |  | H | H | H | OH | H | H |
| 90 |  | H | H | H | OH | H | H |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 99 |  | H | H | H | OH | H | H |
| 106 |  | H | H | H | OH | H | H |

Note: Glc= 

General chemicals were purchased from either Aldrich Chemical Co. (Milwaukee, WI) or Lancaster Synthesis Inc. (Windham, NH). All organic solvents used were of HPLC grade and were supplied by J.P. Baker (Phillips burg, NJ) or Fisher Scientific Company (Pittsburgh, PA). Daidzin (analog 42) and Glycitin (analog 47) were purchased from LC Laboratories (Woburn, MA). Daidzein (analog 40) was first synthesized by Tyger Scientific Inc. (Princeton, NJ) and later obtained from LC Laboratories. 6-Chloro-7-methylisoflavone (analog 37), 7,3',4'-trihydroxyisoflavone (analog 38), formononetin (analog 41), genistein (analog 43), genistin (analog 44), prunetin (analog 45), biochanin A (analog 46), and puerarin (analog 48) were products of Indofine Chemical Company (Somerville, NJ). 7-Acetoxy-2-methylisoflavone (analog 39) was purchased from Aldrich (Milwaukee, WI). The 7-*O*-substituted daidzeins (analog 4-analog 8) were prepared in a previous study. Rooke, N.; Li, D. J.; Li, 1. Q.; Keung, W. M. The mitochondrial monoamine oxidase-aldehyde dehydrogenase pathway: a potential site of action of daidzin. *J. Med. Chem.* 2000, 43, 4169-4179. Serotonin (5-HT) was purchased from Research Biochemical International (Natick, MA) and its metabolic intermediate 5-HIAL was produced in this laboratory by monoamine oxidase (MAO)-catalyzed oxidative deamination of 5-HT with a rat liver mitochondrial membrane preparation used as a source of MAO. Nilsson, G.E.; Tottmar, O. Biogenic aldehydes in brain: on their preparation and reactions with rat brain tissue (*J. Neurochem.* 1987,48, 1566-1572). All other reagents used were the best grade available.

$^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker AMX 500 BQ spectrometer at 500 MHz and Bruker AM-500 spectrometer at 126 MHz (NuMega Resonance Labs. Inc., San Diego, CA), respectively, using DMSO as solvent and as internal standard (2.50 and 39.51 ppm for $^{1}H$ and $^{13}C$, respectively) unless otherwise indicated. Mass spectra were measured on a Perkin Elmer PE-SCIEX API 100 mass spectrometer by infusion. Samples were ionized by electro spray and spectra were recorded in positive and negative mode. Melting points were determined with a Hoover capillary melting point apparatus. Elementary analyses were performed by NuMega Resonance Labs. Inc. Crude synthetic' products were purified by one or a combination of the following methods: chromatography on Sephadex LH-20 (Fluka, 25-100 μm) or Silica Gel 60 (70-230 mesh, EM Science) column and recrystallization from acetone or chloroform/methanol of various proportions. Analytical thin layer chromatography (TLC) was performed on Kiselgel $60F_{254}$ plates (Merck KgaA, Darmstadt, Germany).

**7-Hydroxyisoflavone (Analog 1)**

To a solution of 6.3 g of 2, 4-dihydroxyphenyl-benzyl ketone (27.4 mmol) in 10.5 ml of 2-propanol was added 0.5 ml of morpholine and 6 ml of ethyl orthoformate (36.0 mmol). The mixture was stirred at 80 °C for 7 hours and concentrated by flash evaporation. Residue was dissolved in 30 ml of methanol, stirred at 50-60 °C for 20 min and allowed to precipitate at 4 °c for 24 hours. The yellowish precipitates formed were collected by filtration, washed with small portions of methanol and dried to give 5.16 g of amorphous powder (1), a yield of 81.6 % (w/w): mp 203-205 °C; 1H NMR (DMSO-$d_6$) δ 6.88 (d, 1H, *J*= 2.0 Hz, 8-H), 6.95 (dd, 1H, *J*= 8.80, 1.90 Hz, 6-H), 7.38 (dd, 1H, *J* = 2.01, 7.24 Hz, 4'-H), 7.42 (dd, IH, *J*= 1.70, 7.30 Hz, 3', 5'-H), 7.57 (dd, IH, *J*= 1.55, 7.84 Hz, 2', 6'-H), 7.97 (d, 1H, *J*= 8.85 Hz, 5-H), 8.38 (s, 1H, 2-H), 10.80 (br. 1H, 7-OH). $^{13}C$ NMR (DMSO-$d_6$) δ 102.2 (C-8), 115.3 (C-6), 116.6 (C-10), 123.5 (C-3), 127.3 (C-1'), 127.7 (C-5), 128.1 (C-3', 5'),128.9 (C-2', 6'),132.1 (C-4'), 153.8 (C-2), 157.5 (C9), 162.7 (C-7), 174.4 (C-4). MS (m/z) 239.2 $(M + H)^{+}$, 237.2 (M-H)-. Anal. ($C_{15}H_{10}O_3$) for C, H. Cacld: 75.62, 4.23; found: 75.62, 4.21.

**7-Methoxy-4'-Hydroxyisoflavone (Analog 2)**

To a solution of 5.1 g of daidzein (20.06 mmol) in 40 ml of DMSO was added 3.5 g of anhydrous $K_2CO_3$ (25.4 mmol) and 6 ml of iodomethane (96.4 mmol). The mixture was stirred at room temperature for 2 hours and then poured into ice water to precipitate product. Precipitates were extracted with ethyl acetate, dried by flash evaporation and purified on a Sephadex LH-20 column (chloroform:methanol/7:3). Final product was recrystallized from acetone to give 2.3 g of crystalline analog 2, a yield of 45.1 % (w/w): mp 210-211 °C; $^1$H NMR (DMSO-$d_6$) δ 3.89 (-$OCH_3$), 6.81 (d, 2H, *J*= 8.43 Hz, 3', 5'-H), 7.05 (dd, 1H, *J*= 8.89,2.39 Hz, 6-H), 7.11 (d, 1H, *J*= 2.07 Hz, 8-H), 7.39 (d, 2H, *J*= 8.62 Hz, 2', 6'-H), 8.01 (d, 1H, *J*= 8.87 Hz; 5-H), 8.35 (s, 1H, 2-H), 9.54 (s, 1H, 4'-OH). Anal. ($C_{16}H_{12}O_4$) for C, H. Cacld: 71.64, 4.51; found: 71.24, 4.47.

**7-Methoxyisoflavone (Analog 3)**

This compound was prepared by the same method as analog 2 using 500 mg of analog 1 instead of daidzein as the starting material. The total product obtained was 515 mg, a yield of 97.1 % (mol/mol): mp 156-158 °C; IH NMR (DMSO-$d_6$) δ 3.90 ($OCH_3$), 7.08 (dd, 1H, *J*= 8.94, 2.41 Hz, 6-H), 7.15 (d, 1H, *J*= 2.07 Hz, 8-H), 7.38 (m, 1H, 4'-R), 7.43 (t, 2H, *J*= 7.22, 7.76 Hz, 3', 5'-H), 7.58 (d, 2H, *J*= 7.26 Hz, 2', 6'-H), 8.03 (d, 1H, *J*= 8.86 Hz, 5-H), 8.45 (s, 1H, 2-H). Anal. ($C_{16}H_{12}O_3$) for C, H. Cacld: 76.26, 4.80; found: 76.07, 4.76.

**7-0-Isopropylisoflavone (Analog 5)**

To a solution of 2.50 g of analog 1 (10.5 mmol) in 30 ml of DMF was added 1.80 g of anhydrous $K_2CO_3$ (13.0 mmol) and 3.0 ml of 2-bromopropane (32.0 mmol). The mixture was stirred at 80 °C for 4 hours and then poured into ice water. Precipitates were collected by filtration, washed with small portions of water, and dried to give a residue of crude product. The residue was loaded onto a Sephadex LH-20 column (chloroform:methanol, 7:3) and fractions that contained pure product were pooled, concentrated, and recrystallized from acetone to give 1.05 g of crystalline analog 5, a yield of 42 % (w/w): mp 110-111 °C. $^1$H NMR (DMSO-$d_6$) δ 1.33 (-$CH_3$), 1.34 (-$CH_3$), 4.86 (m, 1H, >$CH_2$-), 7.05 (dd, 1H, *J*= 8.86, 2.20 Hz, 6-H), 7.16 (d, 1H, *J*= 2.50 Hz, 8H), 7.38 (t, 1H, *J*= 6.78 Hz, 4'-H), 7.44 (t, 2H, *J*= 7.76, 1.6 Hz, 3', 5'-H), 7.59 (d, 2H, *J*= 8.09, 1.6 Hz, 2', 6'-H), 8.02 (d, IH, *J*= 8.86 Hz, 5-H), 8.46 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 21.5 (-$CH_3$), 21.5 (-$CH_3$), 70.4 (-$CH_2$-O-), 101.8

(C-8), 115.7 (C-6), 117.3 (C-10), 123.7 (C-3), 127.0 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 132.0 (C4'), 154.0 (C-2), 157.5 (C-9), 162.0 (C-7), 174.1 (C-4). MS (m/z) 281.4 $(M + H)^+$, 303.3 $(M + Na)^+$, 319.4 $(M + K)^+$ ,279.5 $(M - H)^-$. Anal. ($C_{23}H_{24}O_5$) for C, H. Cacld: 77.12, 5.75; found: 76.50, 5.69.

**Daidzein 7-ω-Ethoxycarbonylpentyl Ether (Analog 6)**

To a solution of 7.7 g of daidzein (30.31 mmol), 30 mL of 2 *N* aq. KOH (60 mmol) and 120 mL of acetone was added 12.5 mL of ethyl-6-bromo-1-hexanoate (70.60 mmol). The mixture was refluxed with gentle stirring for 72 hours and products were allowed to precipitate over night in the cold room. Precipitates were collected on a flitted funnel and fractionated on a Sephadex LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, dried and recrystallized from acetone to give 670 mg of analog 64. Analyses: white crystalline needles: yield 5.6%; mp 130-131 °C; $^1$H NMR (DMSO-$d_6$) δ 1.17 [t, 3H, -$CH_3$], 1.45 (m, 2H, -$CH_2$-), 1.58 (m, 2H, $CH_2$-), 1.74 (m, 2H, -$CH_2$-), 2.31 (t, 2H, -$CH_2$-), 4.04 (m, 2H, -$CH_2$-O-), 4.09 (m, 2H, $CH_2$-O-), 6.82 (dd, 2H, *J*=9.76 Hz, 2.7 Hz, H-3', H-5'), 7.04 (dd, 1H, *J*=8.87 Hz, 2.79 Hz, H-6), 7.09 (d, 1H, *J*=2.1 Hz, H-8), 7.40 (dd, 2H, *J*=9.76 Hz, 2.7 Hz, H-2', H-6'), 8.0 (d, 1H, *J*=8.86 Hz, H-5), 8.34 (s, 1H, H-2), 9.55 (s, 1H, OH-4'); $^{13}$C NMR δ 14. 1 (-$CH_3$), 24.2 (-$CH_2$-), 24.9 (-$CH_2$-), 28.1 (-$CH_2$-), 33.4 (-$CH_2$-), 59.7 (-$CH_2$-O-), 68.3 (-$CH_2$-O-), 100.9 (C-8), 114.9 (C-6), 115 (C-3', C-5'), 117.5 (C-10), 122.4 (C-1'), 123.7 (C-3), 126.9 (C-5), 130.1 (C-2', C-6'), 153.1 (C-2), 157.2 (C-9), 157.4 (C-4'), 163.0 (C-7), 172.8 (-OCO-), 174.7 (C-4); MS m/z 397.3 $(M + H)^+$. Elementary analysis ($C_{23}H_{24}O_6$). Cacld: C, 69.68; H, 6.10. Found: C, 69.84; H, 6.15.

**7-*O*-Ethoxycarbonylpentylisoflavone (Analog 7)**

This compound was prepared by the same method described for analog 5 except that ethyl 6-bromohexanoate was used as the alkylating agent. From 2.52 g of analog 1, 3.42 g of analog 7 was obtained, a yield of 85% (mol/mol): mp 112- 114 °C; $^1$H NMR (DMSO-$d_6$) δ 1.17 (t, 3H, -$CH_3$), 1.45 (m, 2H, -$CH_2$-), 1.60 (m, 2H, -$CH_2$-), 1.77 (m, 2H, -$CH_2$-), 2.32 (t, 2H, -$CH_2$-), 4.05 (q, 2H, -$CH_2$-O-), 4.12 (t, 2H, -$CH_2$-O-), 7.07 (dd, 1H, *J*= 8.86, 1.94 Hz, 6-H), 7.16 (d, 1H, *J=1.97* Hz, 8-H), 7.38 (dd, 1H, *J*= 7.75, 1.85 Hz, 4'-H), 7.44 (d, 2H, *J*= 7.67 Hz, 3', 5'-H), 7.58 (d, 2H, *J*= 1.94, 7.19 Hz, 2', 6'-H), 8.02 (d, 1H) =

8.94 Hz, 5-H), 8.47 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-$CH_3$), 24.2 (-$CH_2$-), 25.0 (-$CH_2$-), 28.1 (-$CH_2$-), 33.4 (-$CH_2$-), 59.7 (-$CH_2$-O-), 68.4 (-$CH_2$-O-), 101.1 (C-8), 115.1 (C-6), 117.5 (C-10), 123.7 (C-3), 126.9 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'),132.0 (C-4'), 154.1 (C-2), 157.5 (C-9), 163.1 (C-7), 172.8 (>C=O), 174.4 (C-4). MS (m/z) 381.5 $(M + H)^+$, 403.6 $(M + Na)^+$, 419.3 $(M + K)^+$. Anal. ($C_{23}H_{24}O_5$) for C, H. Cacld: 72.61, 6.36; found: 72.57, 6.33.

**7-(Hydroxyethylethoxy)Ethoxyisoflavone (Analog 9)**

This compound was prepared by the same method described for analog 5 except that 2-[2-(2-chloroethoxy)ethoxy]ethanol was used as the alkylating agent. From 2 g of analog 1,280 mg of analog 9 was obtained, a yield of 14% (w/w): mp 105-107 °C. $^1$H NMR (DMSO-$d_6$) δ 3.43 (m, 2H, -$CH_2$-), 3.50 (m, 2H, -$CH_2$-), 3.56 (m, 2H, -$CH_2$-), 3.61 (m, 2H, -$CH_2$-), 3.80 (m, 2H, -$CH_2$-), 4.26 (m, 2H, -$CH_2$-), 4.58 (t, 2H, -$CH_2$-), 7.10 (dd, 1H, *J*= 8.97, 2.57 Hz, 6-H), 7.18 (d, 1H, *J*= 2.68 Hz, 8-H), 7.37 (t, 1H, *J*= 7.26 Hz, 4' H), 7.44 (t, 2H, *J*= 7.75 Hz, 3', 5'-H), 7.57 (dd, 1H, *J*= 1.60, 7.32 Hz, 2', 6'-H), 8.03 (d, 1H, *J*= 8.88 Hz, 5-H), 8.46 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 60.2 (-$CH_2$-), 68.1 (-$CH_2$-), 68.6 (-$CH_2$-), 69.8 (-$CH_2$-), 70.0 (-$CH_2$-), 72.4 (-$CH_2$-), 101.2 (C-8), 115.1 (C-6), 117.8 (C-10), 123.7 (C-3), 126.9 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 132.1 (C-4'), 154.1 (C-2), 157.4 (C-9), 162.9 (C-7), 174.4 (C-4). MS (m/z) 371.5 $(M + H)^+$, 393.3 $(M + Na)^+$, 409.2 $(M + K)^+$. Anal. ($C_{21}H_{22}O_6$) for C, H. Cacld: 68.10, 5.99; found: 67.92, 6.05.

**7-*O*-Acetylisoflavone (Analog 10)**

To a solution of 500 mg of analog 1 in 15 ml of anhydrous pyridine 2.0 ml of acetic anhydride was added. The mixture was gently stirred for 6 minutes and left at room temperature for 72 hours. Reaction product was precipitated in 100 ml of ice water, collected by filtration, washed with small portions of cold water, and dried under vacuum to give 490 mg of analog 9 (crystalline needle), a yield of 98% (mol/mol): mp 134-135 °C. $^1$HNMR (DMSO-$d_6$) δ 2.34 (-$CH_3$), 7.33 (dd, 1H, *J*= 8.47, 2.47 Hz, 6-H), 7.39 (m, 1H, 4'-H), 7.45 (t, 2H, *J*= 7.75 Hz, 3', 5'-H), 7.58 (d, 1H, *J*= 2.3 Hz, 8-H), 7.60 (d, 2H, *J* = 7.75, 1.79 Hz, 2', 6'-H), 8.18 (d, 1H, *J*= 8.86 Hz, 5-H), 8.55 (s, 1H, 2-H). Anal. ($C_{17}H_{12}O_4$) for C, H. Cacld: 72.85, 4.35; found: 72.36, 4.34.

**7-*O*-[Tetrahydro-2-(H)-Pyran-2-*O*-Propanyl]-Daidzein (Analog 11)**

This compound was prepared by the same method described for analog 5 except that daidzein and 2-(3-bromopropoxy)tetrahydro-2H-pyran were used as starting materials. From 5.1 g of daidzein, 4.20 g of analog 11 (white amorphous powder) was obtained, a yield of 82.4 % (w/w): mp 112-113°C. $^1$H NMR (DMSO-$d_6$) δ 1.39 (m, 2H, $CH_2$), 1.49 (m, 2H, 3"-H), 1.59-1.71 (m, 2H, 2"-H), 2.01 (m, 2H, -O-$CH_2$-), 3.34-3.53 (m, 2H, 4"-H), 3.70-3.82 (m, 2H, 5"-H), 4.19 (t, 2H, -$CH_2$-O-), 4.58 (d, IH, *J*= 3.62 Hz, 1"H), 6.82 (d, 2H, *J*= 8.53, 2.59, Hz, 3',5'-H), 7.06 (dd, IH, *J*= 8.86, 2.42 Hz, 6-H), 7.14 (d, 1H, *J*= 2.13 Hz, 8-H), 7.39 (d, 2H, *J*= 8.64 Hz, 2',6'-H), 8.01 (d, IH, *J*= 8.88 Hz, 5H), 8.35 (s, 1H, 2-H) $^{13}$C NMR (DMSO-$d_6$) δ 19.1 (-$CH_2$-), 25.0 (C-3"), 28.9 (C-4"), 30.2 (C-2"), 61.3 (C-5"), 63.1(-$CH_2$-O-), 65.6 (-$CH_2$-O-), 98.0 (C-1"), 101.0 (C-8), 114.9 (C-6), 114.9 (C-3', 5'), 117.6 (C-10), 122.4 (C-l'), 123.7 (C-3), 126.9 (C-5), 130.0 (C-2', 6'), 153.1 (C-2), 157.2 (C-9), 157.4 (C-4'), 162.9 (C-7), 174.7 (C-4). MS (m/z) 397.2 $(M + H)^+$, 419.4 $(M + Na)^+$, 435.4 $(M + K)^+$, 395.4 $(M - H)^-$. Anal. ($C_{23}H_{24}O_6$) for C, H. Cacld: 69.68, 6.10; found: 69.43, 6.08.

**7-*O*-[Tetrahydro-(2H)-Pyran-2-*O*-]Propanylisoflavone (Analog 12)**

This compound was prepared by the same method described for analog 5 except that 2-(3-bromopropoxy) tetrahydro-2H-pyran was used as the alkylating agent. From 2.38 g of I, 2.67 g of analog 12 (crystalline needle) was obtained, a yield of 70.2 % (mol/mol): mp 113 - 115 °C. $^1$H NMR (DMSO-$d_6$) δ 1.41 (m, 2H, -$CH_2$-), 1.47 (m, 2H, 3"-H), 1.59-1.73 (m, 2H, 2"-H), 2.0 (m, 2H, -O-$CH_2$-), 3.40-3.54 (m, 2H, 4"-H), 3.703.83 (m, 2H, 5"-H), 4.21 (t, 2H, -$CH_2$-O-), 4.58 (d, 1H, *J*= 3.5 Hz, 1"-H), 7.08 (dd, 1H, *J* = 8.9, 1.97 Hz, 6-H), 7.17 (d, 1H, *J*= 1.97 Hz, 8-H), 7.38 (t, IH, *J*= 6.98 Hz, 4'-H), 7.43 (t, 2H, *J*= 7.38 Hz, 3', 5'-H), 7.58 (d, 2H, *J*= 7.56 Hz, 2', 6'-H), 8.03 (d, 1H, *J*= 8.88 Hz, 5-H), 8.46 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 19.1 (-$CH_2$-), 25.0 (C-3"), 28.9 (C-4"), 30.2 (C-2"), 61.3 (C-5"), 63.1 (-$CH_2$-O-), 65.7 (-$CH_2$-O-), 98.0 (C-1"), 101.1 (C-8), 115.1 (C-6), 117.6 (C-10), 123.7 (C-3), 126.96 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 132.0 (C-4'), 154.1 (C-2), 157.4 (C-9), 163.1 (C-7), 174.1 (C-4). MS (m/z) 381.4 $(M + H)^+$, 403.4 $(M + Na)^+$, 419.2 $(M + K)^+$, 379.6 $(M - H)^-$. Anal. ($C_{23}H_{24}O_5$) for C, H. Cacld: 72.61, 6.36; found: 72.02, 6.34.

**7-*O*-(Phthalimide-N-)Butyldaidzein (Analog 13)**

To a suspension of 5.3 g of daidzein (20.85 mmol) and 50 ml of acetone, 11 ml of 2 N KOH (22 mmol) and 5.6g of N-(4-bromobutyl)-phthalimide (19.9 mmol) were added. The mixture was stirred under gentle reflux for 72 hours and concentrated. The residue was dissolved in chloroform-methanol (7:3) and loaded onto a Sephadex LH-20 column (chloroform:methanol,7:3). Fractions that contained pure product were pooled, concentrated, and recrystallized from acetone to give 1.76 g of analog 13, a yield of 33.2 % (w/w): mp 195-197 °C. $^1$H NMR (DMSO-$d_6$) δ 1.78 (-$CH_2$-$CH_2$-), 3.65 (-N-$CH_2$-), 4.13 (-O-$CH_2$-), 6.81 (d, 2H, *J*= 8.5 Hz, 3', 5'-H), 7.01 (dd, IH, *J*= 8.89, 2.5 Hz, 6-H), 7.07 (d, IH, *J*= 2.2Hz, 8-H), 7.39 (d, 2H, *J*= 8.6 Hz, 2', 61-H), 7.80 (m, 2H, 5", 6"-H), 7.83 (m, 2H, 4", 7"-H), 7.96 (d, IH, *J*= 8.89 Hz, 5-H), 8.32 (s, 1H, 2-H), 9.54 (s, 1H, 4'OH). $^{13}$C NMR (DMSO-$d_6$) δ 24.6 (-$CH_2$-), 25.8 (-$CH_2$-), 37.1 (-N-$CH_2$-), 67.9 (-$CH_2$-O), 101.0 (C-8), 114.8 (C-6), 114.9 (C-3', 5'), 117.5 (C-10), 122.4 (C-l'), 122.9 (C-3", 8"), 123.6 (C-3), 126.9 (C-5), 130.0 (C-2', 6'), 131.6 (C-5", 6"), 134.3 (C-4", 7"), 153.0 (C-2), 157.2 (C-9), 157.3 (C-4'), 162.8 (C- 7), 168.0 (C-2", 9"), 174.6 (C-4). MS (m/z) 456.5 $(M + H)^+$. Anal. ($C_{27}H_{21}O_6N$) for C, H, N. Cacld: 71.20, 4.65, 3.08; found: 70.89, 4.61, 3.09.

**Isoflavone 7-ω-(N-phthalimidylbutyl) Ether (Analog 14)**

To a suspension of 1.6 g of 7-hydroxyisoflavone (6.7 mmol) in 50 ml of acetone was added 7 ml of 2 *N* aq. KOH (14 mmol). The resulting mixture was stirred at room temperature until all starting materials were dissolved. To this solution, 4.4 g (15.6 mmol) of N-4-bromobutylphthalimide was added and the reaction mixture was stirred under gentle reflux for 72 hours and left in the cold room overnight. Precipitates were collected by filtration and recrystallized from acetone to give 1.26 g analog 76. Analyses: colorless crystals; yield 78.5%, w/w; mp 167-168 °C. $^1$H NMR (DMSO-$d_6$) δ 1.78 (-$CH_2$-$CH_2$-), 3.65 (-N-$CH_2$-), 4.14 (-O-$CH_2$-), 7.03 (dd, 1H, *J*= 8.9, 2.0 Hz, H-6), 7.10 (d, 1H, *J*= 1.8 Hz, H-8), 7.37 (dd, 1H, *J*= 6.92 Hz, H-4'), 7.42 (dd, 2H, *J*= 7.7 Hz, H-3', 5'), 7.57 (dd, 2H, *J*=7.6, H-2', 6'), 7.80 (m, 2H, H-5", 6"), 7.83 (m, 2H, 4", H- 7"), 7.96 (d, 1H, *J*= 8.89Hz, H-5), 8.32 (s, 1H, H-2), 9.54 (s, 1H, OH-4'). $^{13}$C NMR (DMSO$d_6$) δ 24.6 (-$CH_2$-), 25.8 (-$CH_2$-), 37.1 (-N-$CH_2$-), 67.9 (-$CH_2$-O-), 101.0 (C-8), 115.0 (C6), 117.5 (C-10), 122.9 (C-3", 8"), 123.7 (C-3), 126.9 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 131.6 (C-5", 6"), 132.0 (C-4'), 134.3 (C-4", 7"), 154.1 (C-2), 157.4 (C-9), 163.0 (C-7), 168.0 (C-2", 9"), 174.6 (C-4). MS

(m/z) 440.1 $(M + H)^+$. Elementary analyses ($C_{27}H_{21}O_5N$) for C, H, N: Cacld. 73.79, 4.82, 3.19; found 73.40, 4.81, 3.20.

**2-Ethoxycarbonyl-7-*O*-Phthalimide-N-Butylisoflavone (Analog 15)**

To a solution of 2.53 g (8.16 mmol) of analog 20 (see below for method of synthesis) in 50 ml of DMF, 1.59 g of $K_2CO_3$ (11.49 mmol) and 3.0 g of N-4bromobutylphthalimide (10.63 mmol) were added, respectively. The mixture was stirred at 80 °C for 1 hour and then poured into ice water. Precipitates were collected by filtration, washed with small portions of water, dried, and recrystallized in acetone to give 3.85 g of analog 15, a yield of 92.2 % (mol/mol): mp 163-165 °C. $^1$H NMR (DMSO-$d_6$) δ 0.90 (-$CH_3$), 1.78 (-$CH_2$-$CH_2$-), 3.65 (-N-$CH_2$-), 4.08 (-O-$CH_2$-), 4.16 (-O-$CH_2$-), 7.05 (dd, IH, *J*= 8.97, 2.42 Hz, 6-H), 7.16 (d, 1H, *J*= 2.44 Hz, 8-H), 7.24 (d, 2H, *J*= 8.53 Hz, 3', 5'-H), 7.39 (m, 3H, 2', 4', 6'-H), 7.79-7.81 (m, 2H, 5", 6"-H), 7.82-7.85 (m, 2H, 4", 7"H), 7.92 (d, 1H, *J*= 8.89 Hz, 5-H). $^{13}$C NMR (DMSO-$d_6$) δ 13.2 (-$CH_3$), 24.5 (-$CH_2$-), 25.8 (-$CH_2$-), 37. 1 (-N-$CH_2$-), 62.1 (-$CH_2$-O-), 68.1 (-$CH_2$-O-), 101.0 (C-8), 115.9 (C-6), 116.7 (C-10), 122.9 (C-3", 8"), 125.2 (C-1'), 126.9 (C-3), 127.8 (C-3', 5'), 127.9 (C-S), 129.7 (C-2', 6'), 131.5 (C-5", 6"), 134.3 (C-4", 1"), 150.0 (C-2), 156.7 (C-9), 160.9 (>C=O), 163.7 (C- 7), 168.0 (C-2", 9"), 175.3 (C-4). MS (m/z) 533.7 $[(M-1) + Na]^+$, 549.6 $[(M-1) + K]^+$,439.1 $[M-COOC_2H_5-1]^-$. Anal. ($C_{30}H_{25}O_7N$) for C, H, N. Cacld: 70.44, 4.93, 2.74; found: 70.35, 4.92, 2.76.

**2-Carboxy-7-*O*-Phthalimide-N-Butylisoflavone (Analog 16)**

This compound was prepared by hydrolyzing analog 15. To a solution of 1.0 g of analog 15 in 20 ml of methanol was added 80 ml of 0.02 N aqueous KOH. The mixture was stirred under gentle reflux for 3 hours until all samples were hydrolyzed (TLC). Methanol was removed by flash evaporation. The remaining solution was acidified with 1 N HCl to pH 2-3 to precipitate produce. Precipitates were collected by filtration and washed with small portions of water until pH of filtrate became neutral. The solution was then dried and residue was recrystallized from acetone to give 630 mg of analog 16 (white crystals): mp 143-145 °C. MS (m/z) 484.5 $(M + H)^+$, 506.4 $(M + Na)^+$, 482.5 $(M-H)^-$. Anal. ($C_{28}H_{21}O_7N$) for C, H, N. Cacld: 69.56, 4.38, 2.90; found: 69.56, 4.42, 2.89.

**7-*O*-Phthalimide-N-Butylpuerarin (Analog 17)**

This compound was prepared by the same method described for analog 13 except that puerarin was used instead of daidzein. From 500 mg of puerarin, 512 mg of analog 17 (white powder) was obtained, a yield of 69.0%: mp 68-69 °C. $^1$H NMR (DMSO-$d_6$) δ 1.78 (-$CH_2$-CH2-), 3.65 (-N-$CH_2$-), 4.14 (-O-$CH_2$-), 4.82 (d, 1H, *J*= 9.7 Hz, glucosyl 1'''-H), 7.03 (dd, 1H, *J*= 8.9, 2.0 Hz, 6-H), 7.10 (d, 1H, *J*= 1.8 Hz, 8-H), 7.37 (dd, 1H, *J* = 6.92 Hz, 4'-H), 6.81 (dd, 2H, *J*= 7.7 Hz, 3', 5'-H), 7.41 (dd, 2H, *J*= 7.6, 2', 6'-H), 7.80 (m, 2H, 5", 6"-H), 7.83 (m, 2H, 4", 7"-H), 7.96 (d, 1H, *J* = 8.89 Hz, 5-H), 8.40 (s, 1H, 2H), 9.54 (s, 1H, 4'-OH). $^{13}$C NMR (DMSO-$d_6$) δ 24.6 (-$CH_2$-), 25.8 (-$CH_2$-), 37.1(-$NCH_2$-), 61.5 (glucosyl C-6'''), 67.9 (-$CH_2$-O-), 70.7 (glucosyl C-4'''), 70.75 (glucosyl C2'''), 73.39 (glucosyl C-1'''), 78.71 (glucosyl C-3'''), 81.79 (glucosyl C-5'''), 112.6 (C-8), 115.0 (C-6), 117.5 (C-10), 122.9 (C-3", 8"), 123.7 (C-3), 126.9 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 131.6 (C-5", 6"), 132.0 (C-4'), 134.3 (C-4", 7"), 154.1 (C-2), 157.4 (C-9), 163.0 (C-7), 168.0 (C-2", 9"), 174.6 (C-4). MS (m/z) 618.4$(M+H)^+$, 640.5 $(M + Na)^+$, 616.7 $(M - H)^-$. Anal. ($C_{33}H_{31}O_{11}N$) for C, H, N. Cacld: 64.18, 5.06, 2.27; found: 63.89, 5.05, 2.24.

**Daidzein 7-*O*-1H-Benzotriazole-1-Methyl ether (Analog 18)**

This compound was prepared by the same method described for analog 5 except that daidzein and 1-chloromethyl-1H-benzotriazole were used as the starting material. From 5.1 g of daidzein, 4.35 g of analog 18 was obtained, a yield of 85.3% (w/w): mp 227-228 °C. $^1$H NMR (DMSO-$d_6$) δ 6.83 (dd, 2H, *J*= 8.63, 2.62 Hz, 3', 5'-H), 6.98 (s, 2H, -$CH_2$-), 7.21 (dd, 1H, *J*=8.89, 2.31 Hz, 6-H), 7.41 (dd, 2H, 2', 6'-H), 7.8 (t, 1H, *J*=7.84 Hz, 1"-H), 7.54 (d, 1H, *J*= 2.02 Hz, 8-H), 7.66 (t, 1H, *J*= 7.69, 2.02 Hz, 4"-H), 8.03 (t, 1H, *J*= 8.85, 8.04 Hz, 2", 3"-H), 8.11 (d, 1H, *J*= 8.52 Hz, 5-H), 8.40 (s, 1H, 2-H), 9.56 (s, 1H, 4'-OH). $^{13}$C NMR (DMSO-$d_6$) δ 73.7 (-N-$CH_2$-O-), 103.2 (C-8), 110.7 (C1"), 115.0 (C-3', 5'), 115.4 (C-6), 118.9 (C-4"), 119.5 (C-10), 122.2 (C-1'), 123.8 (C-3), 124.8 (C-2"), 127.3 (C-5), 128.5 (C-3"), 130.1 (C-2', 6'), 132.7 (C-6"), 145.3 (C-5"), 153.4 (C-2), 156.9 (C-9), 157.3 (C-4'), 159.9 (C-7), 174.7 (C-4). MS (m/z), 386.4 $(M + H)^+$. Anal. ($C_{29}H_{20}O_4N_3$) for C, H, N, Cacld: 68.57, 3.92, 10.90, found: 68.28, 3.92, 10.94.

**2-Ethoxycarbonyl-7-*O*-1H-Benzotriazole-l-Methylisoflavone (Analog 19)**

This compound was prepared by the same method described for analog 18 except that analog 20 (see below for method of synthesis) was used instead of daidzein. From 3.1 g of analog 20, 1.43 g of analog 19 was obtained, a yield of 46 % (w/w): mp 182-183 °C. $^1$H NMR (DMSO-$d_6$) δ 0.92 (-$CH_3$), 4.09 (-$CH_2$-O-), 7.03 (s, 2H, -N-$CH_2$-O-), 7.23 (dd, 1H, *J*= 8.78, 2.79 Hz, 6-H), 7.25 (dd, 2H, *J*= 8.78, 2.77 Hz, 3', 5'-H), 7.40 (dd, 2H, *J*= 8.53, 1.50 Hz 2', 6'-H), 7.40 (d, 1H, *J=1.5* Hz, 8-H), 7.49 (t, 1H, *J=7.99,* 7.46 Hz, 4'H), 7.65-7.68 (m, 2H, 1 ", 4"-H), 7.99-8.04 (m, 2H, 2", 3"-H), 8.12 (d, IH, *J* = 8.38 Hz, 5H). $^{13}$C NMR (DMSO-$d_6$) δ 13.2 (-$CH_3$), 62.1 (-$CH_2$-O-), 73.6 (-N-$CH_2$-O-), 103.2 (C8), 110.7 (C-1"), 116.3 (C-6), 118.1 (C-10), 119.5 (C-4"), 124.8 (C-3), 125.5 (C-1'), 127.3 (C-5), 127.8 (C-3', 5'), 128.0 (C-2"), 128.6 (C-3"), 129.7 (C-2', 6'), 131.4 (C-6"), 132.7 (4'-H), 145.3 (C-5"), 150.1 (C-2), 156.3 (C-9), 160.8 (>C=O, C-7), 175.5 (C-4). MS (m/z) 442.2 $(M + H)^+$, 463.9 $(M + Na)^+$, 479.8 $(M + K)^+$. Anal. ($C_{25}H_{19}O_5N_2$) for C, H, N. Cacld: 68.02, 4.34, 9.52; found: 68.66, 4.36, 9.58.

**2-Ethoxycarbonyl-7-Hydroxyisoflavone (Analog 20)**

To a solution of 4.56 g of 2,4-dihrdroxyphenylbenzylketone (20.24 mmol) in 20 ml of pyridine was added 3.5 ml of ethyl chlorooxoacetate (31.28 mmol). The mixture was stirred gently at room temperature for 1 hour and yellowish precipitates were fanned. The precipitates were collected by filtration and dried, redissolved in chloroform:methanol (7:3), and loaded onto a Sephadex LH-20 (chloroform:methanol, 7:3) column. Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 4 g of analog 20, a yield of 87.61 %: mp 213-214 °C. 1H NMR (DMSO-$d_6$) δ 0.88 (t, 3H, -$CH_3$), 4.05 (-$CH_2$-), 6.90 (d, 1H, *J*= 1.91 Hz, 8-H), 6.98 (dd, 1H, *J*= 8.82, 1.91 Hz, 6-H), 7.23 (d, 1H, *J*= 7.15 Hz, 3', 5'- H), 7.39-7.42 (m, 3H, 2', 4', 6'-H), 7.92 (d, 1H, *J*= 8.84 Hz, 5-H), 11.02 (s, 1H, 7-0H). $^{13}$C NMR (DMSO-$d_6$) d 13.2 (-$CH_3$), 62.0 (-$CH_2$-O-), 102.2 (C-8), 115.9 (C-6), 115.9 (C-10), 125.1 (C-1'), 127.4 (C-3), 127.8 (C-3', 5'), 127.9 (C-5), 129.8 (C-2', 6'), 131.7 (C-4'), 149.8 (C-2), 156.8 (C-9), 161.0 (>C=O), 163.5 (C-7), 175.3 (C-4). MS (m/z) 311.3 $(M + H)^+$, 333.1 $(M + Na)^+$, 309.2 $(M - H)^-$. Anal. ($C_{18}H_{14}O_5$) for C, H. Cacld: 69.67, 4.55; found: 69.27, 4.59.

**2-Carboxy-7-Hydroxyisoflavone (Analog 21)**

This compound was obtained by hydrolyzing analog 20. To a solution of 1 g of analog 20 in 10 ml of methanol was added 20 ml of 0.2 N aqueous KOH. The mixture was stirred under gentle reflux for 2 hours until analog 20 was completely hydrolyzed (monitored by TLC). Methanol was removed on a rotary evaporator. Hydrolyzed product in remaining solution was precipitated by acidification (HCl, pH 2-3). Precipitates were collected by filtration, washed with small portions of water until pH of filtrate approached neutral, and recrystallized from acetone to give 630 mg of pure analog 21, a yield of 63 % (w/w): mp 257-258 °C. MS (m/z) 283.2 $(M + H)^+$, 305.3 $(M + Na)^+$, 321.3 $(M + K)^+$, 237.4 $(M–COOH)^+$, 281.5 $(M - H)^-$. Anal. ($C_{16}H_{10}O_5$) for C, H. Cacld: 68.09, 3.57; found: 68.17, 3.54.

**2-Carboxy-7-ω-Carboxylpentyl Isoflavone (Analog 22)**

To a solution of 5.0 g (16.1 mmol) of analog 20 in 60 ml of DMF was added 2.6 g of $K_2CO_3$ (18.8 mmol) and 3 ml of ethyl6-bromo hexanoate (16.86 mmol). The mixture was refluxed for 1 hour and poured into 100 ml of ice water. Products were extracted with ethyl acetate and evaporated to dryness. Dry residue was dissolved in 20 ml of methanol followed by addition of 20 ml of 1*N* KOH (20 mmol). The mixture was stirred under gentle reflux for 2 hours until the ethyl ester was completely hydrolyzed (monitored by TLC). Solvent was removed under reduced pressure. Residue was dissolved in chloroform-methanol (7:3) and purified on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 5.34 g of analog 22, a yield of 83.7 %: mp 157-159 °C. $^1$H NMR (DMSO-$d_6$) δ 1.44 (m, 2H, -$CH_2$), 1.60 (m, 2H, -$CH_2$), 1.76 (m, 2H, -$CH_2$), 2.33 (m, 2H, -$CH_2$), 4.14 (-$CH_2$-), 7.09 (d, 1H, *J*= 1.8 Hz, 6-H), 7.21 (d, 1H, *J*= 2.5 Hz, 8-H), 7.26 (dd, 1H, *J*= 7.54, 1.52 Hz, 3', 5'- H), 7.34-7.41 (m, 3H, 2', 4', 6'-H), 7.96 (d, 1H, *J*= 8.88 Hz, 5-H).13C NMR (DMSO-$d_6$) 8 24.1 (-$CH_2$), 24.9 ($CH_2$), 28.0 (-$CH_2$), 33.1 (-$CH_2$), 33.4 (-$CH_2$), 68.5 (-$CH_2$-O-), 101.0 (C-8), 115.8 (C-6), 116.7 (C-10), 123.9 (C-1'), 126.9 (C-3), 127.8 (C-31, 51), 127.9 (C-5), 129.8 (C-2', 6'), 131.7 (C-41), 151.7 (C-2), 156.8 (C-9), 162.3 (>C=O), 163.8 (C-7), 173.3 (C-4), 175.5 (C-4). Anal. ($C_{22}H_{20}O_7$) for C, H. Cacld: 66.67, 5.09; found: 66.89, 5.14.

**7-Hydroxy-4'-Fluoroisoflavone (Analog 23)**

A mixture of 1.54 g of 4-fluorophenylacetic acid (90.5 mmol), 11.0 g of resorcinol (99.9 mmol) and 50ml of boron trifluoride diethyl etherate was stirred at 80 °C for 5 hours. The reaction mixture was cooled to room temperature and then washed rust with aqueous $K_2CO_3$ and then with water until pH of the water layer approached 7. The product was extracted with ethyl acetate and concentrated. The residue was dissolved and loaded onto a Sephadex-LH-20 column (chloroform:methanol, 7:3). Fractions that contained pure analog 23 were pooled and dried to give 3.5g of crude 2, 4dihydroxyphenyl-4'-fluorobenzylketone (I). To 3.5 g on in 20 ml of2-propanol was added 1.0 ml of morpho line and 2.5 ml of triethyl orthoformate. The mixture was stirred at 80 °C for 7 hours. Solvents were removed and residue was dissolved in 30 ml of methanol and stirred at 50 °C for 30 min. The solution was cooled to room temperature and kept at 4 °C overnight. White crystals were collected by filtration, washed with small portions of methanol, and dried to give 1.53 g of analog 23, a yield of 43.7% (w/w): mp 235-236 °C. $^1$H NMR (DMSO-$d_6$) δ 6.88 (d, 1H, *J*= 1.73 Hz, 8-H), 6.95 (dd, 1H, *J*= 8.80, 1.85 Hz, 6-H), 7.25 (t, 2H, *J*= 8.82 Hz, 3', 5'-H), 7.61(dd, 2H, *J*= 2.52, 8.21 Hz, 2', 6'-H), 7.97 (d, 1H, *J*= 8.80 Hz, 5-H), 8.40 (s, 1H, 2-H), 10.8 (s. 1H, 7-OH). $^{13}$C NMR (DMSO-$d_6$) δ 102.2 (C-8), 114.9 (C-6), 115.0 (C-3', 5'), 116.5 (C-10), 122.5 (C-1'), 127.3 (C-3), 128.4 (C-5), 130.9 (C-2', 6'), 153.8 (C-2), 157.5 (C-9), 160.8 (C-4'), 162.7 (C-7), 174.3 (C-4). MS (m/z) 257.2 $(M + H)^+$, 279.3 $(M + Na)^+$, 255.4 $(M - H)^-$. Anal. ($C_{15}H_9O_3F$) for C, H. Cacld: 70.31, 3.54; found: 70.56, 3.51.

**7-*0*-Ethoxycarbonylpentyl-4'-Fluoroisoflavone (Analog 24)**

This compound was prepared by the same method described for analog 5 except that the starting materials were analog 23 and ethyl 6-bromohexanoate. From 1.28 g of analog 23, 964 mg of analog 24 (crystalline needles) was obtained, a yield of 75.3 % (w/w): mp 91-93 °C. $^1$H NMR (DMSO-$d_6$) δ 1.16 (t, 3H, -$CH_3$), 1.43 (m, 2H, -$CH_2$-), 1.60 (m, 2H, -$CH_2$-), 1.76 (m, 2H, -$CH_2$-), 2.31 (t, 2H, -$CH_2$-), 4.05 (q, 2H, -$CH_2$-O-), 4.10 (t, 2H, -$CH_2$-O-), 7.05 (d, 1H, *J*= 8.88, Hz, 6-H), 7.14 (d, 1H, *J*=1.97 Hz, 8-H), 7.26 (d, 2H, *J*= 8.78 Hz, 3', 5'-H), 7.63 (t, 2H, *J*= 8.78 Hz, 2', 6'-H), 8.01 (d, 1H, *J*= 8.84 Hz, 5-H), 8.46 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-CH3), 24.2 (-$CH_2$-), 24.9 (-$CH_2$-), 28.1 (-$CH_2$-), 33.4 (-$CH_2$-), 59.6 (-$CH_2$-O-), 68.3 (-$CH_2$-O-), 101.0 (C-8), 114.9 (C-6), 115.0 (C-3', 5'), 117.4 (C-10), 122.7 (C-1'), 126.9 (C-3), 128.3 (C-5), 130.9 (C-2', 6'),154.1 (C-2),

157.4 (C-9), 160.9 (C-4'), 163.1 (C-7), 172.8 (>C=O), 174.3 (C-4). MS (m/z) 399.4 $(M + H)^+$, 421.4 $(M + Na)^+$. Anal. ($C_{23}H_{23}O_5F$) for C, H. Cacld: 69.34, 5.82; found: 69.23, 5.79.

**7-Hydroxy-4'-Bromoisoflavone (Analog 25)**

The synthesis was similar to that described for analog 23 except that 4-fluorophenylacetic acid was replaced with 4-bromophenylacetic acid. This synthesis resulted in 1.41 g of analog 25, a yield of 42 % (based on resorcinol, w/w): mp 266-268 °C. $^1$H NMR (DMSO-$d_6$) δ 6.87 (d, 1H, *J*= 1.65 Hz, 8-H), 6.94 (dd, 1H, *J* = 8.80, 1.78 Hz, 6-H), 7.54 (d, 2H, *J*= 8.43 Hz, 3', 5'-H), 7.61 (d, 2H, *J*= 8.43 Hz, 2" 6'-H), 7.97 (d, 1H, *J*= 8.63 Hz, 5-H), 8.42 (s, 1H, 2-H), 10.8 (s, br. 1H, 7-OH). $^{13}$C NMR (DMSO-$d_6$) δ 102.2 (C-8), 115.4 (C-6), 116.4 (C-10), 121.0 (C-1'), 122.3 (C-3), 127.3 (C-5), 130.9 (C 3',51),131.0 (C-2', 6'),131.4 (C-4'), 154.0 (C-2), 157.5 (C-9), 162.7 (C-7), 174.1 (C-4). MS (m/z) 317.1 $(M)^+$, 340.8 $(M + Na)^+$. Anal. ($C_{15}H_9O_3Br$) for C, H. Cacld: 56.81, 2.86; found: 56.56, 2.85.

**4'-Bromoisoflavone 7-ω-Ethoxycarbonylpentyl Ether (Analog 26)**

A reaction mixture containing 4.36 g of 4-bromophenylacetic acid (20.3 mmol), 3.36 g of resorcinol (30.5 mmol) and 50 ml of boron trifluoride diethyl etherate was stirred at 80°C for 5h. The reaction mixture was cooled to room temperature and then washed with aqueous $K_2CO_3$ followed by water until pH of the water layer reached ≈7. The product was extracted with ethyl acetate and concentrated by flash evaporation. Residue was dissolved and fractionated on a Sephadex-LH-20 column (chloroform:methanol/7:3). Fractions that contained the pure product 2, 4-dihydroxyphenyl-4'-bromobenzylketone were pooled and evaporated to dryness. To this residue, 50 ml of 2-propanol, 7.0 ml of morpholine and 10 ml of triethyl orthoformate were added. The reaction mixture was stirred at 80°C for 24h. Solvents were evaporated and residue was dissolved in 20 ml of methanol and stirred at 50°C for another 20min. The solution was cooled to room temperature and kept at 4°C overnight. The yellow precipitates of 4'-bromo-7-hydroxylisoflavone was collected by filtration (2.8 g). The precipitate was dissolved in 50 ml of DMF and to this solution was added 2.76 g of $K_2CO_3$ (20.0 mmol), followed by 6.0 ml of ethyl 6-bromohexanoate (33.7 mmol). The mixture was stirred and heated at 80°C for 3.5 h and poured into 200 ml of ice water. Precipitates formed were collected by filtration and fractionated on a Sephadex LH-20 column (chloroform:methanol/7:3). Fractions that contained purified product were pooled,

concentrated and recrystallized from acetone to give 1.62 g pure analog 104. Analyses: white crystals; yield, 57.1 %; mp 122-124 °C. $^1$H NMR (DMSO-$d_6$) δ 1.17 (t, 3H, -$CH_3$), 1.43 (m, 2H, -$CH_2$-), 1.60 (m, 2H, -$CH_2$-), 1.76 (m, 2H, -$CH_2$-), 2.31 (m, 2H, -$CH_2$-), 4.04 (q, 2H, -$CH_2$-O-), 4.11 (t, 2H, -$CH_2$-O-), 7.06 (dd, 1H, *J*= 8.88, 2.56 Hz, H-6), 7.15 (d, 1H, *J*= 1.93 Hz, H-8), 7.55 (d, 2H, *J*= 8.68 Hz, H-3', 5'), 7.62 (d, 2H, *J*= 8.68 Hz, H-2', 6'), 8.01 (d, 1H, *J*=8.87 Hz, H-5), 8.50 (s, 1H, H-2). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-$CH_3$), 24.1 (-$CH_2$-), 24.9 (-$CH_2$-), 28.0 (-$CH_2$-), 33.4 (-$CH_2$-), 59.6 ($CH_2$-O-), 68.4 (-$CH_2$-O-), 101.0 (C-8), 115.2 (C-6), 117.4 (C-10), 121.1 (C-1'), 122.5 (C3),126.9 (C-5), 130.9 (C-3', 5'),131.0 (C-2', 6'), 131.2 (C-4'), 154.3 (C-2), 157.4 (C-9), 163.2 (C-7), 172.8 (>C=O), 174.1 (C-4). MS (m/z), 460.0 (M + H)+. Elementary analyses ($C_{23}H_{23}O_5Br$) for C, H: Cacld: 60.14, 5.05; found: 59.74, 4.66.

**7-Hydroxy-4'-Nitroisoflavone (Analog 27)**

This compound was prepared by the same method described for analog 23 except that the starting materia14-fluorophenylacetic acid was replaced by 4-nitrophenyl acetic acid. A total of 1.35g of analog 27 was obtained, a yield of 38.6 % (w/w): mp 270 °C (decomposes). $^1$H NMR (DMSO-$d_6$) δ 6.86 (d, 1H, *J*= 2.0 Hz, 8-H), 6.94 (dd, 1H, *J*= 8.84, 1.93 Hz, 6-H), 7.88 (d, 2H, *J*= 8.76,1.48 Hz, 2', 6'-H), 7.97 (d, 1H, *J*= 8.79 Hz, 5H), 8.27 (d, 2H, *J*= 8.78, 1.70 Hz, 3', 51-H), 8.56 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 102.3 (C-8), 115.9 (C-6), 116.0 (C-10), 121.5 (C-3), 123.2 (C-2', 6'), 127.3 (C-5), 129.9 (C-31, 51), 139.4 (C-1'), 146.7 (C-4'), 155.3 (C-2), 157.5 (C-9), 163.8 (C-7), 173.8 (C-4). MS (m/z) 283.9 ($M^+$), 306.3 $(M + Na)^+$, 282.4 $(M - H)^-$. Anal. ($C_{15}H_9O_5N$) for C, H, N. Cacld: 63.61, 3.20, 4.95; found: 63.48, 3.21, 4.91.

**7-*O*-Ethoxycarbonylpentyl-4'-Nitroisoflavone (Analog 28)**

This compound was prepared by the same method described for analog 24 except that analog 27 was used as the starting material. A total of 270mg analog 28 was obtained from this synthesis, a yield of 13.5% (w/w): mp 173-175 °C. $^1$H NMR (DMSO-$d_6$) δ 1.17 (t, 3H, -$CH_3$), 1.44 (m, 2H, -$CH_2$-), 1.59 (m, 2H, -$CH_2$-), 1.60 (-$CH_2$-), 1.77 (-$CH_2$-), 2.32 (-$CH_2$-), 4.04 (-$CH_2$-O-), 4.13 (-$CH_2$-O-), 7.10 (dd, 1H, *J*= 8.85, 1.94 Hz, 6-H), 7.20 (d, 1H, *J*= 2.32 Hz, 8-H), 7.92 (d, 2H, *J*= 8.73 Hz, 2', 6'-H), 8.04 (d, 1H, *J*= 8.87 Hz, 5-H), 8.29 (d, 2H, *J*= 8.74 Hz, 3', 5'-H), 8.68 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-$CH_3$), 24.2 (-$CH_2$-), 24.9

(-$CH_2$-), 28.0 (-$CH_2$-), 33.4 (-$CH_2$-), 59.7 ($CH_2$-O-), 68.4 (-$CH_2$-O-), 101.2 (C-8), 115.4 (C-6), 117.4 (C-10), 121.8 (C-3), 123.2 (C2', 6'), 126.9 (C-5), 129.9 (C-3', 5'), 139.1 (C-1'), 146.7 (C-4'), 155.7 (C-2), 157.4 (C-9), 163.4 (C-7), 172.8 (-C=O), 173.9 (C-4). Anal. ($C_{23}H_{23}O_7N$) for C, H, N. Cacld: 64.93, 5.54, 3.29; found: 64.63, 5.48, 3.31.

**7-Hydroxy-4'-Methylisoflvone (Analog 29)**

This compound was prepared by a similar method described for analog 23 except that 4-flurophenylacetic acid was replaced by p-tolylacetic acid. A total of 780 mg analog 29 was obtained from this procedure, a yield of 21.3 % (based on resorcinol, w/w): mp 241-243 °C. $^1$H NMR (DMSO-$d_6$) δ 2.33 (s, 3H, -$CH_3$), 6.87 (d, $^1$H, *J*= 1.94 Hz, 8-H), 6.94 (dd, 1H, *J*= 8.72, 2.68 Hz, 6-H), 7.21 (t, 2H, *J*= 7.92 Hz, 3', 5'-H), 7.45 (d, 2H, *J*= 8.28 Hz, 2', 6'-H), 7.97 (d, 1H, *J*= 8.85 Hz, 5-H), 8.33 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 20.8 (-$CH_3$), 102.1 (C-8), 115.2 (C-6), 116.6 (C-10), 123.4 (C-1'), 127.3 (C-3), 128.7 (C-3', 5'), 128.7 (C-2', 6'), 129.1 (C-5), 137.0 (C- 4'), 153.4 (C-2), 157.4 (C-9), 162.6 (C- 7), 174.5 (C-4). MS (m/z) 253.4 $(M + H)^+$, 275.2 $(M + Na)^+$, 251.4 $(M - H)^-$. Anal. ($C_{16}H_{12}O_3$) for C, H. Cacld: 76.18, 4.79; found: 75.39, 4.82.

**7-*O*-Ethoxycarbonylpentyl-4'-Methylisoflavone (Analog 30)**

This compound was prepared by the same method as described for analog 5 except that starting materials were analog 29 and ethyl 6-bromohexanoate. The synthesis resulted in 900 mg of analog 30, a yield of 35.71% (w/w): mp 141-142 °C. $^1$H NMR (DMSO-$d_6$) δ 1.16 (t, 3H, -$CH_3$), 1.44 (m, 2H, -$CH_2$-), 1.59 (m, 2H, -$CH_2$-), 1.76 (m, 2H, -$CH_2$-), 2.31 (m, 2H, -$CH_2$-), 2.33 (-$CH_3$), 4.04 (q, 2H, -$CH_2$-O-), 4.11 (t, 2H, -$CH_2$-O-), 7.06 (dd, IH, *J*= 8.79, 2.53 Hz, 6-H), 7.14 (d, 1H, *J=1.89* Hz, 8-H), 7.23 (d, 2H, *J*=8.04 Hz, 3', 5'-H), 7.47 (d, 2H, *J*= 7.63 Hz, 2', 6'-H), 8.01 (d, 1H, *J*= 8.86 Hz, 5-H), 8.42 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-$CH_3$), 20.8 (-$CH_3$), 24.1 (-$CH_2$-), 24.9 ( $CH_2$-), 28.0 (-$CH_2$-), 33.4 (-$CH_2$-), 59.6 (-$CH_2$-O-), 68.3 (-$CH_2$-O-), 101.0 (C-8), 115.0 (C-6), 117.5 (C-10), 123.6 (C-1'), 126.9 (C-3), 128.7 (C-3', 51),128.7 (C-2', 6'), 129.0 (C-5), 137.1 (C-4'), 153.7 (C-2), 157.4 (C-9), 163.1 (C- 7), 172.8 (>C=O), 174.5 (C-4). Anal. ($C_{24}H_{26}O_5$) for C, H. Cacld: 73.08, 6.64; found: 73.11, 6.61.

**7, 4'-Dimethoxyisoflavone (Analog 31)**

To a solution of 1.28 g of daidzin (5.0 mmol) in 40 ml of DMSO was added 3.84 g of NaOH pellets. The mixture was stirred at RT for 6 min and 3 ml of iodomethane (48.2 mmol) was added dropwise. The mixture was stirred at RT for another 6 min and poured into ice water. Product in the water was extracted with chloroform and dried. Residue was fractionated on a Sephadex LH-20 column (chloroform-methanol, 7:3). Fractions that contained pure product were pooled, concentrated, and recrystallized from acetone to give 450 mg of analog 31 (crystalline needles), a yield of 35.2 (w/w): mp 162-163 °C. $^1$H NMR (DMSO-$d_6$) δ 3.79 (-$OCH_3$), 3.90 (-$OCH_3$), 6.99 (d, 2H, *J*= 8.36 Hz, 3', 5'-H), 7.07 (dd, IH, *J*= 8.88, 2.51 Hz, 6-H), 7.14 (d, 1H, *J*= 1.82 Hz, 8-H), 7.52 (d, 2H, *J*= 8.67 Hz, 2', 6'-H), 8.02 (d, 1H, *J*= 8.88 Hz, 5-H), 8.40 (s, 1H, 2-H). Anal. ($C_{17}H_{14}O_4$) for C, H. Cacld: 72.33, 4.99; found:.72.47, 4.95.

**7-Hydroxy-4'-Aminoisoflavone (Analog 32)**

This compound was prepared by the reduction of analog 27. To a suspension of 500 mg of analog 27 and 50 ml of ethanol was added 1.0 g of zinc powder. The mixture was stirred at 50 °C while 10 ml of glacial acetic acid was added slowly through a span of 30 min. After analog 27 was completely reduced, reaction mixture was filtered and filtrate was concentrated. The concentrate was suspended in 20 ml of water and extracted with ethyl acetate. The organic layer was evaporated to dryness and residue was recrystallized from methanol:chloroform:petroleum ether (30-60 °C) (1:5:20) to give 153 mg of yellow amorphous powder of analog 32, a yield of 30.6% (w/w): mp 250 °C (decomposes). $^1$H NMR (DMSO-$d_6$) δ 6.59 (d, 2H, *J*= 8.62 Hz, 3', 5'-H), 6.81 (d, 1H, *J* = 2.63 Hz, 8-H), 6.89 (dd, 1H, *J*= 8.93, 2.56 Hz, 6-H), 7.24 (d, 2H, *J*= 8.08 Hz, 2', 6'-H), 7.94 (d, 1H, *J*= 8.78 Hz, 5-H), 8.21 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 102.1 (C-8), 113.4 (C-3', 5'), 115.2 (C-6), 117.5 (C-10), 119.0 (C-1'), 123.9 (C-3), 127.2 (C-5), 129.5 (C-2', 6'), 148.5 (C-4'), 152.3 (C-2), 157.4 (C-9), 162.9 (C-7), 174.9 (C-4). MS (m/z) 254.3 $(M + H)^+$, 276.2 $(M + Na)^+$, 252.6 $(M - H)^-$, Anal. ($C_{15}H_{10}O_3N$) for C, H, N. Cacld: 71.14, 4.38, 5.53; found: 70.89, 4.40, 5.49.

**7-Ethoxycarbonylpentyl-4'-Aminoisoflavone (Analog 33)**

This compound was prepared by reducing analog 28 under the same conditions described in analog 32. A total of 863mg of analog 33 was obtained, a yield of 43.2 % (w/w)

mp 147-148 °C. $^1$H NMR (DMSO-$d_6$) δ 1.17 (t, 3H, -$CH_3$), 1.44 (m, 2H, -$CH_2$-), 1.60 (-$CH_2$-), 1.74 (-$CH_2$-), 2.31 (-$CH_2$-), 4.04 (-$CH_2$-O-), 4.13 (-$CH_2$-O-), 6.60 (d, 2H, *J* = 8.74 Hz, 3', 5'-H), 7.03 (dd, 1H, *J*= 8.93, 2.56 Hz, 6-H), 7.09 (d, 1H, *J*= 2.63 Hz, 8-H), 7.26 (d, 2H, *J*= 6.52, 1.61 Hz, 2', 6'-H), 7.99 (d, 1H, *J*= 8.88 Hz, 5-H), 8.29 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-CH3), 24.2 (-$CH_2$-), 24.9 (-$CH_2$-), 28.0 (-$CH_2$-), 33.4 (-$CH_2$-), 59.7 (-$CH_2$-O-), 68.3 (-$CH_2$-O-), 100.8 (C-8), 113.4 (C-3', 5'), 114.8 (C-6), 117.5 (C-10), 118.8 (C-1'), 124.1 (C-3), 126.9 (C-5), 129.5 (C-2', 6'), 148.5 (C-4'), 152.3 (C-2), 157.3 (C-9), 162.9 (C-7), 174.9 (C-4). MS (m/z) 396.5 $(M + H)^+$, 418.4 $(M + Na)^+$, 394.1 $(M - H)^-$. Anal. ($C_{23}H_{25}O_5N$) for C, H, N. Cacld: 69.86, 6.37, 3.54; found: 69.34, 6.29, 3.53.

**7, 8-Dimethoxyisoflavone (Analog 34)**

This compound was prepared as described in the synthesis of analog 23. A mixture of 2.76 g of phenylacetic acid (20.08 mmol), 5.0 g of 2, 3-dimethoxylphenol (32.43mmol) and 50 ml of boron trifluoride diethyl ether ate was stirred at 80 °C for 19 hours. Reaction mixture was then cooled to RT and washed with aqueous $K_2CO_3$ and water sequentially until pH of the filtrate approached neutrality. Product was extracted with ethyl acetate and concentrated to dryness. The residue, which contained mostly 2-hydroxy-3,4-dimethoxyphenylbenzylketone, was dissolved in 50 ml of 2-propanol and 3.0 ml of morpho line and 6.0 ml of triethyl orthoformate (36.07 mmol) were added. The mixture was stirred at 80 °C for 20 hours and evaporated to dryness. The residue was fractionated on a silica gel (chloroform-methanol system) and fractions that contained pure product were pooled, dried, and recrystallized from acetone to give 300 mg of analog 34 (crystalline plates), a yield of 6% (based on 2,3-dimethoxylpheno1, w/w): mp 143-144 °C. $^1$HNMR (DMSO-$d_6$) δ 3.89 (8-$OCH_3$), 3.96 (7-$OCH_3$), 7.30 (d, 1H, *J*= 9.03, Hz, 6-H), 7.39 (t, 1H, *J*= 6.94 Hz, 4'-H), 7.43 (t, 2H, *J*= 7.06, 7.72 Hz, 3', 5'-H), 7.57 (dd, 2H, *J*= 7.26, 1.44 Hz, 2', 6'-H), 7.87 (d, 1H, *J*= 8.88 Hz, 5-H), 8.50 (s, 1H, 2H). $^{13}$C NMR (DMSO-$d_6$) δ 56.5 (-$OCH_3$), 60.9 (-$OCH_3$), 111.0 (C-6), 118.5 (C-10), 120.9 (C-3), 123.3 (C-1'), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 131.9 (C-4'), 136.1(C-8), 149.9(C-7), 154.1 (C-2), 156.2 (C-9), 174.6 (C-4). MS (m/z) 283.3 $(M + H)^+$, 305.4 $(M + Na)^+$, 281.9 $(M - H)^-$. Anal. ($C_{17}H_{14}O_4$) for C, H. Cacld: 72.33, 4.99; found: 72.21, 4.97.

**7-Ethylisoflavone (Analog 35)**

This compound was prepared by the same method described for the synthesis of analog 23. To a solution of 6.51 g of phenylacetic acid (47.81 mmol) in 50 ml of boron trifluroide diethyl etherate was added 6.5 ml of 3-ethylphenol (53.20 mmol). The mixture was stirred at 80 °C for 21 hours, cooled to RT, and then washed with aqueous $K_2CO_3$ and water until the pH of wash approached neutrality. The product was extracted with ethyl acetate and concentrated. Residue was dissolved in 50 ml of 2-propanol and 8 ml of triethyl formate (48.1 mmol) and 3 ml of morpho line were added. The mixture was stirred at 80 °C for 7 hours and solvent was removed by flash evaporation. The syrup obtained was dissolved in 30 ml of methanol, stirred at 50 °C for 30 min, cooled to room temperature, and kept at 4 °C overnight. Crystals were collected by filtration and washed with small portions of acetone to give 790 mg of analog 35, a yield of 12.2% (based on 3-ethylphenol, w/w): mp 103-104 °C. $^1$H NMR (DMSO-$d_6$) δ 1.35 (t, 3H, -$CH_3$), 2.78 (q, 2H, -$CH_2$-), 7.38 (m, 4'-H), 7.39 (dd, 1H, *J*= 8.10, 1.91 Hz, 6-H), 7.44 (t, 2H, *J*= 7.77 Hz, 3', 5'-H), 7.52 (d, 1H, *J*= 2.0 Hz, 8-H), 7.59 (dd, 2H, *J*= 7.35, 1.38 Hz, 2', 6'-H), 8.05 (d, 1H, *J*= 8.54 Hz, 5-H), 8.51 (s, 1H, 2-H). $^{13}$C NMR (DMSO-$d_6$) δ 14.9 (-$CH_3$), 28.2 ($CH_2$-), 116.7 (C-6, 8), 121.8 (C-10), 123.8 (C-3), 125.4 (C-1'), 125.8 (C-7), 127.8 (C-5), 128.1 (C-3', 5'), 128.9 (C-2', 6'), 131.9 (C-4'), 154.4 (C-2), 155.9 (C-9), 174.9 (C-4). MS (m/z), 251.4 (M + H)$^+$, 273.2 (M + Na)$^+$, 249.3 (M - H)$^-$. Anal. ($C_{17}H_{14}O_2$) for C, H. Cacld: 81.58, 6.91; found: 80.92, 6.87.

**7-Ethoxylcarbonylpentoxypuerarin (Analog 36)**

This compound was prepared by the same method described for analog 5. To a solution of 3.5 g of puerarin (8.41 mmol) in 60 ml of DMF, 2.76g of anhydrous $K_2CO_3$ (20.0 mmol) and 6.0 ml of ethyl 6-bromohexanoate (33.6 mmol) were added. The mixture was stirred at 80 °C for 6 hours, poured into ice water, and extracted with ethyl acetate. Solvent was evaporated and residue was fractionated on a on Silica gel column (chloroform:methanol, 8:2) followed by a Sephadex LH-20 column (chloroform:methanol, 7:3). Final product was recrystallized from petroleum ether:chloroform:methanol (10:0.5:0.1) to give 3.19 g of analog 36 (white amorphous powder), a yield of 91.14% (w/w): mp 165-168 °C. $^1$H NMR (DMSO-$d_6$) δ 1.18 (-$CH_3$), 1.47 (-$CH_2$-), 1.58 (-$CH_2$-), 1.77 (-$CH_2$-), 2.32 (-$CH_2$-), 4.07 (-$CH_2$-O-), 4.12 (-$CH_2$-O-), 3.08 - 5.23 (m, H from

glucose), 6.81 (dd, 2H, *J*= 7.29, 2.11 Hz, 3', 5'-H), 7.22 (d, IH, *J*=8.57, 6-H), 7.41 (dd, 2H, *J*= 8.6, 2.1 Hz, 2', 6'-H), 8.08 (d, 1H, *J*= 9.0 Hz, 5-H), 8.41 (s, 1H, 2-H), 9.53 (s, 1H, 4'-OH). $^{13}$C NMR (DMSO-$d_6$) δ 14. 1 (-$CH_3$), 24.1 (-$CH_2$-), 25.0 (-$CH_2$-), 28.3 (-$CH_2$-), 33.5 (-$CH_2$-), 59.7 (-$CH_2$-O-), 61.7 (C-6"), 68.4 (-$CH_2$-O-), 70.2 (C-4"), 70.6 (C-2"), 73.0 (C-1"), 78.8 (C-3"), 81.8 (C-5"), 111.3 (C-8), 114.8 (C-6), 114.9 (C-3', 5'), 117.2 (C-10), 122.4 (C-1'), 123.1 (C-3), 126.8 (C-5), 130.0 (C-2', 6'), 155.1 (C2), 157.1 (C-9), 157.4 (C-4'), 162.1 (C-7), 172.9 (-C=O), 175.0 (C-4). MS (m/z) 559.6 $(M + H)^+$, 581.5 $(M + Na)^+$, 557.9 $(M - H)^-$. Anal. ($C_{29}H_{34}O_{11}$) for C, H. Cacld: 62.36, 6.14; found: 61.92, 6.17.

**Daidzein 7-ω-Hydroxyhexyl Ether (Analog 50)**

Analog 50 was synthesized according to the method described for analog 75 using 6-bromo-l-hexanol (3 ml, 22.93 mmol) as the alkylating agent. After reflux, the reaction mixture was refrigerated for 12 hours. Precipitates formed during refrigeration were collected and fractionated on a Sephadex LH-20 column in methanol. Fractions that contained pure product were pooled, dried and recrystallized from acetone to give 1.16 g of analog 50. Analyses: white amorphous powder; yield 16.4%; mp 177-178.5 °C; $^1$H NMR (DMSO-$d_6$) δ 1.35 (m, 2H, -$CH_2$-), 1.43 (m, 4H, -$CH_2$-$CH_2$-), 1.75 (m, 2H, -$CH_2$-), 3.40 (m, 2H, -$CH_2$-), 4.1 (t, -$CH_2$-O-), 6.81 (dd, 2H, *J*=8.7Hz, 1.6 Hz, H-3', H-5'), 7.04 (dd, 1H, *J*=8.86 Hz, 1.98 Hz, H-6), 7.10 (d, 1H, *J*=1.99, H-8), 7.40 (dd, 2H, *J*=8.7 Hz, 1.58 Hz, H-2', H-6'), 8.0 (d, 1H, *J*=8.86 Hz, H-5), 8.34 (s, 1H, H-2), 9.53 (s, 1H, OH-4'); $^{13}$C NMR δ 25.2 (-$CH_2$-), 25.3 (-$CH_2$-), 28.5 (-$CH_2$-), 32.5 (-$CH_2$-), 60.6 (-$CH_2$-O-), 68.4 (-O-$CH_2$-), 100.9 (C-8), 114.9 (C-6), 114.9 (C-3', C-5'), 117.5 (C-10), 122.4 (C-1'), 123.7 (C-3), 126.9 (C-5), 130.1 (C-2', C-6'), 153.1 (C-2), 157.2 (C-9), 157.4 (C-4'), 163.0 (C7), 174.7 (C-4); MS m/z 355.3 $(M+H)^+$. Elementary analysis ($C_{21}H_{22}O_5$). Cacld: C, 71.17; H, 6.26. Found: C, 70.82, H, 6.44.

**Daidzein 7-ω-Ethoxycarbonylbutyl Ether (Analog 53)**

To a solution of 5.1 g of daidzein (20.08 mmol), 8.0 mL of 2 *N* aq. KOH (16 mmol) and 50 mL of acetone was added 3.5 mL of ethyl-5-bromovalerate (22.1 mmol). The mixture was refluxed with gentle stirring for 72 hours and cooled and kept at 4 °c overnight. Precipitates were collected on a fritted funnel and fractionated on a Sephadex LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were

pooled, dried and recrystallized from acetone to give 670 mg of analog 53. Analyses: colorless crystalline plates; yield 8.8%; mp 146-148 °C; $^1$H NMR (DMSO-$d_6$) δ 1.17 [t, 3H, -$CH_3$], 1.69 (m, 2H, -$CH_2$-), 1.77(m, 2H, -$CH_2$-), 2.38 (t, 2H, -$CH_2$-), 4.05 (m, 2H, -$CH_2$-O-), 4.12 (m, 2H, -$CH_2$-O-), 6.81 (dd, 2H, *J*=8.67 Hz, 1.5 Hz, H-3', H-5'), 7.04 (dd, 1H, *J*=8.89 Hz, 2.1 Hz, H-6), 7.11 (d, 1H, *J*=2.1Hz, H-8), 7.39 (dd, 2H, *J*=8.7 Hz, 1.5 Hz, H-2', H-6'), 8.0(d, IH, *J*=8.89 Hz, H-5), 8.35 (s, 1H, H-2), 9.55 (s, 1H, OH-4'); $^{13}$C NMR δ 14.1 (-$CH_3$), 21.1 (-$CH_2$-), 27.7 (-$CH_2$-), 59.7 (-$CH_2$-O-), 68.1 (-$CH_2$-O-), 100.9 (C-8), 114.9 (C-6), 114.9 (C-3', C-5'), 117.5 (C-10), 122.4 (C-1'), 123.7 (C-3), 126.9 (C-5), 130.1 (C-2', C-6'), 153.1 (C-2), 157.3 (C-9), 157.4 (C-4'), 162.9 (C- 7), 172.7 (O=C-O-), 174.7 (C-4). MS m/z 383.3 $(M + 1)^+$. Elementary analysis ($C_{22}H_{22}O_6$). Cacld: C, 69.09; H, 5.80. Found: C, 68.60; H, 5.84.

**Daidzein 7-ω-Hydroxynonyl Ether (Analog 57)**

To a suspension of 5.1 g of daidzein (20.08 mmol) in 60 ml of acetone was added 15 ml of 2 *N* aq. KOH (30 mmol). The mixture was stirred at room temperature until daidzein was completely dissolved. Five g of 9-bromo-l-nonanol (22.41 mmol) was then added and the mixture was stirred under gentle reflux for 72 hours. Reaction mixture was cooled and kept at 4 °C overnight. Precipitates were collected by filtration and were fractionated on a Sephadex LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to yield 305 mg of analog 57. Analyses: colorless crystalline plates; yield 5.98%; mp 165-167 °C; $^1$H NMR (DMSO-$d_6$) δ 1.27 [m, 8H, (-$CH_2$-)$_4$], 1.40 (m, 4H, -$CH_2$-$CH_2$-), 1.73 (m, 2H, -$CH_2$-), 3.37 (m, 2H, -$CH_2$-), 4.09 (t, -$CH_2$-O-), 6.81 (d, 2H, *J*=8.75 Hz, 1.58 Hz, H-3', H-5'), 7.04 (dd, 1H, *J*=8.87 Hz, 2.1 Hz, H-6), 7.10 (d, 1H, *J*=2.1 Hz, H-8), 7.39 (d, 2H, *J*=8.75 Hz, 1.58 Hz, H-2', H-6'), 8.0 (d, 1H, *J*=8.86 Hz, H-5), 8.34 (s, 1H, H-2), 9.53 (s, 1H, OH-4'); $^{13}$C NMR 825.4 (-$CH_2$-), 25.5 (-$CH_2$-), 28.4 (-$CH_2$-), 28.7 (-$CH_2$-), 28.9 (-$CH_2$-), 29.0 (-$CH_2$-), 32.5 (-$CH_2$-), 60.7(-$CH_2$-O-), 68.4 (-O-$CH_2$-), 100.9 (C-8), 114.9 (C-6), 114.9 (C-3', C-5'), 117.5 (C-10), 122.4 (C-1'), 123.7 (C-3), 126.9 (C-5), 130.0 (C2', C-6'), 153.0 (C-2), 157.2 (C-9), 157.4 (C-4'), 163.0 (C-7), 174.7 (C-4); MS m/z 397.5 $(M + H)^+$, 419.4 $(M + Na)^+$, 435.4 $(M + K)^+$, 395.3 $(M-H)^-$. Elementary analyses ($C_{24}H_{28}O_5$). Cacld: C, 72.71, H, 7.12. Found: C, 73.11; H, 7.13.

**Daidzein 7-ω-Hydroxydodecyl Ether (Analog 63)**

Analog 63 was synthesized and purified according to the procedures described for analog 57 using 12-bromo-l-dodecanol (5 g, 18.85 mmol) as the alkylating agent. Analyses: white amorphous powder; yield 20.5%; mp 142-144 °C; $^1$H NMR (DMSO-$d_6$) δ 1.23 [m, 14H, (-$CH_2$-)$_7$], 1.37 (m, 4H, -$CH_2$-$CH_2$-), 1.73 (m, 2H, -$CH_2$-), 3.36 (m, 2H, $CH_2$-O-), 4.08 (t, -$CH_2$-O-), 6.81 (dd, 2H, *J*=8.69 Hz, 1.58 Hz, H-3', H-5'), 7.04 (dd, 1H, *J*=8.86 Hz, 1.9 Hz, H-6), 7.09 (d, IH, *J*=1.9 Hz, H-8), 7.40 (dd, 2H, *J*=8.69 Hz, 1.58 Hz, H-2', H-6'), 7.98 (d, 1H, *J*=8.86 Hz, H-5), 8.34 (s, 1H, H-2), 9.55 (s, 1H, OH-4'); $^{13}$C NMR δ 25.4 [(-$CH_2$-)$_2$], 25.5 (-$CH_2$-), 28.4 (-$CH_2$-), 28.7 (-$CH_2$-), 28.9 (-$CH_2$-), 29.1 (-$CH_2$-), 32.5 (-$CH_2$-), 60.7 (-$CH_2$-O-), 68.4 (-O-$CH_2$-), 100.9 (C-8), 114.8 (C-6), 114.8 (C3', C-5'), 117.5 (C-10), 122.4 (C-1'), 123.7 (C-3), 126.9 (C-5), 130.0 (C-2', C-6'), 153.0 (C-2), 157.2 (C-9), 157.4 (C-4'), 163.0 (C-7), 174.7 (C-4); MS m/z 439.6 $(M + H)^+$. Elementary analysis ($C_{27}H_{34}O_5$). Cacld: C, 73.95; H, 7.81. Found: C, 74.20; H, 7.61.

**Daidzein 7-(1"*H*-indole-3"-yl)-Ethyl Ether (Analog 73)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 7.7 g of $K_2CO_3$ (55.71mmol) and 5.0 g of 3-(2-bromoethyl)indole (22.31 mmol) successively. The mixture was stirred and heated at 80 °C for 4 hours and precipitated by pouring reaction mixture into 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 2.5 g of analog 73. Analyses: white crystalline product; yield, 49%; mp 203-205 °C. $^1$H NMR (DMSO-$d_6$) δ 4.35 (t, 2H, -O-$CH_2$-), 6.84 (d, 2H, *J*= 8.7 Hz, H-3', 5'), 7.01 (dd, 1H, *J*= 7.4 Hz, H-7"), 7.05 (dd, 1H, *J*= 8.9, 2.6 Hz, H-6), 7.10 (dd, 1H, *J*= 8.2, 2.1, H5"), 7.29 (d, 1H, *J*= 1.8 Hz, H-8), 7.38 (d, 1H, *J*= 8.0 Hz, H-4"), 7.41 (d, 2H, *J*= 8.7 Hz, H-2', 6'), 7.62 (d, 1H, *J=7.9* Hz, H-6"), 7.95 (s, 1H, H-2"), 8.01 (d, 1H, *J=8.9* Hz, H-5), 8.32 (s, 1H, H-2), 9.54 (s, 1H, OH-4'). $^{13}$C NMR (DMSO-$d_6$) δ 24.7 (-$CH_2$-), 68.9 (-$CH_2$-O-), 101.0 (C-8), 110.2 (C-7"), 111.4 (C-5"), 114.9 (C-3', 5'), 115.0 (C-6), 117.6 (C-10), 118.4 (C-3"), 121.0 (C-8"), 122.4 (C-1'), 123.3 (C-4"), 123.7 (C-3), 127.3 (C-5), 130.1 (C-2', 6'), 136.2 (C-2"), 153.0 (C-2), 153.0 (C-9"), 157.3 (C-9); 157.4 (C-4'), 162.9 (C-7), 174.4 (C-4). MS (m/z) 397.9 $(M)^+$. Elementary analyses ($C_{25}H_{19}O_4N$) for C, H, N: Cacld. 75.55, 4.82, 3.52; found 72.02, 5.60, 6.08.

**Daidzein 7-Hydroxyethyl Ether (Analog 75)**

To a suspension of 5.3 g of daidzein (20.87 mmol) in 60 ml of acetone was added with vigorous stirring 13 ml of 2 *N* aq. KOH (26 mmol). After daidzein was completely dissolved, 2 ml of 2-bromoethanol (28.21 mmol) was added and the reaction mixture was refluxed with gentle stirring for 72 hours. Volatile solvent was evaporated and residue was partitioned in water and ethyl acetate. Product in the ethyl acetate layer was further purified by a silica gel column in petroleum ether:ethyl acetate:methanol (6:3:1) followed by a Sephadex LH-20 column in methanol. Fractions that contained pure product were pooled, dried and recrystallized from acetone to give 1.7 g of analog 75. Analyses: white amorphous powder; yield 28.5%; mp 210-212 °C; MS m/z 299.1 $(M + H)^+$. Elementary analysis ($C_{17}H_{14}O_5$): Cacld: C, 68.45; H, 4.73. Found: C, 67.86; H, 4.23.

**Daidzein 7-[2,4-(1H, 3H)-quinazolinedione-3-N-yl]-ethoxyl ether (Analog 78)**

To the suspension of daidzein (5.1g, 20.06 mmol) and 50 ml of acetone was added 30 ml of 2N aq. KOH (60.0 mmol) and 5.0g of 3-(2-chloroethyl)-2, 4 (1*H*, 3*H*) quinazolinedione (22.26 mmol). The mixture was stirred at room temperature for 48h. Precipitates were filtered, dried and fractionated first on a silica gel column (chloroform-methanol, 9.25 : 0.75) followed by a Sephadex-LH-20 column (chloroform-methanol, 7:3). Fractions containing pure product were pooled, concentrated and recrystallized from acetone to yield 617 mg HPLC-pure product. Analyses: white crystals; yield, 12.9%; mp 270 °C (decompose). $^1$HNMR (DMSO-$d_6$) δ, 4.33-4.38 (m, 4H, -N-$CH_2$- $CH_2$-O-), 6.82 (dd, 2H, $J$ = 8.65, 3.2 Hz, 3', 5'-H), 7.03 (dd, 1H, $J$ = 9.01, 2.44 Hz, 6-H), 7.18 (d, 1H, $J$ = 7.27 Hz, 7"-H), 7.19 (d, 1H, 8-H), 7.21 (d, 1H, $J$ = 7.72 Hz, 8"-H), 7.38 (d, 2H, $J$ = 8.55 Hz, 2', 6'-H), 7.66 (t, 1H, $J$ = 7.43, 1.36, 9"-H), 7.94 (d, 1H, $J$ = 7.75, 6"-H), 7.99 (d, 1H, $J$ = 9.0 Hz, 5-H), 8.35 (s, 1H, 2-H). 9.58 (4'-OH). $^{13}$CNMR (DMSO-$d_6$) δ, 38.5 (-N-$CH_2$-), 65.0 (-$CH_2$-O-), 101.1 (C-8), 113.7( C-9"), 115.0( C-7"), 115.0 (C-3', 5'), 115.2(C-6), 117.8 (C-10), 122.3 ( C-5"), 122.6 (C-1'), 123.7 (C-3), 127.0 ( C-6"), 127.4 (C-5), 130.1 (C-2', 6'), 135.1 ( C-8"), 139.5 ( C-10"), 150.1 ( C-2"), 153.1 (C-2), 157.3 (C-9), 157.3 (C-4'), 162.1 (C-4"), 162.5 (C-7), 174.7 (C-4). Anal. ($C_{25}H_{18}O_6N_2$) for C, H, N. Cacld, 67.87, 4.10, 6.33; found, 64.60, 4.13, 6.40.

**Daidzein 7-(4", 6"-dimethoxy-1", 3", 5"-triazine)-2"-yl Ether (Analog 81) and daidzein 7,4'-di-(4", 6"-dimethoxy-1", 3", 5"-triazine)-2"-yl Ether (Analog 82)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 3.2 g of $K_2CO_3$ (23.1 mmol) and 5.0 g of 2-chloro-4,6-dimethoxy-1,3,5-triazine (28.5 mmol) successively. The mixture was stirred and heated at 80 °C for 4 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform: methanol (7:3). Fractions that contained pure products were pooled accordingly and concentrated and recrystallized from acetone to give 1.29 g analog 81 and 7.5 g analog 82. Analyses: analog 81, white crystals; yield, 25.3%; mp 226-228 °C. $^1$H NMR (DMSO-$d_6$) δ 3.91 (s, 6H, 2 X-$OCH_3$), 6.82 (dd, 2H, *J*= 8.7, 1.7 Hz, H-3', 5'), 7.41 (dd, 1H, *J*= 8.8, 1.8 Hz, H-6), 7.42 (d, 2H, *J*= 8.8, 1.8 Hz, H-2', 6'), 7.69 (d, 1H, *J*= 1.96 Hz, H-8), 8.18 (d, 1H, *J*=*8.8* Hz, H-5), 8.45 (s, 1H, H-2), 9.58 (s, 1H, OH-4'). $^{13}$C NMR (DMSO-$d_6$) 055.3 (-$OCH_3$), 55.4 (-$OCH_3$), 111.2 (C-8), 115.0 (C-3', 5'), 119.9 (C-10), 121.3 (C-6), 121.7(C-8), 122.0 (C-1'), 124.0 (C-3), 127.0 (C-5), 130.1 (C-2', 6'), 153.8 (C-2), 155.3 (C-6"), 156.1 (C-4"), 157.4 (C-9), 157.5 (C-4'), 172.2 (C-7), 173.3(C-2"), 174.8 (C-4). MS (m/z) 394.3 $(M + H)^+$, 416.6 $(M + Na)^+$, 432.4 $(M + K)^+$. Elementary analyses ($C_{20}H_{15}O_6N_3$) for C, H, N: Cacld. 61.07, 3.84, 10.68; found 60.90, 4.06, 10.76. Analog 82, white amorphous powder; yield, 70.2%; mp 265°C (decompose). $^1$H NMR, $^{13}$C NMR and MS similar to analog 81. Elementary analyses ($C_{25}H_{20}O_8N_6$) for C, H, N: Cacld. 56.39, 3.79, 15.78; found 57.06, 3.99, 15.71.

**Daidzein 7-N-(2"(3"H)-Benzothiazolonyl)-Methyl Ether (Analog 83)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 3.0 g of $K_2CO_3$ (21.7 mmol) and 5.0 g of3-chloromethyl-2(3H)-benzothiazolone (25.0 mmol) successively. The mixture was stirred and heated at 80 °C for 14 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 5.02 g of analog 83. Analyses: colorless crystals; yield, 98.4%; mp 234-235 °C. $^1$H NMR (DMSO-$d_6$) δ 6.1 (s, 2H, -O-$CH_2$-), 6.82 (d, 2H, *J*= 8.7, 2.5 Hz, H-3', 5'), 7.15 (dd, 1H, *J* = 8.9, 1.9 Hz, H-6), 7.27 (t, 1H, *J*= 7.34, 7.75 Hz, H-7"), 7.41 (d, 2H, *J*= 8.7 Hz, H-2', 6'), 7.42 (d, 1H, *J*= 8.8 Hz, H-4"), 7.43 (d, 1H, *J*= 2.7 Hz, H-8), 7.50 (d, 1H, *J*=8.2 Hz, H-5"), 7.70 (d, 1H, *J*= 7.82 Hz,

H-6"), 8.04 (d, 1H, $J$= 8.9 Hz, H-5), 8.39 (s, 1H, H-2). $^{13}$C NMR (DMSO-$d_6$) δ 69.5 (-$CH_2$-O-), 102.6 (C-8), 112.0 (C-1"), 115.0 (C-3', 5'),115.3 (C-6), 118.6 (C-10), 121.1 (C-4"), 122.2 (C-1'), 123.1 (C-5"), 123.8 (C-3), 124.2 (C-6"), 126.9 (C-9"), 127.3 (C-5), 130.1 (C-2', 6'), 135.6 (C-8"), 153.3 (C-2), 157.0 (C-9), 157.3 (C-4'), 160.0 (C-7), 169.6 (-S-C=O),174.4 (C-4). MS (m/z) 440.4 $(M + Na)^+$, 416.8 $(M-H)^-$. Elementary analyses ($C_{23}H_{15}O_5NS$) for C, H, N, S: Cacld.66.18, 3.62, 3.36, 7.68; found 65.80, 3.6, 3.42, 7.52.

**Daidzein 4'-(1 "-Phenyl-1 "*H*-Tetrazole-5"-yl) Ether (Analog 86)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 3.0 g of $K_2CO_3$ (21.74 mmol) and 3.6 g of 5-chloro-1-phenyl-1H-tetrazole (20.0 mmol) successively. The mixture was stirred and heated at 80 °C for 4 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 4.4 g of analog 86. Analyses: colorless crystals; yield, 86.3%; mp 265-267 °C. $^1$H NMR (DMSO-$d_6$) δ 6.89 (d, 1H, $J$= 2.2 Hz, H-8), 6.96 (d, 2H, $J$= 8.8, 2.1 Hz, H-6), 7.58 (dd, 2H, $J$=9.31, 1.64Hz, H-6", 10"), 7.61 (d, 1H, $J$= 7.4Hz, H-8"), 7.67 (dd, 2H, $J$= 7.8, H 7", 9"), 7.70 (dd, 2H, $J$= 8.7, 1.6 Hz, H-3', 5'), 7.87 (d, 2H, $J$= 7.83 Hz, H-2', 6'), 7.99 (d, 1H, $J$= 8.8 Hz, H-5), 8.47 (s, 1H, H-2), 10.8 (s, 1H, OH-7). $^{13}$C NMR (DMSO-$d_6$) δ 102.2 (C-8), 115.4 (C-6), 116.5 (C-10), 119.7 (C-1'), 122.5 (C-3), 123.2 (C-2', 6'), 127.3 (C-5), 129.9 (C-3', 5'), 129.9 (C-6", 10"), 130.4 (C-8"), 130.5 (C-7", 9"), 132.6 (C-11 "), 152.9 (C-2), 154.1 (C-5"), 157.5 (C-9), 159.6 (C-4'), 162.8 (C-7), 174.3 (C-4). MS (m/z) 421.3 $(M + Na)^+$, 437.2 $(M + K)^+$, 397.2 $(M - H)^-$. Elementary analyses ($C_{22}H_{14}O_4N_4$) for C, H, N. Cacld. 66.32, 3.54, 14.06; found 65.67, 3.62, 14.12.

**Daidzein 7-(6"-Chloropiperonyl) Ether (Analog 87)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 2.7 g of $K_2CO_3$ (19.6 mmol) and 5.0 g of 6-chloropiperonylchloride (24.4 mmol) successively. The mixture was stirred and heated at 80 °C for 15 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 6.4 g of analog 87. Analyses: colorless crystals; yield, 75.5%; mp 254-256 °C. $^1$H NMR (DMSO-$d_6$) δ 5.18 (-O-$CH_2$-), 6.10 (s, 2H, -

O-$CH_2$-O-), 6.81 (d, 2H, *J*= 8.6,1.6 Hz, H-3', 5'), 7.13 (dd, 1H, *J*= 8.9, 2.6 Hz, H-6), 7.16 (s, 1H, H-5"), 7.23 (s, 1H, H-2"), 7.27 (d, 1H, *J*= 2.0 Hz, H-8), 7.40 (d, 2H, *J*= 8.7 Hz, H-2', 6'), 8.03 (d, 1H, *J*= 8.9 Hz, H-5), 8.37 (s, 1H, H-2), 9.47 (s, 1H, 4'-OH). $^{13}$C NMR (DMSO-$d_6$) δ 67.7 (-$CH_2$-O-), 101.5 (C-8), 102.3 (-O-$CH_2$-O-), 109.8 (C-2"), 110.3 (C-5"), 115.0 (C-3', 5'), 115.0 (C-6), 117.9 (C-10), 122.3 (C-1'), 123.7 (C-3), 125.4 (C-1"), 127.0 (C-5), 130.0 (C-2', 6'), 146.7 (C-3"), 148.4 (C4"), 153.2 (C-2), 153.2 (C-2"), 157.2 (C-9), 157.3 (C-4'), 162.4 (C-7), 174.4 (C-4). MS (m/z) 423.2 $(M + H)^+$, 445.3 $(M + Na)^+$, 461.3 $(M + K)^+$. Elementary analyses ($C_{23}H_{15}O_6Cl$): Cacld C, 65.34; H, 3.58. Found: C, 64.31; H, 3.61.

**Daidzein 7-ω-(3"-Chlorophenylpiperozine)-4-N-Propyl Ether (Analog 88)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 3.0 g of $K_2CO_3$ (21.74 mmol) and 6.2 g of 1-(3-chlorophenyl)-4-(3-chloropropyl) piperazine monohydrochloride (20.02 mmol) successively. The mixture was stirred and heated at 80 °C for 4 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform: methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 3.48 g of analog 88. Analyses: pure white amorphous powder; yield, 68.2%; mp 183-185 °C. $^1$H NMR (DMSO-$d_6$) δ 1.94 (-$CH_2$-), 2.48 (-$CH_2$N-), 2.50 (H-b, d), 3.15 (H-a, c), 4.16 (-O-CH2-), 6.76 (dd, 1H, *J*= 7.81, 1.4 Hz, H-4"), 6.83 (d, 2H, *J*=8.6, 1.6 Hz, H-3', 5'),6.87 (dd, 1H, *J*= 8.5, 2.0 Hz, H-6"), 6.91 (d, IH, *J* = 1.85 Hz, H-2"), 7.05 (dd, 1H, *J* = 8.9, 1.97 Hz, H-6), 7.11 (d, 1H, *J* = 2.5 Hz, H-8), 7.19 (t, 1H, *J*= 8.4 Hz, H-5"), 7.39 (d, 2H, *J*= 8.7, 1.6 Hz, H-2', 6'), 8.01 (d, 1H, *J*= 8.9 Hz, H-5), 8.34 (s, 1H, H-2), 9.55 (s, 1H, OH-4'). $^{13}$C NMR (DMSO-$d_6$) 025.9 (-$CH_2$-), 47.6 (C-b, d), 52.6 (C-c), 54.2 (C-a), 66.8 (-$CH_2$-O-), 101.0 (C-8), 113.6 (C-2"), 114.4 (C-6"), 114.9 (C-6), 115.0 (C-3', 5'), 117.5 (C-10), 117.9 (C-4"), 122.4 (C-1'), 123.7 (C3),126.9 (C-5), 130.1 (C-2', 6'),130.4 (C-5"), 133.8 (C-1"), 152.2 (C-3"), 153.1 (C-2), 157.2 (C-9), 157.4 (C-4'), 163.0 (C-7), 174.4 (C-4). MS (m/z) 491.3 $(M + H)^+$. Elementary analyses ($C_{28}H_{27}O_4N_2Cl$): Cacld. C, 68.5; H, 5.54. Found: C, 65.63; H, 5.54.

**Daidzein 7-(2"-Ethoxycarbonylfurfuryl) Ether (Analog 89)**

To a solution of 5.1 g of daidzein (20.08 mmol) in 60 ml of DMF was added 2.95 g of $K_2CO_3$ (21.38 mmol) and 5.0 g of ethyl5-(chloromethyl)-2-furancarboxylate (26.51

mmol) successively. The mixture was stirred and heated at 80 °C for 1.5 hours and products were precipitated in 200 ml of ice water. Precipitates were collected by filtration and fractionated on a Sephadex-LH-20 column in chloroform:methanol (7:3). Fractions that contained pure product were pooled, concentrated and recrystallized from acetone to give 5.89 g of analog 89. Analyses: white amorphous powder; yield, 72.3%; mp 208-210 °C. $^1$H NMR (DMSO-$d_6$) δ 1.27 (-$CH_3$), 4.28 (-$CH_2$-O-), 5.34 (-O-$CH_2$-), 6.81 (d, 2H, *J*= 8.7, 1.68 Hz, H-3', 5'), 6.87 (d, 1H, *J*= 3.2 Hz, H-8), 7.15 (dd, 1H, *J*= 8.9, 2.7 Hz, H-6), 7.31 (d, 1H, *J*=7.5 Hz, H-4"), 7.32 (d, 1H, *J*= 6.8 Hz, H-3"), 7.40 (dd, 2H, *J*=8.2, 1.8 Hz, H-2', 6'), 8.03 (d, 1H, *J*= 8.9 Hz, H-5), 8.38 (s, 1H, H-2), 9.55 (s, 1H, OH-4'). $^{13}$C NMR (DMSO-$d_6$) δ 14.1 (-$CH_3$), 60.8 (-$CH_2$-O-), 62.1 (-$CH_2$-O-), 101.6 (C8), 113.1 (C-4"), 115.0 (C-6), 115.0 (C-3', 5'), 118.1 (C-10), 118.9 (C-3"), 122.3 (C-1'), 123.8 (C-3), 127.1 (C-5), 130.1 (C-2', 6'), 144.4 (C-2"), 153.2 (C-5"), 153.4 (C-2), 157.2 (C-9), 157.3 (C-4'), 157.8 (-C = 0), 161.9 (C-7), 174.8 (C-4). MS (m/z) 407.5 $(M + H)^+$, 429.4 $(M + Na)^+$, 445.4 $(M + K)^+$. Elementary analyses ($C_{23}H_{18}O_7$) for C, H: Cacld. 67.98, 4.46; found 67.30, 4.62.

**Daidzein 7-(1, 8-naphthalimide-N-yl)-ethoxyl ether Analog (90)**

Daidzein (5.1g, 20.06 mmol) was dissolved in 60 ml of DMF, and 3.0 g of $K_2CO_3$ (21.74 mmol) and 5.0g of 1-(chloromethyl)-1*H*-benzotriazole (19.25 mmol) were added successively. The mixture was stirred at 80°C for 4h and then poured into 200 ml ice water. Precipitates were collected, dried, and purified on Sephadex-LH-20 column (chloroform:methanol/7:3). Fractions containing product were pooled, concentrated and recrystallized from acetone to give 562 mg crystalline product. Analyses: yield, 11.0% w/w; mp 278-280 °C. $^1$HNMR (DMSO-$d_6$) δ, 4.42 (t, 2H, -N-$CH_2$-), 4.49 (t, 2H, -$CH_2$-O-), 6.82 (dd, 2H, *J* = 8.73, 2.57 Hz, 3', 5'-H), 7.01(dd, 1H, *J* = 8.86, 2.57 Hz, 6-H), 7.18 (d, 1H, *J* = 1.98 Hz, 8-H), 7.37 (dd, 2H, *J* = 6.4, 1.71 Hz, 2', 6'-H), 7.85 (t, 2H, *J* = 8.01, 7.60 Hz, 3", 6"-H), 7.95 (d, 1H, *J* = 8.91 Hz, 5-H), 8.32 (s, 1H, 2-H), 8.42 (d, 2H, *J* = 7.91 Hz, 4", 5"-H), 8.49 (d, 2H, *J* = 6.86 Hz, 2", 7"-H), 9.55 (s, 1H, 4'-OH). $^{13}$CNMR (DMSO-$d_6$) δ, 38.1 (-N-$CH_2$-), 65.1 (-$CH_2$-O-), 101.0 (C-8), 114.95 (C-6), 115.0 (C-3', 5'), 117.7 (C-10), 121.9 (C-1", 8"), 122.3 (C-1'), 123.7 (C-3), 126.96 (C-9"), 127.2 (C-5), 127.4 (C-3", 6"), 130.1 (C-2', 6'), 130.9 (C-4",5"), 131.3 (C-10"), 134.5 (C-2", 7" ), 153.1 (C-2), 157.2 (C-9), 157.3 (C-4'), 162.4 (C-7), 163.5 (N-C=O),174.6 (C-4). MS (m/z), 478.1 $(M + H)^+$, 516 $(M + K)^+$. Anal. ($C_{29}H_{19}O_6N$) for C, H, N. Cacld, 72.95, 4.01, 2.93, found, 71.35, 4.18, 2.85.

**Daidzein 7-(2"-Carboxyfurfuryl) Ether (Analog 99)**

The compound was prepared by hydrolysis of analog 89. To a solution of 1.0 g of analog 89 in 20 ml of methanol was added 2 ml of 2 *N* aq. KOH and 10 ml of water. The mixture was stirred and gently refluxed for 2 hours until all reactants were hydrolyzed (monitored by TLC). The reaction mixture was concentrated by flash evaporation and products were precipitated by adjusting the pH to 3-4. Precipitates were collected by filtration and purified by recrystallization from acetone to give 730 mg of crystalline analog 99. Analyses: yield, 73%. MS (m/z) 379.2 $(M + H)^+$, 401.2 $(M + Na)^+$, 417.3 $(M + K)^+$, 377.3 $(M-H)^-$. Elementary analyses ($C_{21}H_{14}O_7$) for C, H: Cacld 66.67, 3.73; found 65.78, 3.84.

## EXAMPLE V

### MAO and ALDH-2 Assays

Several of the synthesized compounds were tested for MAO and ALDH-2 suppression activity. The membrane and supernatant fractions of the lysate of a gradient purified hamster liver mitochondria preparation were used as sources of MAO and ALDH-2, respectively. ALDH-2 and MAO activity assays were performed according to established procedures (Rooke et al. (2000) *J. Med. Chem.* 43:4169).

## EXAMPLE VI

### Ethanol Drinking Experiments

Effects of the newly synthesized analogs on hamster ethanol intake were determined according to the method described below. Animals were male adult golden hamsters (outbred, Lakeview Lak: LVG[SYR]), purchased from Charles River Laboratories, Wilmington, MA 01887 or Harlan Sprague Dawley, Inc., Indianapolis, IN 46229. Upon arrival, hamsters were housed in a room maintained at 23°C, 35-45% humidity, with a 12/12 hr light/dark cycle (light on 0600-1800 hr), *ad libitum* access to Purina Chow (5001) and a 15% v/v ethanol solution. After a week, each hamster was transferred to an individual stainless steel cage (26 x 18 x 17.5 cm). Two 50 ml drinking bottles, one containing water and the other a 15% ethanol solution, were provided continuously. The bottles were placed in holders equipped with tilted platforms which direct spillage to tubes placed outside of the cages. The positions of the two drinking bottles on each cage were altered daily to prevent

development of positional preference. Fluid intake was measured at 0900hr each day. Hamsters that drank significant (>8 ml/day) and consistent (daily variance < ±20%) amounts of ethanol solution were selected for testing. To establish baseline ethanol and water intake, 1 ml sterile saline was administered daily to each hamster between 1500-1600 hr for 6 days (saline control period, Day 1 to Day 6). A daily dose of 0.07 mmol of test compound was then administered for 5 consecutive days (treatment period, Day 7 to Day 11). After the last dose, ethanol and water intake were monitored for another 6 days (post treatment period, Day 12 to Day 17). The results demonstrated that these analogs, at a dose of 0.07 mmol/day/hamster, suppress hamster ethanol intake. Among the 17 active analogs tested, 5 of them (Analogs 81, 87, 88, 89, and 99) were more potent than daidzin. At an equivalent dose, daidzin suppressed ethanol intake by about 55% (Table 1).

**Table 1: Ethanol Intake Suppressive Activity of Analogs of Daidzin**

| Daidzin & Analog | EtOH intake suppression, % |
|---|---|
| 42 (Daidzin) | 55±12 |
| 40 (Daidzein) | 22±5 |
| Hexzein | 69±12 |
| Hepzein | 69±8 |
| Deczein | 84±5 |
| Undeczein | 86±7 |
| 50 | 46±7 |
| 53 | 49±8 |
| 57 | 57±8 |
| 63 | 55±10 |
| 6 | 54±4 |
| 13 | 58±5 |
| 73 | 44±6 |
| 75 (half dose) | 36±12 |
| 75 | 53±4 |
| 14 | 37±3 |
| 81 | 91±2 |
| 83 | 44±8 |
| 86 | 50±4 |
| 87 | 73±5 |
| 88 | 78±9 |
| 89 | 83±11 |
| 99 | 81±9 |
| 26 | 32±16 |
| 33 | 49±7 |

## EXAMPLE VII

### Structure-Activity Relationship (SAR)

The effects of substitutions at the 2, 3', 4', 5, 6 and 8 positions of an isoflavone on the potencies for ALDH-2 and MAO inhibition were evaluated and results are set forth below.

Substitution of 4'-OH

Nine 4', 7-*O*-disubstituted analogs of daidzin were synthesized, and their ALDH-2 and MAO inhibitory activities were tested and compared with those of their respective 7-*O*-mono-substituted analogs. While all the mono-substituted analogs inhibited ALDH-2 ($IC_{50}$= 0.08 to 9 μM), none of the di-substituted analogs were inhibitory (Gao et al. (2001) *J Med. Chem.* 44:3328). Among the mono-substituted analogs studied, three inhibited MAO and di-substitution abolished this activity as well. This indicated that either a free 4'-hydroxyl and/or a smaller 4'-substituent imparted antidipsotropic activity.

To further evaluate the SAR of 4'-substitution, two series of 7-*O*-substituted analogs were synthesized: the 7-*O*-substituted 4'-hydroxyisoflavones (analogs 2, 4, 6, 8, 11, and 13) and the 7-*O*-substituted isoflavones (analogs 3, 5, 7, 9, 12, and 14). All members of the 7-*O*-substituted 4'-hydroxyisoflavone series were potent ALDH-2 inhibitors, with $IC_{50}$ values ranging from 0.04 μM to 0.28 μM. While the 7-*O*-substituted isoflavone derivatives also inhibited ALDH-2, they were less potent than their 4'-hydroxy counterparts, with $IC_{50}$ values ranging from 0.1 μM to 1.5 μM. In all cases, the 4'-OH/4'H substitutions decreased potencies for ALDH-2 inhibition, raising $IC_{50}$ values from 2- (analog 6 vs. analog 7) to 19-fold (analog 4 vs. analog 5). The effect of 4'-OH/4'H substitution on MAO inhibition, however, was more dramatic. While all members of the 4' hydroxyl derivatives (analogs 2, 4, 6, 8, 11 and 13) inhibited MAO, none of the 4'-H series (analogs 3, 5, 7, 9, 12 and 14) had any effect on MAO. The fact that the effect of 4'-OH/4'-H substitution was independent of the nature of the 7-*O*-substituents indicated that the 4'-hydroxyl can influence MAO inhibition.

While not intending to be bound by theory, replacing the 4'-OH with an H can serve to decrease the polarity and hydrogen bonding ability of an isoflavone molecule. Moreover, it can induce redistribution of electron density on the B-ring. These factors, alone or combined, can contribute to decreased ALDH-2 and/or MAO inhibition. To explore the effects of polarity, hydrogen bonding ability, and the electron donating and withdrawing

property of the 4'-substituent on the potencies for MAO and ALDH-2 inhibition, two series of 4'-substituted analogs were synthesized and studied: the 7-*O*-ω-ethoxycarbonylpentyl- and the 7-hydroxy-isoflavones. Among the 7-*O*-ω-ethoxycarbonylpentylisoflavones, the 4'-$NH_2$ (analog 33), 4'-OH (analog 6), and 4'-H (analog 7) were more potent ALDH-2 inhibitors than the 4'-F (analog 24), 4'-Br (analog 26), and 4'-$CH_3$ (analog 30) derivatives. The 4'-$NO_2$ derivative (analog 28) was not inhibitory. This indicated that polarity, hydrogen bonding ability and electron withdrawing and releasing properties of the 4'-substituent affect potency for ALDH-2 inhibition. The most potent inhibitors were those that had polar (low hydrophobic constants), electron releasing, and hydrogen bond forming 4'-substituents such as analog 33 (4'-$NH_2$) and analog 6 (4'-OH). The 4'-$CH_3$ (analog 30) and 4'-Br derivatives (analog 26) were highly lipophilic having π values of 0.56 and 0.86 (Table 2), respectively, and were therefore, much less potent ALDH-2 inhibitors.

**Table 2. Hydrophobic Constants of, and Changes in Chemical Shifts Induced by Various 4'-Substituted-7-*O*-ω-Ethoxycarbonylpentylisoflavones**

| Analog | 4'-Substituent | π[b] | $^{13}$C-NMR[a] 1' | $^{13}$C-NMR[a] 2', 6' | $^{13}$C-NMR[a] 3', 5' | $^{13}$C-NMR[a] 4' |
|---|---|---|---|---|---|---|
| 4b | H | 0.00 | 0 (126.9) | 0 (128.9) | 0 (128.1) | 0 (131.9) |
| 2b[c] | H | 0.00 | 0.1 | -0.1 | 0 | -0.2 |
| 11b | F | 0.14 | *0* | *2.0* | *-13.1* | *28.9* |
| 12b | Br | 0.86 | 0 | 2.0 | 2.9 | -0.7 |
| 13b | $NO_2$ | -0.28 | 12.2 | -5.7 | 1.8 | 14.9 |
| 14b | $CH_3$ | 0.56 | -3.3 | -0.2 | 0.6 | 5.2 |
| 4a | OH | -0.67 | *-4.5* | *1.1* | *-13.2* | *25.5* |
| 2a[c] | OH | -0.67 | *-4.5* | *0.8* | *-13.0* | *25.6* |
| 16b | $NH_2$ | -1.23 | *-8.1* | *0.6* | *-14.7* | *16.6* |

[a]4'-Substituents-induced chemical shifts, relative to those of analog 7. Actual δ values (ppm) for the analog 7 B-ring $^{13}$C signals are shown in parentheses. [b]Hydrophobic constants, taken from Fujita et al. ((1964) *J. Am. Chem. Soc.* 86:5175). [c]Analogs 2 and analog 3 are 4'-substituted-7-*O*-methylisoflavones.

Despite the presence of the 1'-benzopyran function, electron density distributed rather evenly on the six carbons of the B-ring of analog 7 and analog 3 as indicated by the $^{13}$C-NMR data (Table 2). Substitution of 4'-H changed electron density distribution. Interestingly, 4'-substitutions that increased the electron densities at the C-1', C-3' and C5' (shifting the $^{13}$C-1', $^{13}$C-3' and $^{13}$C-5' signals up field) and decreased the electron densities at the C-4' (shifting the

$^{13}$C-4' signal down field) had increased potencies for ALDH-2 inhibition (analog 2, analog 6, analog 24, analog 33), whereas those that only decreased electron density at the C-4' had decreased potencies for ALDH-2 inhibition (analog 28, analog 30).

All members of the 4'-substituted 7-hydroxyl derivatives inhibited MAO, with the 4'-$NO_2$ (analog 27) being most potent ($IC_{50}$=0.1μm), followed by 4'-F (analog 23) (1.5 μm), 4'-Br (analog 25) (1.0 μm), 4'-H (1) (8.6 μm), 4'-$CH_3$ (analog 29) (9 μm), 4'-OH (analog 40) (12 μm), and 4'-$NH_2$-7-hydroxyisoflavone (analog 32) (12 μm). This indicated that potencies for MAO were linked positively to the electron withdrawing properties of the 4'-substituents: the analog that had the strongest electron withdrawing 4' -substituent ($NO_2$) also inhibited MAO most potently ($IC_{50}$ = 0.1 μM), whereas that with the strongest electron donating 4'-substituent ($NH_2$) was least inhibitory ($IC_{50}$ > 9 μm).

Substitution of 8-H

To evaluate the effect of C-8 substituents on ALDH-2 and MAO inhibition, two 8-C-glucosylated 7-*O*-substituted analogs were synthesized: 7-ethoxycarbonylpentoxy-puerarin (analog 36), 7-*O*-phthalimide-N-butylpuerarin (analog 17). One 8-methoxylated 7-*O*-substituted analog was synthesized: 7,8-dimethoxyisoflavone (analog 34). Their potencies for ALDH-2 and MAO inhibition were determined and compared with their 8-H substituted counterparts, 7-ethoxycarbonyl-pentoxydaidzein (analog 6), 2-carboxy-7-*O*-phthalimide-N-butyldaidzein (analog 13), and 7-methoxy-isoflavone (analog 3), respectively. Results indicated that replacing the 8-H with either a glucosyl (analog 36 and analog 17) or a methoxy function (analog 34) (i) completely abolished ALDH-2 inhibitory activities, and (ii) substantially decreased potencies for MAO inhibition.

Substitutions at 5, 2, 6, and 3' Positions

The 5-hydroxyl substituted 7-methoxydaidzein (analog 2 vs. analog 45) had decreased potency for ALDH-2 inhibition. This, together with the finding that hydroxyl substitution at the 5 position increased potency for MAO inhibition (analog 40 vs. analog 43, analog 2 vs. analog 45, analog 41 vs. analog 46), indicated that a 5-OH function can contribute negatively to antidipsotropic activity.

One 2-methyl derivative (analog 39), one 2-carboxy derivative (analog 16), and two 2-ethoxycarbonyl derivatives (analog 15, analog 19) were prepared, and their potencies for

ALDH-2 inhibition were determined and compared with their respective non-substituted counterparts, analogs analog 10, analog 14, and analog 18. While analog 10, analog 14, and analog 18 inhibited ALDH-2 fairly potently, with $IC_{50}$ values ranging from 8 μM to 0.14 μM, none of their 2-substituted derivatives exhibited inhibitory activity toward ALDH-2. Three additional 2-substituted analogs, analog 20, analog 21 and analog 22, were also tested and shown to have no effect on ALDH-2 activity. Without wishing to be bound by theory, these results indicated that the 2 position of daidzin or its active analogs occupies an important area on the enzyme binding site.

A series of daidzin analogs having 4'-OR groups replaced with H (analogs 3, 5, 7, 9, 10, 12, 14, and 19), F (analog 23, 24), Br (analog 25, 26), $CH_3$ (analog 29,30), $NH_2$ (analog 32, 33), $NO_2$ (analog 27, 28), or $OCH_3$ (analog 41) substituents were prepared. Their potencies for ALDH-2 and MAO inhibition were determined and compared among each other and with those of their respective 7-*O*-substituted derivatives of daidzein. Results revealed that the potency for ALDH-2 inhibition of a daidzin analog was associated with a polar 4'-substituent with electron donating and hydrogen bonding ability such as -$NH_2$ and -OH, whereas that for MAO inhibition was positively linked to the electron withdrawing property of the 4'-substituent. In ALDH-2, the 2, 3', 5, 6 and 8 positions of a bound inhibitor were located in relatively restricted areas and attempts to replace the -H atom on any of these positions resulted in complete loss of ALDH-2 inhibitory activity. Thus, a potent antidipsotropic analog of daidzin is a 4', 7-disubstituted isoflavone. In one embodiment, a 4'-substituent is small, polar, can release one or more electrons, and/or can bind hydrogen. In another embodiment, a 4'-substituent is -OH or $NH_2$. In yet another embodiment, a 7-substituent is a straight-chain alkyl optionally having a terminal polar function. In yet another embodiment, a 7-substituent is -$(CH_2)_n$-OH, $(CH_2)_n$-COOH or -$(CH_2)_n$-$NH_2$, wherein $2 \leq n \leq 6$, $5 \leq n \leq 10$, $n \geq 4$, respectively.

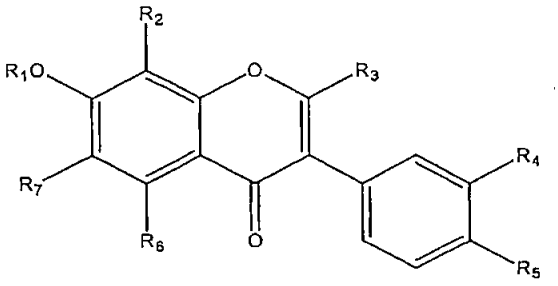
In one embodiment, an antidipsotropic compound does not inhibit MAO activity. In another embodiment, an antidipsotropic compound partially inhibits MAO activity. As used herein, the term "partially inhibits" refers to an inhibition of 75%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or less of the wild-type activity.

## OTHER EMBODIEMNTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims. All publications and patent applications cited above are incorporated by reference herein in their entirety for all purposes to the same extent as if each individual publication or patent application were specifically and individually indicated to be so incorporated by reference.

What is claimed is:

1. A method for inhibiting ALDH-2 comprising contacting ALDH-2 with a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched $(C_1-C_6)$haloalkyl, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_3-C_6)$cycloalkylcarbonyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, and heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1-C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

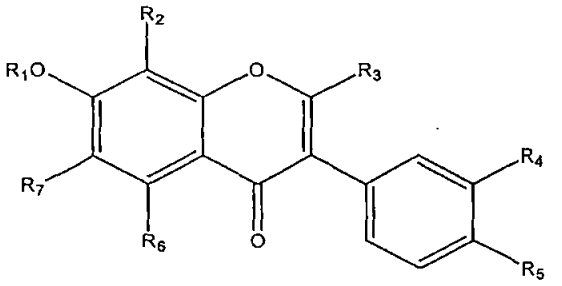
$R_5$ is selected from the group consisting of hydrogen, carboxy, hydroxy, halo, branched or unbranched $(C_1-C_6)$alkyl, $(C_1-C_6)$haloalkyl, $(C_2-C_6)$alkenyl, $(C_3-C_6)$alkadienyl, $(C_1-C_6)$alkoxy, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_2-C_6)$alkynyloxy, $(C_1-C_6)$alkoxy$(C_1-C_6)$alkyl, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_1-C_6)$alkylcarbonyl, $(C_3C_6)$cycloalkylcarbonyl, $(C_1-C_6)$alkoxycarbonyl, $(C_4-C_6)$alkoxycarbonylalkyl, $(C_1C_6)$hydroxyalkyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from

one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy.

2. A method for inhibiting ALDH-2 comprising contacting ALDH-2 with a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyc1yl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy.

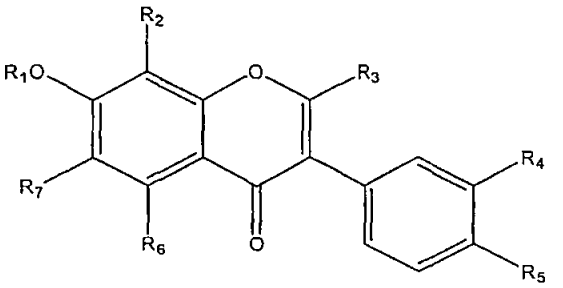
3. The method of claim 1, wherein $R_5$ is OH or $NH_2$.

4. The method of claim 2, wherein $R_1$ is a straight chain alkyl.

5. The method of claim 4, wherein the straight chain alkyl is selected from the group consisting of -$(CH_2)_n$-OH with $2 \leq n \leq 6$, $(CH_2)_n$-COOH with $5 \leq n \leq 10$, and -$(CH_2)_n$-$NH_2$, with $n \geq 4$.

6. The method of claim 1, wherein the ALDH-2 is human ALDH-2.

7. A method of modulating alcohol consumption in mammal comprising administering a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched $(C_1-C_6)$haloalkyl, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_3-C_6)$cycloalkylcarbonyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, and heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1-C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, hydroxy, halo, branched or unbranched $(C_1-C_6)$alkyl, $(C_1-C_6)$haloalkyl, $(C_2-C_6)$alkenyl, $(C_3-C_6)$alkadienyl, $(C_1-C_6)$alkoxy, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_2-C_6)$alkynyloxy, $(C_1-C_6)$alkoxy$(C_1-C_6)$alkyl, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_1-C_6)$alkylcarbonyl, $(C_3C_6)$cycloalkylcarbonyl, $(C_1-C_6)$alkoxycarbonyl, $(C_4-C_6)$alkoxycarbonylalkyl, $(C_1C_6)$hydroxyalkyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino,

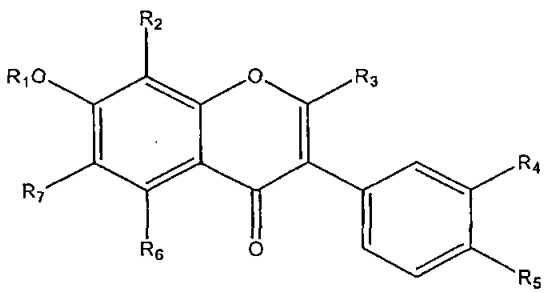
$(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1-C_6$ alkoxy,

in an amount effective to increase a concentration of an aldehyde formed during catabolism of a neurotransmitter.

8. A method of modulating alcohol consumption in mammal comprising administering a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched $(C_1-C_6)$alkyl, $(C_1-C_6)$haloalkyl, $(C_2-C_6)$alkenyl, $(C_3-C_6)$alkadienyl, $(C_1-C_6)$alkoxy, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_2-C_6)$alkynyloxy, $(C_1-C_6)$alkoxy$(C_1-C_6)$alkyl, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_1-C_6)$alkylcarbonyl, $(C_3-C_6)$cycloalkylcarbonyl, $(C_1-C_6)$alkoxycarbonyl, $(C_4-C_6)$alkoxycarbonylalkyl, $(C_1-C_6)$hydroxyalkyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1-C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy,

in an amount effective to increase a concentration of an aldehyde formed during catabolism of a neurotransmitter.

9. The method of claim 7, wherein the mammal is a human.

10. The method of claim 7, wherein the neurotransmitter is serotonin or dopamine.

11. The method of claim 7, wherein the aldehyde is 5-hydroxyindoleacetaldehyde or 3,4-dihydroxyphenylacetaldehyde.

12. The method of claim 7, wherein the compound does not inhibit monoamine oxidase.

13. The method of claim 7, wherein $R_5$ is OH or $NH_2$.

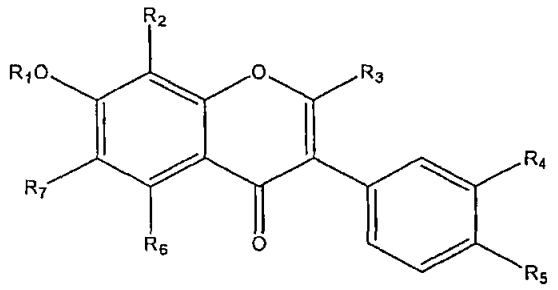
14. The method of claim 8, wherein $R_1$ is a straight chain alkyl.

15. The method of claim 14, wherein the straight chain alkyl is selected from the group consisting of $-(CH_2)_n-OH$ with $2 \leq n \leq 6$, $(CH_2)_n-COOH$ with $5 \leq n \leq 10$, and $-(CH_2)_n-NH_2$, with $n \geq 4$.

16. The method of claim 7, wherein the compound is administered intraperitoneally, intramuscularly or orally.

17. A method for identifying a compound that modulates ALDH-2 comprising the steps of:

i) providing a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched $(C_1-C_6)$haloalkyl, $(C_3-C_6)$cycloalkoxy, $(C_1-C_6)$haloalkoxy, $(C_3-C_6)$cyclohaloalkoxy, $(C_3-C_6)$cycloalkoxyalkyl, $(C_1-C_6)$alkoxy$(C_3-C_6)$cycloalkyl, $(C_3-C_6)$cycloalkylcarbonyl, substituted or unsubstituted phenyl, phenyl$(C_1-C_6)$alkyl, heterocyclyl, and heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, $(C_1-C_3)$alkyl, $(C_1-C_3)$haloalkyl, $(C_1-C_3)$alkoxy, $(C_1-C_3)$haloalkoxy, $(C_1-C_3)$alkylamino, di$(C_1-C_3)$alkylamino, $(C_1-C_2)$alkoxy$(C_1-C_2)$alkyl, $(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, di$(C_1-C_2)$alkylamino$(C_1-C_2)$alkyl, $(C_1-C_3)$alkylcarbonyl, $(C_1-C_3)$alkoxycarbonyl, $(C_1C_3)$alkylaminocarbonyl, and di $(C_1-C_3)$alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, hydroxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy;

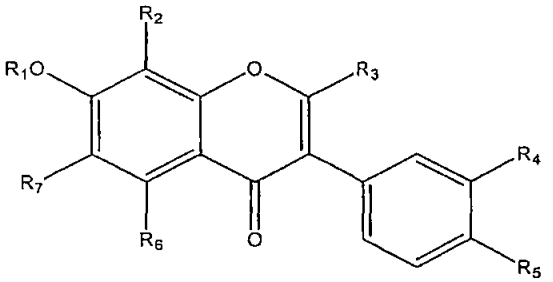
ii) contacting ALDH-2 with the compound;

iii) assaying the ability of the compound to modulate ALDH-2 activity; and

iv) selecting a compound that modulates ALDH-2 activity as a modulator of ALDH-2 activity.

18. A method for identifying a compound that modulates ALDH-2 comprising the steps of:

i) providing a compound of Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyc1yl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl,

phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy;

ii) contacting ALDH-2 with the compound;

iii) assaying the ability of the compound to modulate ALDH-2 activity; and

iv) selecting a compound that modulates ALDH-2 activity as a modulator of ALDH-2 activity.

19. The method of claim 17, wherein the modulation is inhibition.

20. The method of claim 17, wherein the compound is further capable of increasing a concentration of an aldehyde.

21. The method of claim 20, wherein the aldehyde is 5-hydroxyindoleacetaldehyde or 3,4-dihydroxyphenylacetaldehyde.

22. The method of claim 17, wherein the compound does not inhibit monoamine oxidase.

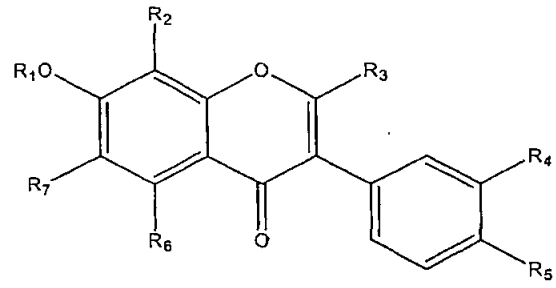
23. The method of claim 17, wherein $R_5$ is OH or $NH_2$.

24. The method of claim 18, wherein $R_1$ is a straight chain alkyl.

25. The method of claim 24, wherein the straight chain alkyl is selected from the group consisting of -$(CH_2)_n$-OH with $2 \leq n \leq 6$, $(CH_2)_n$-COOH with $5 \leq n \leq 10$, and -$(CH_2)_n$-$NH_2$, with $n \geq 4$.

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26. A compound for inhibiting ALDH-2 comprising Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)haloalkyl, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_3$-$C_6$)cycloalkylcarbonyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, and heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

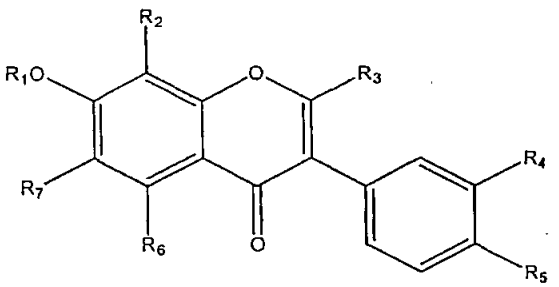
$R_5$ is selected from the group consisting of hydrogen, carboxy, hydroxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-

$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy.

27. A compound for inhibiting ALDH-2 comprising Formula I




Formula I

wherein:

$R_1$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyc1yl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl;

$R_2$ is selected from the group consisting of hydrogen and alkoxy;

$R_3$ is selected from the group consisting of hydrogen $C_1$-$C_6$ alkoxycarbonyl, carboxy and sugar;

$R_4$ is selected from the group consisting of hydrogen and hydroxide;

$R_5$ is selected from the group consisting of hydrogen, carboxy, halo, branched or unbranched ($C_1$-$C_6$)alkyl, ($C_1$-$C_6$)haloalkyl, ($C_2$-$C_6$)alkenyl, ($C_3$- $C_6$)alkadienyl, ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkoxy, ($C_1$-$C_6$)haloalkoxy, ($C_3$-$C_6$)cyclohaloalkoxy, ($C_2$-$C_6$)alkynyloxy, ($C_1$-$C_6$)alkoxy($C_1$-$C_6$)alkyl, ($C_3$-$C_6$)cycloalkoxyalkyl, ($C_1$-$C_6$)alkoxy($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkylcarbonyl, ($C_3$-$C_6$)cycloalkylcarbonyl, ($C_1$-$C_6$)alkoxycarbonyl, ($C_4$-$C_6$)alkoxycarbonylalkyl, ($C_1$-$C_6$)hydroxyalkyl, substituted or unsubstituted phenyl, phenyl($C_1$-$C_6$)alkyl, heterocyclyl, heterocyclyloxy, heterocyclylcarbonyl, wherein substituents are from one to four and are selected from the group consisting of halo, aminocarbonyl, aminothiocarbonyl, carboxy, formyl, hydroxy, amino, carbamoyl, ($C_1$-$C_3$)alkyl, ($C_1$-$C_3$)haloalkyl, ($C_1$-$C_3$)alkoxy, ($C_1$-$C_3$)haloalkoxy, ($C_1$-$C_3$)alkylamino, di($C_1$-$C_3$)alkylamino, ($C_1$-$C_2$)alkoxy($C_1$-$C_2$)alkyl, ($C_1$-$C_2$)alkylamino($C_1$-$C_2$)alkyl, di($C_1$--$C_2$)alkylamino($C_1$-$C_2$)alkyl, ($C_1$-$C_3$)alkylcarbonyl, ($C_1$-$C_3$)alkoxycarbonyl, ($C_1$-$C_3$)alkylaminocarbonyl, and di ($C_1$-$C_3$)alkylaminocarbonyl

$R_6$ is selected from the group consisting of hydrogen and hydroxide; and

$R_7$ is selected from the group consisting of hydrogen, halogen, and $C_1$-$C_6$ alkoxy.

28. The compound of claim 26, wherein $R_5$ is OH or $NH_2$.

29. The compound of claim 27, wherein $R_1$ is a straight chain alkyl.

30. The compound of claim 29, wherein the straight chain alkyl is selected from the group consisting of -$(CH_2)_n$-OH with $2 \leq n \leq 6$, $(CH_2)_n$-COOH with $5 \leq n \leq 10$, and -$(CH_2)_n$-$NH_2$, with $n \geq 4$.

31. The compound of claim 26, wherein the compound further inhibits alcohol consumption in a mammal.

32. The compound of claim 31, wherein the mammal is a human.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/20584

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/352; C07D 311/34
US CL : 514/456 ; 435/184 ; 549/403
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/456 ; 435/184 ; 549/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KEUNG, W. et al. Daidzin inhibits mitochondrial aldehyde dehydrogenase and suppresses ethanol intake of syrian golden hamsters. Proc. Natl. Acad. Sci., USA. March 1997, Vol. 94, pages 1675-1679, especially table 4 on page 1678. | 1-32 |
| X | ROOKE, N. et al.: The mitochondrial monoamine oxidase-aldehyde dehydrogenase pathway: A potential site of action of Diadzin. J. Med. Chem. November 2000, Vol. 43, pages 4169-4179, especially figure 2 and table 1 on page 4171, table 4 on page 4173. | 1-32 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search
25 September 2003 (25.09.2003)

Date of mailing of the international search report
31 OCT 2003

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Form PCT/ISA/210 (second sheet) (July 1998)

XP-002462450

Phytochemistry, 1970, Vol. 9, pp. 1735 to 1744. Pergamon Press. Printed in England.

# THE DEGRADATION OF FORMONONETIN AND DAIDZEIN IN *CICER ARIETINUM* AND *PHASEOLUS AUREUS**

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**Abstract**—To investigate the fate of isoflavones in *Cicer arietinum L.* and *Phaseolus aureus Roxb.*, experiments with formononetin-(methyl-$^{14}$C) and daidzein-(4-$^{14}$C) have been carried out. Using intact plants and separated or sliced plant parts extensive degradation of the substrates in both plants was observed showing catabolic reactions to be involved. The degree of assimilation observed is discussed in relation to the theoretically expected turnover. Data are presented that rhizosphere microorganisms contributed to the observed degradation. Experiments with sterile *Phaseolus aureus* plants revealed significant differences in the metabolism of daidzein and formononetin.

## INTRODUCTION

PREVIOUS communications from this laboratory[1,2] presented evidence that formononetin (I) in *Cicer arietinum* L. as well as daidzein (7,4'-dihydroxyisoflavone) (II) and coumestrol (III) in *Phaseolus aureus* Roxb. are not metabolically inactive end products but are further metabolized, with biological half-lives in the order of 50 hr. Further experiments with *C. arietinum* revealed that on approximately the eighth day after germination the amount of formononetin had reached a steady-state level characterized by comparable rates of biosynthesis and turnover.[3] In the case of biochanin A (IV) in *C. arietinum* however, rapid synthesis occurred in young plants only between the third and the ninth day after germination and then production gradually decreased with no comparable turnover.

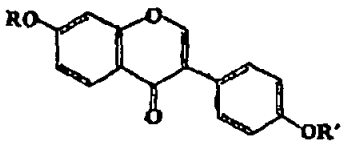
These findings lend further support to the hypothesis that accumulation of secondary plant products is regulated by the induction or repression of enzyme systems involved in the formation or metabolism of such plant products.[4] From our earlier observations, we could draw no conclusions whether anabolic or catabolic processes are involved. To further investigate the turnover of the above mentioned compounds, formononetin-methyl-$^{14}$C[5-7] (I) and daidzein-4-$^{14}$C[8-10] (II) were synthesized and fed to *C. arietinum* and *P. aureus*. This paper describes various experiments designed to determine the fate of isoflavones in these plants.

* Part III in the series "Metabolism of aromatic plant constituents".

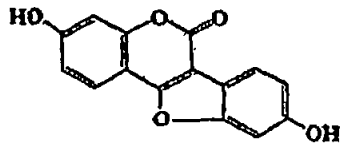
[1] W. BARZ, *Z. Naturforsch.* 24b, 234 (1969).
[2] W. BARZ and B. ROTH-LAUTERBACH, *Z. Naturforsch.* 24b, 638 (1969).
[3] H. GRISEBACH and W. BARZ, *Naturwiss.* 56, 538 (1969).
[4] M. H. ZENK, *Ber. Deut. Botan. Ges.* 80, 573 (1967).
[5] L. JURD, *J. Am. Chem. Soc.* 80, 5531 (1958).
[6] L. JURD, *J. Org. Chem.* 1786 (1959); 1294 (1962).
[7] E. WALZ, *Liebigs Ann. Chem.* 489, 118 (1931).
[8] L. YODER, E. CHENG and W. BURROUGH, *Proc. Iowa Acad. Sci.* 61, 271 (1954).
[9] P. M. DEWICK, W. BARZ and H. GRISEBACH, *Phytochem.* 9, 775 (1970).
[10] R. N. IYER, K. H. SHAH and K. VENKATARAMAN, *Proc. Indian Acad. Sci.* 33A, 116 (51).



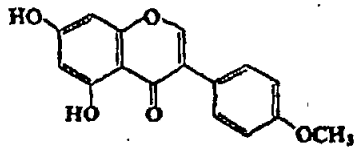
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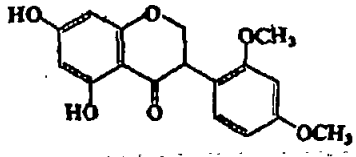
(I) R = H; R' = $CH_3$
(II) R = R' = H
(V) R = —$CH_2C_6H_5$; R' = H
(VI) R = R' = —$COCH_3$
(VII) R = —$CH_2C_6H_5$; R' = —$COCH_3$
(VIII) R = —$CH_2C_6H_5$; R = $CH_3$



(III)



(IV)



(IX)

## RESULTS

### *Feeding Experiments with Intact Plants*

In a first series of experiments, formononetin-(methyl-$^{14}$C) (·07 $\mu$C) was root-fed to 9-day-old hydroponically grown plants[2] of *Cicer arietinum* and *Phaseolus aureus*. Mung bean plants have not been found so far to contain formononetin or other methylated flavonoids. They were, however, included in the experiments in order to obtain an indication as to variation in isoflavonoid metabolism. Assuming that the applied substrate can freely enter the metabolically active isoflavone pool and that the biological half-life of (I) is 48–50 hr,[1] a 24-hr feeding period was chosen. One could then expect that *ca.* 25% of the substrate taken up by the plants should be metabolized.

The plants were fractionated according to the scheme outlined in Fig. 1, which also shows the distribution of label in both plants. By dilution analysis, it was demonstrated that only 8·5 and 6·0%, respectively, of the absorbed formononetin-(methyl-$^{14}$C) could be reisolated. This fact, together with the randomization of radioactivity between label in all fractions obtained and the high percentage of $^{14}CO_2$ evolved led us to assume that strong demethylating activity might be present in both plants. By chromatographing and counting aliquots of the light petroleum extract[11] and of the three ether fractions it could be shown that substantial radioactivity had been incorporated into the xanthophyll, lutein, and into several phenolic compounds. The almost complete assimilation of formononetin and the high production of $^{14}CO_2$ is in contrast to the previously determined rate of isoflavone turnover in these plants[1] considering the duration of the experiment. Possible explanations are that the higher rate of degradation might be due either to unspecific oxidations[12] of substrate by tissue not normally involved in isoflavone turnover or to degradative processes by rhizosphere micro-organisms.

[11] A. Hagen and T. Meyer-Bertenrath, *Planta* 69, 198 (1966).
[12] D. Neumann and K. H. Tschoepe, *Flora Jena* 156, 521 (1966).

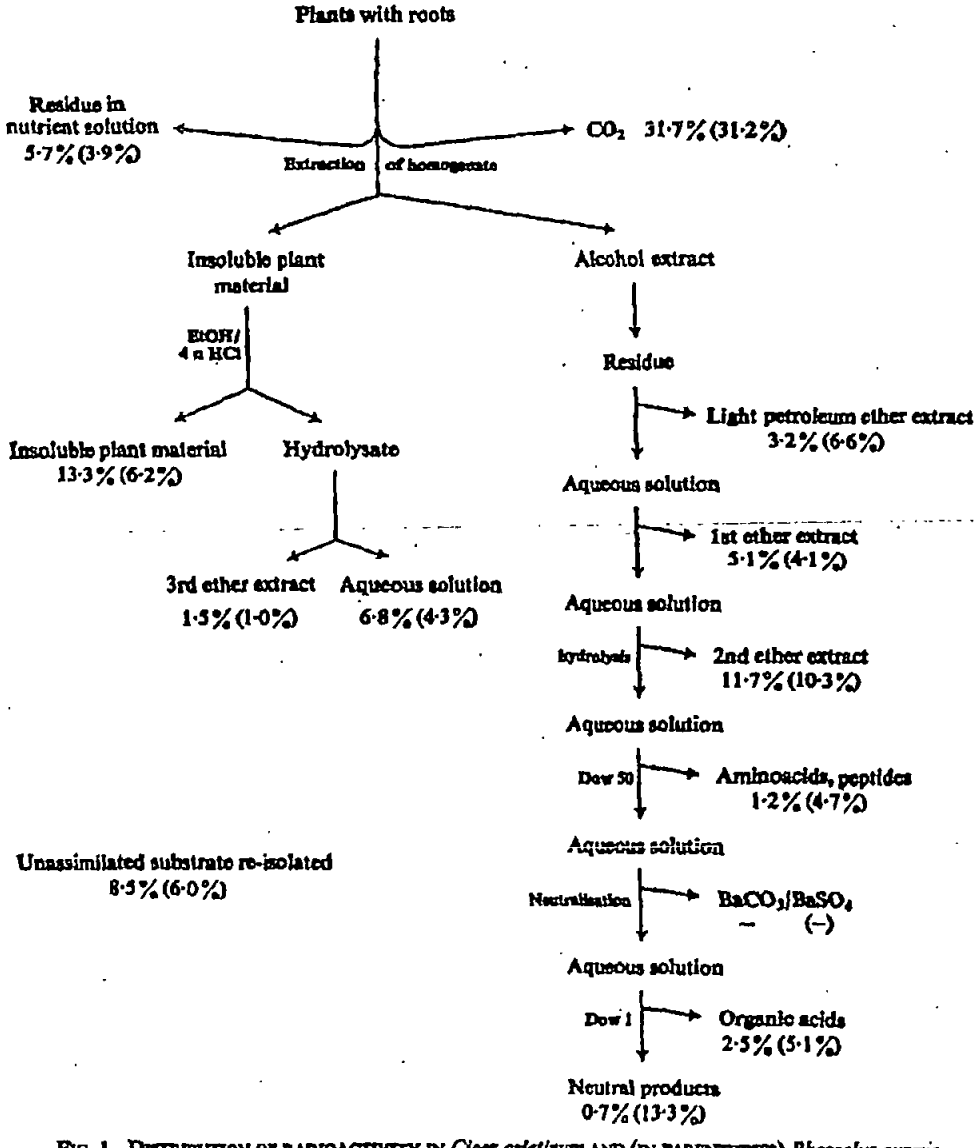



FIG. 1. DISTRIBUTION OF RADIOACTIVITY IN *Cicer arietinum* AND (IN PARENTHESES) *Phaseolus aureus* ROXB. AFTER FEEDING OF FORMONONETIN-(METHYL-$^{14}$C).

### *Feeding Experiments with Plant Slices*

To test the first-mentioned possibility, separate intact roots or stems and thinly sliced (3–4 mm thick) root or green parts (stems and leaves) of *C. arietinum* were separately incubated with formononetin-(methyl-$^{14}$C) with efficient aeration. This should ensure better

[13] M. H. ZENK, *Z. Pflanzenphysiol.* 53, 404 (1965).
[14] H. GRISEBACH and G. BRANDNER, *Experientia* 18, 400 (1962).

penetration and uptake[13] and decrease the degree of unspecific and irreversible binding of fed phenols to insoluble polymeric structures.[14]

After incubation for 24 hr the plant material was fractionated according to the scheme outlined previously (Fig. 1). The results obtained are summarized in Table 1.

Table 1. Distribution of radioactivity when formononetin-(methyl-$^{14}$C) is fed to separated or sliced parts of *Cicer arietinum*

| Fraction \ Plant part | Intact green parts | Sliced green parts | Intact roots | Sliced roots |
|---|---|---|---|---|
| % Residue in nutrient solution | 61·3 | 33·1 | 13·3 | 11·0 |
| % $CO_2$ | 1·0 | 9·8 | 29·4 | 35·5 |
| % Insoluble plant material | 3·5 | 3·6 | 13·6 | 26·9 |
| % Light petrol extract | 6·8 | 8·3 | 2·4 | 0·8 |
| % 1st ether extract | 14·7 | 40·6 | 9·8 | 15·7 |
| % 2nd ether extract | 9·6 | 2·1 | 12·1 | 6·5 |
| % Amino acids<br>organic acids<br>neutral products | 5·1 | 0·7 | 6·8 | 2·9 |
| % Total | 102·0 | 98·2 | 87·4 | 99·3 |
| % Unassimilated substrate re-isolated | 42·5 | 60·4 | 13·1 | 22·1 |

In the experiments with either intact or sliced roots, we again observed a relatively high production (*ca.* 30%) of $^{14}CO_2$ and label was again widely distributed in all fractions obtained. Though formononetin was found in the fractions containing the free and glycosidated phenols—only 13 and 22%, was re-isolated from the intact or sliced root tissue respectively.

When formononetin was absorbed by the intact stems, on the other hand, a very much smaller (1·0%)$^{14}CO_2$ production was observed. Although appreciable randomization of label was found in the phenolic and the non-phenolic fractions, 42·5% of the absorbed formononetin was re-isolated. In the experiment with the sliced leaves, 60·4% of formononetin was not assimilated. Although there was a somewhat greater production of $^{14}CO_2$, the overall distribution of label agreed with that expected if the formononetin was catabolically metabolized.

While we had found considerable labelling of carotenoids and xanthophylls in the experiments with the intact plants mentioned earlier (Fig. 1), significantly less radioactivity appeared in these compounds when separated leaves were used. Root-feeding of intact *Cicer*-plants, however, resulted in significant labelling of carotenoid compounds. This lead us to assume that in the roots it was $^{14}CO_2$, formed from the labelled isoflavone, that entered the plants metabolism via $CO_2$-fixation and was incorporated into the carotenoid fraction. This is corroborated by the observation that when intact *Cicer arietinum* plants are fed with comparable amounts of $H^{14}CO_3^-$ (1,5 $\mu$c) to the amount of formononetin-$^{14}$C given above, similar high specific activities are found in the carotenoids. Table 2 shows the distribution of label in the main carotenoid constituents of *Cicer arietinum* which had been separated as described by Egger[15] and identified by their absorption spectra.[16]

It was earlier observed[17] that liberated *O*-methyl groups can be re-used via the $C_1$-pool. Therefore biochanin A (IV) and homoferreirin (IX) were isolated from the phenolic fractions

[15] K. Egger, *Phytochem.* 4, 609 (1965).
[16] A. Hager and T. Meyer-Bertenrath, *Planta* 76, 149 (1967).
[17] W. Barz and H. Grisebach, *Z. Naturforsch.* 22b, 627 (1967).

TABLE 2. SPECIFIC RADIOACTIVITY OF CAROTENOIDS ISOLATED FROM *Cicer arietinum* AFTER APPLICATION OF EITHER FORMONONETIN-(METHYL-$^{14}$C) OR $H^{14}CO_3'$

| Experiment (substrate) | Activity (cpm/μmole × $10^{-6}$) | | | |
|---|---|---|---|---|
| | β-Carotene | Lutein | Violaxanthin | Neoxanthin |
| Intact root-fed *C. arietinum* (formononetin-methyl-$^{14}$C) | 76·3 | 6·61 | 80·0 | 85·7 |
| Green parts of *C. arietinum* (formononetin-methyl-$^{14}$C) | 0·21 | 0·034 | 1·0 | 0·83 |
| Intact *C. arietinum* ($H^{14}CO_3'$) | 8·2 | 7·55 | 21·0 | 75·0 |

of *C. arietinum* in the experiments described above (Fig. 1 and Table 1) in order to examine the extent of re-methylation processes. Biochanin A was found both in the roots and the green parts, while IX could only be isolated from the root extracts. The results in Table 3 demonstrate that all samples proved to give radioactive biochanin A. However, the highest radioactivity was obtained from the experiment using the intact green parts.

TABLE 3. SPECIFIC AND TOTAL RADIOACTIVITY OF BIOCHANIN A AND HOMOFERREIRIN ISOLATED FROM *Cicer arietinum* AFTER FEEDING FORMONONETIN-(METHYL-$^{14}$C) AND $H^{14}C_3'$

| Compound isolated | Plant part used: Intact plants | Green parts | Sliced green parts | Intact roots | Sliced roots | Intact plants fed $H_{14}CO_3'$ |
|---|---|---|---|---|---|---|
| Biochanin A (IV) | | | | | | |
| Sp. act. (cpm/mmole) × $10^{-4}$ | 4·8 | 100 | 4·5 | 3·2 | 14 | (0·9) |
| Total act. (cpm) | 84 | 836 | 132 | 194 | 414 | (8) |
| Homoferreirin (IX) | | | | | | |
| Sp. act. (cpm/mmole) × $10^{-4}$ | 28·0 | — | — | 56·0 | 60·0 | (0·01) |
| Total act. (cpm) | 441 | — | — | 582 | 630 | (0) |

A comparison of the data from Tables 2 and 3 leads to the conclusion that feeding of intact plants with either formononetin or $H^{14}CO_3'$ results in significant labelling of carotenoids with little or no radioactivity in other isoflavonoids, while incubation of intact green parts with the isoflavone shows the opposite pattern of labelling.

*Evidence for Rhizosphere Microorganisms*

The conflicting data presented so far led us to test the roots and the green parts of both our hydroponically grown *Cicer arietinum* and *Phaseolus aureus* plants for the presence of isoflavone-metabolizing microorganisms.

Microorganisms which could be washed off either the roots or the green parts of both plants were separately incubated with formononetin-(methyl-$^{14}$C). While all preparations from both roots and stems contained micro-organisms as indicated both by microscopic inspection and plating on nutritional agar, only the microbial suspension isolated from the roots completely degraded formononetin-(methyl-$^{14}$C) within 24 hr. In this case 84–90 per cent of the applied radioactivity was liberated as $^{14}CO_2$. The microorganisms isolated from the green parts produced only negligible amounts of $^{14}CO_2$ (0·01 %), practically all the formononetin being left intact. These results strongly suggest that rhizosphere microorganisms are

mainly responsible for the degradation of formononetin in the above experiments with the roots or whole root-fed plants. The high production of $^{14}CO_2$ in these cases is presumably partly due to microbial oxidation of formononetin-(methyl-$^{14}$C), and the results in Figure 1 and Table 1 suggested that part of the liberated $^{14}CO_2$ enters the plant via some $CO_2$-fixation mechanism.

*Experiments with Sterile Mung Bean Plants*

The isolation of an isoflavone-degrading microorganism from the roots of *Cicer arietinum* and *Phaseolus aureus* as described in a following communication[18] necessitated the use of aseptically grown plants for further feeding experiments. Small batches of ethanol-washed seeds of *Phaseolus aureus* were rigorously surface sterilized with hypochlorite solution, repeatedly washed with water and grown under sterile conditions. The seeds were kept individually in small vials placed in larger test tubes with a layer of nutritional agar at the bottom. Thus it was possible to inspect each plant for microbial contamination prior to use.

The results of experiments with formononetin-(methyl-$^{14}$C) and daidzein-(4-$^{14}$C) using these intact sterile plants are shown in Table 4. While in the experiment with the non-sterile

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN ASEPTICALLY GROWN *Phaseolus aureus* PLANTS AFTER FEEDING OF LABELLED ISOFLAVONES

| Fraction \ Substrate | Formononetin-(methyl-$^{14}$C) % | Daidzein-(4-$^{14}$C) % |
|---|---|---|
| Residue in nutrient solution | 32·1 | 5·7 |
| $CO_2$ | 0·5 | 1·4 |
| Petrol-ether extract | 1·4 | 5·6 |
| 1st ether extract | 61·6 | 6·9 |
| 2nd ether extract | 2·9 | 3·6 |
| Amino acids | 0·44 | 1·2 |
| Organic acids, neutral products | 0·01 | 0·4 |
| Insoluble plant material | 3·0 | 71·4 |
| Ether soluble material liberated from insoluble plant material | — | 4·4 |
| Amino acids liberated from insoluble plant material | — | 6·3 |
| Carbohydrates, organic acids from insoluble plant material | — | 0·7 |
| "Lignin" | — | 60·0 |
| Total | 101·95 | 96·2 |
| Unassimilated substrate reisolated | 95·0 | 8·5 |

mung bean plants only 6·0% unassimilated formononetin was observed (Fig. 1), the sterile plants produced only negligible amounts of $^{14}CO_2$ or other nonphenolic radioactive products. Approximately 95 per cent of the substrate which had been taken up by the plants was reisolated after 24 hr. These data clearly show the contribution of the rhizosphere microorganisms to isoflavonoid degradation in the experiment with the non-sterile plants and also indicate that *P. aureus*, apart from glycosidases, does not possess enzymes for the metabolism of the methylated isoflavone. This distinct substrate specificity is further indicated by the results obtained with daidzein-(4-$^{14}$C). In this case the applied isoflavone was rapidly and extensively assimilated, being incorporated into lipid material, phenols, amino acids, organic acids and

[18] W. Barz, *Phytochem.* 9, 1745 (1970).

carbohydrates. A major portion of the substrate was incorporated into the ethanol-insoluble plant material which upon acid hydrolysis released three radioactive phenolic compounds. One was identified as daidzein and the other two are more polar compounds exhibiting an intensive greenish-blue fluorescence when exposed to ammonia fumes. A substantial part of the radioactivity cannot be chemically differentiated from lignin or unhydrolysable polymeric phenolic material. In the fraction of the free phenols, coumestrol (III), was again shown to be radioactive thus giving additional proof of the earlier established biogenetic relationship between isoflavones and coumestanes.[9,19] This experiment with daidzein-(4-$^{14}$C) again indicates that the observed turnover of isoflavones[1,2] can be explained by assuming catabolic pathways to be operating.

## DISCUSSION

The experiments reported in this paper were designed to investigate the general fate of isoflavones and to show, that in contrast to the traditional view,[20] these compounds are not metabolically inactive end products. To distinguish between anabolic[21] and catabolic[4] pathways, we have not only tried to determine the overall quantitative assimilation, but, presuming free transport of the phenols into the reactive pools have assumed one should be able to calculate roughly the percentage of the metabolized products from the known biological half-lives[1,2] of turnover and the duration of the experiment. Taking the results presented in Fig. 1 and Table 1, this approach seems to have been helpful, though steady-state kinetics in this field are far more complicated than our present knowledge.

The results of the experiments with the root-fed plants (Fig. 1) and the separated or sliced roots (Table 1) showed extensive degradation of formononetin-(methyl-$^{14}$C). However, the extensive assimilation of the methyl-labelled isoflavone in the experiments with the roots must partly be explained by participation of rhizosphere microorganisms because of the finding that there is complete degradation of formononetin-(methyl-$^{14}$C) using microbial preparations washed off the roots of our experimental plants. The rhizosphere microorganisms obviously degraded formononetin-(methyl-$^{14}$C) to $^{14}CO_2$ part of which re-entered the plant via fixation processes and led to the strong labelling of carotenoid compounds (Table 2). The significant extent of radioactivity in carotenoids in the experiments with $H^{14}CO_3^-$ and the reduction in activity when plants without roots were used with formononetin-(methyl- $^{14}$C) (Table 2) can be taken as a proof for this assumption.

Unequivocal proof for the contribution of microorganisms was obtained by a comparison of the two experiments with non-sterile and sterile mung bean plants. Sterile plants degraded very little formononetin (Table 4) so that the complete assimilation observed with non-sterile plants (Fig. 1) clearly shows the contribution of microbes located at the roots. However, the assimilation of formononetin-(methyl-$^{14}$C) observed in the experiments with the intact or sliced green parts of *C. arietinum* (Table 1) cannot be explained by assuming microbial contaminations, because the leaf microbial contaminants did not degrade formononetin to any measurable extent. In addition, in these last two cases, the extent of assimilation was quite close to our theoretical expectations based on the degree of turnover.

The isolation of labelled samples of biochanin A (IV) and homoferreirin (IX) from *C. arientinum* (Table 3) again[17] indicates that methyl-ether groups removed from phenols can

[19] W. Barz and H. Griesbach, *Z. Naturforsch.* 21b, 1113 (1966).
[20] K. Mothes, *Experientia* 25, 225 (1969).
[21] J. W. Fairbairn and S. El-Masry, *Phytochem.* 6, 499 (1967); 7, 181 (1968).

enter the $C_1$-pool and be reused for further methylation reactions. It is suggested that the labelling of IV and IX does not originate from $^{14}CO_2$ via photosynthesis, because under our experimental conditions photosynthesis did not lead to labelled isoflavones.

Altogether these results strongly indicate the absolute necessity to test the experimental plants for microbial contamination, especially when experiments with roots are carried out. This seems to be of great importance in all kinds of feeding experiments.[9]

Our results with formononetin suggest that anabolic pathways or extensive binding of this isoflavone to membranes or other polymeric structures such as lignin are not involved in the observed assimilation of formononetin in *C. arietinum*. The very small degree of incorporation of label into any insoluble material (Table 1) is sufficient proof for this.

The results of feeding experiments with daidzein and formononetin to sterile mung bean (Table 4) clearly show a significant difference between these two isoflavones. Although *P. aureus* can degrade daidzein, enzymes for assimilating the methyl ether seem to be absent showing the distinct selectivity of the enzymes involved in daidzein degradation. The results can be taken as additional proof that daidzein is not a metabolic end product as assumed by Miller.[22] The extensive binding of daidzein to insoluble material (Table 4 "lignin") may be due to the presence of polyphenol oxidases in the more woody tissue such as roots and stems where rather large amounts of polymeric leucoanthocyanins and condensed phenols are formed.[23] Experiments with leaves only[24] show that this process seems to be rather specific for root tissue. This is further indicated by the results in Table 1 where such binding to ethanol-insoluble root tissue was found much higher than in the experiments with green tissue.

## EXPERIMENTAL

*Formononetin-(methyl-$^{14}$C)*

Carefully dried daidzein-diacetate[7] (300 mg) was refluxed for 20 hr with anhydrous $C_6H_5CH_2Cl$ (3·0 ml), KI (400 mg) and anhydrous $K_2CO_3$ (1·0 g) in dry acetone (20 ml). After filtration and evaporation of the solvent, excess reagent was removed with light petroleum and the residue repeatedly crystallized from MeOH. Daidzein-7-*O*-benzyl-ether-4'-acetate formed colourless needles, m.p. 173°. U.v. spectrum: $\lambda^{max}_{MeOH}$ 228$^{sh}$, 250, 297, 308$^{sh}$ nm, unaffected by sodium acetate; $\lambda^{max}_{0.002\,NaOMe}$ 240, 248, 258, 280$^{sh}$ nm. I.r. spectrum: $\nu$ CO 1742, 1637 cm$^{-1}$.

Daidzein-7-*O*-benzylether-4'-acetate when treated under reflux with conc. HCl (1·2 ml) in 30 ml acetone for 1 hr afforded daidzein-7-*O*-benzylether as white prisms from methanol/water, m.p. 169–170°. U.v. spectrum: $\lambda^{max}_{MeOH}$ 232, 249, 260, 308 nm unaffected by sodium-acetate; $\lambda^{max}_{0.002\,NaOMe}$ 238, 246, 5$^{sh}$, 281 nm. I.r. spectrum: $\nu_{OH}$ 3330 cm$^{-1}$; $\nu_{CO\,1613}$ cm$^{-1}$. Both daidzein-7-*O*-benzylether-4'-acetate and daidzein-7-*O*-benzylether afforded daidzein when treated with conc. HCl in acetic acid at 120° for 1 hr as indicated by u.v. and i.r. spectra.

Daidzein-7-*O*-benzylether (60 mg, 0·174 mmole) was stirred at 60° under anhydrous conditions with dry $K_2CO_3$ (450 mg), acetone (30 ml), MeI (0·01 ml, 0·16 mmole) and MeI-$^{14}$C (0·47 mg, sp. activity 30 mC/mmole; (Radiochemical Centre Amersham) for 90 min. After the addition of further "cold" MeI (altogether 0·6 ml) the reaction mixture was refluxed for a total of 4 hr. After filtration and evaporation of the solvent, formononetin-7-*O*-benzylether-(methyl-$^{14}$C) was obtained in quantitative yield. The product was identical in all respects with a sample prepared by benzylation of formononetin with benzyl chloride in acetone in the presence of $K_2CO_3$. Formononetin-7-*O*-benzyl-ether formed shiny plates from methanol, m.p. 179–180°. U.v. spectrum: $\lambda^{max}_{MeOH}$ 210, 235, 249, 259, 297, 306 nm unaffected by sodium acetate and sodium methoxide.

Formononetin-7-*O*-benzylether-(methyl-$^{14}$C) (15 mg) was debenzylated to formononetin-(methyl-$^{14}$C) by solution in hot acetic acid (4 ml) and heating with conc. HCl (3 ml) at 120° for 35 min. After evaporation of the solvents, the residue was purified on silica gel TLC with (1) benzene–ethyl acetate–MeOH–light petroleum (60–70°), (6:4:1:3;) (2) $CHCl_3$ isopropanol, (10:1); (3) benzene–EtOH, (92:8). Sp. act. 0·547 mC/mmole.

[22] C. D. Miller, *Planta* 87, 26 (1969).
[23] G. I. Forrest and D. S. Bendall, *Biochem. J.* 113, 757 (1969).
[24] J. Berlin and W. Barz, unpublished.

*Formononetin-(4-$^{14}$C)*

2,4-Dihydroxyphenyl-4-methoxybenzylketone-(carbonyl-$^{14}$C)[9] (60 mg) was treated under anhydrous conditions with pyridine (6 ml), piperidine (0·8 ml) and triethyl-ortho-formate (4 ml) at 115° for 22 hr. The dark solution was poured into ice/water, acidified (50% HCl) and continuously extracted with $Et_2O$ for 24 hr. From the $Et_2O$ extract, formononetin was isolated and purified by TLC chromatography as described above. Yield: 42 mg, sp. act. 0·84 mC/mmole.

*Daidzein-(4-$^{14}$C)*

Formononetin-(4-$^{14}$C) (5·3 mg) was demethylated with conc. HI (1·0 ml, sp. gr. 1·6) for 4 hr. at 120°. After dilution with water and decolourization with $Na_2S_2O_4$, the solution was continuously extracted with ether and daidzein purified by TLC with the solvents: $CHCl_3$–isopropanol (10:1), benzene–MeOH (5:1) Yield 4·78 mg, sp. act. 0·87 mc/mmole.

*Feeding Experiments*

The cultivation of *Cicer arietinum* and *Phaseolus aureus* in hydroponic solution was essentially as described previously.[1] Before each experiment the roots were repeatedly rinsed with sterile water. For all experiments 9-day-old plants were used. For each experiment with formononetin-(methyl-$^{14}$C), 1·02 mg (2·07 $\mu$c) was dissolved in 0·5 ml ethylene glycol mono-methyl ether and added to an appropriate amount of phosphate buffer (0·05 m, pH 8·5). Daidzein-(4-$^{14}$C), 0·61 mg, 2·1 $\mu$c, was administered similarly. $H^{14}CO_3^-$ solution (1·5 $\mu$c, sp. act. 51 mC/mmole) was placed in a small beaker in the feeding tank and $^{14}CO_2$ evolved by injection of dilute $H_2SO_4$. The plants were fed in small beakers placed in a large desiccator closed with a flat glass lid and—except for the $H^{14}CO_3^-$ experiment—moist air was bubbled through. $CO_2$ was absorbed in ethylene glycol monomethylether/ethanolamine as described by Jeffay.[33]

*Sterile Mung Bean Plants*

Batches of 40 $H_2O$- and EtOH-washed seeds were treated for 20 min with a NaOCl solution (12·5% free chlorine). The seeds were washed free from $Cl_2$ with sterile $H_2O$ and, under sterile conditions, individually placed in large sterile test tubes (30 × 200 mm) which contained small vials embedded in a layer of nutritional agar (Merck, 2·0%). The test tubes were stoppered with sterile stoppers and kept in a phytochamber under the normal growth conditions.[1] The seeds were given only small amounts of sterile tap water (0·2 ml) as required. 48 hr before the experiments started, the tubes were gently shaken to allow the water to contact the agar. Bacterial contaminations could easily be detected as turbidity on the agar surface.

*Fractionation Procedure*

After the incubation period the plant material was washed with water, dropped into boiling 70% EtOH and after homogenizing in a Waring blender, repeatedly extracted with hot 70% EtOH. The filtrate was collected, the solvent evaporated under vacuum at 30° and the dry residue thoroughly extracted with light petroleum. After the addition of $H_2O$ (20 ml), phenols were extracted with $Et_2O$ (5 × 50 ml). Hydrolysis of phenolic glycosides was accomplished with 2 N $H_2SO_4$ at 100° for 2 hr.

The EtOH-insoluble plant material was partly hydrolysed with EtOH–4 N HCl (3:1) at 100° for 4 hr and insoluble material collected by centrifugation.

*Isolation of Isoflavonoids*

Aliquots of the $Et_2O$ extracts were chromatographed on silica gel TLC with benzene–EtOH (92:8). Formononetin was further purified as mentioned above. The band of biochanin A and homoferreirin was eluted with MeOH and the two compounds separated by paper chromatography with water–isopropanol (4:1). Concentrations were determined spectrophotometrically[1,3] in a Zeiss PMQ II spectrophotometer. All three compounds were diluted with carrier material and purified by repeated crystallizations from MeOH and EtOH and by high vacuum sublimation.

*Isolation of Carotenoids*

To aliquots of the light petroleum extracts (15 ml), NaOEt (0·1 m, 5 ml) was added and the mixture kept at room temp. for 20 min. After the addition of $H_2O$ (20 ml), carotenoids were extracted with ether–light petroleum (1:1, v/v). The organic layer was washed with $H_2O$, dried ($Na_2SO_4$) and concentrated to a small volume. Carotenoids were separated by TLC on cellulose plates which had been impregnated with triglycerides[10] using MeOH–acetone–water (15:5:1) as solvent. The bands of $\beta$-cartone, lutein, violaxanthin and neoxanthin were eluted with EtOH and the compounds further purified by two TLC separations on silica gel. For $\beta$-carotene light petroleum–benzene (20:1) was used as solvent and for the other three compounds

[33] H. Jeffay and J. Alvarez, *Anal. Chem.* 33, 612 (1961).

light petroleum–acetone (8:5). Absorption spectra were determined in ethanol with a Cary 14 recording spectrophotometer. Extinction coefficients used were as published by Hager.[16] All operations with carotenoids were carried out under dim light.

*Radioactivity Assay*

Radioactivity of soluble compounds was measured by scintillation spectrometry with a Beckman LS 100 scintillation counter using either dioxane or toluene scintillator. Counting efficiencies were determined by internal standardization (toluene-$^{14}C$ standards). Insoluble material was combusted to $CO_2$ and counted in the gas phase according to Simon[35] using a Berthold anti-coincidence counter.

*Acknowledgements*—We acknowledge with thanks the support by the Deutsche Forschungsgemeinschaft, the helpful discussions with Prof. H. Grisebach and the suggestions by Dr. H. Kleinig.

[35] H. SIMON, H. DANIEL and I. F. KLEBE, *Angew. Chem.* 71, 303 (1959).

DUCCION OFICIAL No. 2009-06-19-365
UN DOCUMENTO ESCRITO EN INGLES

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530

| TRASPASO DE PATENTE | |
|---|---|
| Versión Electrónica v1.1<br>Versión Hoja Estilo v1.1 | |
| TIPO DE PRESENTACION<br>NATURALEZA DEL TRASPASO | NUEVO TRASPASO<br>TRASPASO |

| DATOS DE LA PARTE QUE TRASPASA | |
|---|---|
| Nombre | Fecha de Ejecución |
| Jeff Zablocki | 10/17/2007 |
| Matthew Abelman | 10/18/2007 |
| Michael Organ | 10/17/2007 |
| Yaroslav Bilokin | 10/18/2007 |
| Tetsuya Kobayashi | 10/10/2007 |
| Ivan Diamond | 10/22/2007 |
| Peidong Fan | 10/22/2007 |
| Thao Perry | 10/18/2007 |
| Xiaofen Li | 10/18/2007 |
| Rao Kalla | 10/22/2007 |
| Maria Pia Adolfo | 10/22/2007 |
| Lina Yao | 10/22/2007 |
| Robert Jiang | 10/17/2007 |
| Elfatih Elzein | 10/23/2007 |

| DATOS DE LA PARTE QUE RECIBE | |
|---|---|
| Nombre: | CV Therapeutics, Inc. |
| Dirección: | 3172 Porter Drive |
| Ciudad: | Palo Alto |
| Estado/País: | CALIFORNIA |
| Código Postal: | 94304 |
| Total NUMEROS PROPIEDAD: 3 | |

| Tipo de Propiedad | Número |
|---|---|
| Número de la Solicitud: | 60834083 |
| Número de la Solicitud: | 60846428 |
| Número de la Solicitud: | 11829836 |

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Nombre Corresponsal: CV Therapeutics, Inc.

Dirección Línea 1: 3172 Porter Drive

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|---|---|
| NUMERO EXPEDIENTE APODERADO | 06-0223, PRO, & P2 |
| NOMBRE DEL PRESENTADOR: | J. Elin Hartrum |

Total anexos: 45

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# TRASPASO

Caso No.: 06-0223-PRO, 06-0223-P2, & 06-0223
Fecha de Presentación: 27 de julio de 2006, 21 de septiembre de 2006, & 27 de julio de 2007
No. de Serie: 60/834.083, 60/846.428, & 11/829.836

Inventores: Jeff Zablocki
Matthew Abelman
Michael Organ
Yaroslav Bilokin
Ivan Diamond
Maria Pia Adolfo
Lina Yao
Peidong Fan
Elfatih Elzein
Rao Kalla
Thao Perry
Tetsuya Kobayashi
Xiaofen Li
Robert Jiang

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El suscrito por el presente autoriza y le solicita el Comisionado de Patentes y Marcas que expida dicha Patente de Invención a dicho cesionario.

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Este traspaso tiene vigencia desde la fecha de presentación de la solicitud indicada anteriormente.

EN TESTIMONIO DE LO CUAL, he suscrito mi firma.

(firma: ilegible) este día 17 de octubre de 2007; y
Jeff Zablocki

ESTADO DE CALIFORNIA )
CONDADO DE SANTA CLARA )

El 17 de octubre de 2007, ante mí, Marjory Jill Darrow, un Notario Público, compareció personalmente Jeff Zablocki, a quien conozco personalmente que es la persona cuyo nombre está suscrito en el documento adjunto y reconoció ante mí que él firmó el mismo como persona autorizada, y que por medio de su firma en el documento la persona, o entidad, en nombre de quien la persona actúo, firmó el documento.

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(firma: ilegible) este día 22 de octubre de 2007; y

Ivan Diamond

ESTADO DE CALIFORNIA )

CONDADO DE SANTA CLARA )

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(firma: ilegible) este día 22 de octubre de 2007; y
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CONDADO DE SANTA CLARA )

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(firma: ilegible) este día 22 de octubre de 2007; y
Lina Yao

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(firma: ilegible) este día 22 de octubre de 2007; y
Peidong Fan

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(firma: ilegible) este día 23 de octubre de 2007; y
Elfatih Elzein

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EN TESTIMONIO DE LO CUAL, he suscrito mi firma.

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Rao Kalla

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CONDADO DE SANTA CLARA )

El 22 de octubre de 2007, ante mí, Marjory Jill Darrow, un Notario Público, compareció personalmente Rao Kalla, a quien conozco personalmente que es

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(firma: ilegible) este día 18 de octubre de 2007; y
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Mi Comisión Expira el 4 de octubre de 2011

EN TESTIMONIO DE LO CUAL, he suscrito mi firma.

(firma: ilegible) este día 18 de octubre de 2007; y
Thao Perry

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FIRMA DEL NOTARIO PÚBLICO

Marjory Jill Darrow
Comisión # 1771508
Notario Público – California
Condado de Santa Clara
Mi Comisión Expira el 4 de octubre de 2011

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(firma: ilegible) este día 10 de octubre de 2007; y
Tetsuya Kobayashi

ESTADO DE CALIFORNIA )
CONDADO DE SANTA CLARA )

El 10 de octubre de 2007, ante mí, Susan Ahearn, un Notario Público, compareció personalmente Tetsuya Kobayashi, a quien conozco

personalmente que es la persona cuyo nombre está suscrito en el documento adjunto y reconoció ante mí que él firmó el mismo como persona autorizada, y que por medio de su firma en el documento la persona, o entidad, en nombre de quien la persona actúo, firmó el documento.

TESTIFICA mi firma y sello oficial.
(firma: ilegible) (SELLO)
FIRMA DEL NOTARIO PÚBLICO

Susan Ahearn
Comisión # 1526494
Notario Público – California
Condado de Santa Clara
Mi Comisión Expira el 13 de noviembre de 2008

EN TESTIMONIO DE LO CUAL, he suscrito mi firma.

(firma: ilegible) este día 18 de octubre de 2007; y
Xiaofen Li

ESTADO DE CALIFORNIA )
CONDADO DE SANTA CLARA )

El 18 de octubre de 2007, ante mí, Marjory Jill Darrow, un Notario Público, compareció personalmente Xiaofen Li, a quien conozco personalmente que es la persona cuyo nombre está suscrito en el documento adjunto y reconoció ante mí que ella firmó el mismo como persona autorizada, y que por medio de su firma en el documento la persona, o entidad, en nombre de quien la persona actúo, firmó el documento.

TESTIFICA mi firma y sello oficial.
(firma: ilegible) (SELLO)
FIRMA DEL NOTARIO PÚBLICO

Marjory Jill Darrow
Comisión # 1771508
Notario Público – California
Condado de Santa Clara
Mi Comisión Expira el 4 de octubre de 2011

EN TESTIMONIO DE LO CUAL, he suscrito mi firma.

(firma: ilegible) este día 17 de octubre de 2007; y
Robert Jiang

ESTADO DE CALIFORNIA )
CONDADO DE SANTA CLARA )

El 17 de octubre de 2007, ante mí, Marjory Jill Darrow, un Notario Público, compareció personalmente Robert Jiang, a quien conozco personalmente que es la persona cuyo nombre está suscrito en el documento adjunto y reconoció ante mí que él firmó el mismo como persona autorizada, y que por medio de su firma en el documento la persona, o entidad, en nombre de quien la persona actúo, firmó el documento.

TESTIFICA mi firma y sello oficial.
(firma: ilegible) (SELLO)
FIRMA DEL NOTARIO PÚBLICO[1]

Marjory Jill Darrow
Comisión # 1771508
Notario Público – California
Condado de Santa Clara
Mi Comisión Expira el 4 de octubre de 2011



Traducido según mi mejor saber y entender, este día 19 de junio de 2009, en la ciudad de Bogotá, D.C., por Anita Escobar de Tamayo, Traductora Oficial, Res. 2177 de 1989, Ministerio de Justicia, República de Colombia.

[1] Nota del Traductor: Se repite el documento de traspaso con las firmas de cada traductor en hojas aparte.

TRADUCTORA OFICIAL
RES. 2177/89 MINJUSTICIA
ANITA ESCOBAR DE TAMAYO

A 7167492

# THE UNITED STATES OF AMERICA

## TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

January 26, 2009

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF A DOCUMENT RECORDED ON October 25, 2007.

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

P. SWAIN
Certifying Officer

# PATENT ASSIGNMENT

Electronic Version v1.1
Stylesheet Version v1.1

| | |
|---|---|
| SUBMISSION TYPE: | NEW ASSIGNMENT |
| NATURE OF CONVEYANCE: | ASSIGNMENT |

CONVEYING PARTY DATA

| Name | Execution Date |
|---|---|
| Jeff Zablocki | 10/17/2007 |
| Matthew Abelman | 10/18/2007 |
| Michael Organ | 10/17/2007 |
| Yaroslav Bilokin | 10/18/2007 |
| Tetsuya Kobayashi | 10/10/2007 |
| Ivan Diamond | 10/22/2007 |
| Peidong Fan | 10/22/2007 |
| Thao Perry | 10/18/2007 |
| Xiaofen Li | 10/18/2007 |
| Rao Kalla | 10/22/2007 |
| Maria Pia Arolfo | 10/22/2007 |
| Lina Yao | 10/22/2007 |
| Robert Jiang | 10/17/2007 |
| Elfatih Elzein | 10/23/2007 |

RECEIVING PARTY DATA

| | |
|---|---|
| Name: | CV Therapeutics, Inc. |
| Street Address: | 3172 Porter Drive |
| City: | Palo Alto |
| State/Country: | CALIFORNIA |
| Postal Code: | 94304 |

PROPERTY NUMBERS Total: 3

| Property Type | Number |
|---|---|
| Application Number: | 60834083 |
| Application Number: | 60846428 |
| Application Number: | 11829836 |

CORRESPONDENCE DATA

CH $120.00 60834083



550

| | |
|---|---|
| Fax Number: | (650)475-0359 |
| *Correspondence will be sent via US Mail when the fax attempt is unsuccessful.* | |
| Phone: | 650-384-8755 |
| Email: | patentsmailbox@cvt.com |
| Correspondent Name: | CV Therapeutics, Inc. |
| Address Line 1: | 3172 Porter Drive |
| Address Line 4: | Palo Alto, CALIFORNIA 94304 |

| | |
|---|---|
| ATTORNEY DOCKET NUMBER: | 06-0223, PRO, & P2 |
| NAME OF SUBMITTER: | J. Elin Hartrum |

Total Attachments: 45
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# ASSIGNMENT

Case No.: 06-0223-PRO, 06-0223-P2, & 06-0223
Filing Date: July 27, 2006, September 21, 2006, & July 27, 2007
Serial No.: 60/834,083, 60/846,428, & 11/829,836

Inventors: Jeff Zablocki
Matthew Abelman
Michael Organ
Yaroslav Bilokin
Ivan Diamond
Maria Pia Arolfo
Lina Yao
Peidong Fan
Elfatih Elzein
Rao Kalla
Thao Perry
Tetsuya Kobayashi
Xiaofen Li
Robert Jiang

In consideration of One Dollar ($1.00) and other good and valuable considerations in hand paid, the receipt and sufficiency whereof are hereby acknowledged, the undersigned hereby assign to:

**CV Therapeutics, Inc.**

its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in an application for Letters Patent of the United States, entitled:

**ALDH-2 INHIBITORS IN THE TREATMENT OF ADDICTION**

and identified as:

**Case Numbers:** **06-0223-PRO**
**06-0223-P2**
**06-0223**

in the offices of CV Therapeutics, Inc. and in said application and any and all other applications, both United States and foreign, which the undersigned may file, either solely or jointly with others, on said invention or improvements, and in any and all Letters Patent of the United States and foreign countries, which may be obtained on any of said applications, and in any reissue or extension of such patents, and further assigns to said assignee the priority right provided by the International Convention.

The undersigned hereby authorize and request the Commissioner of Patents and Trademarks to issue said Letters Patent to said assignee.

The undersigned hereby authorize and request the attorneys of record in said application to insert in this assignment the filing date and serial number of said application when officially known, and the date of execution of the application.



PATENT
REEL: 020017 FRAME: 0526

The undersigned warrant themselves to be the owners of the entire right, title and interest in said invention or improvements and to have the right to make this assignment, and further warrant that there are no outstanding prior assignments, licenses, or other encumbrances on the interest herein assigned.

For said considerations the undersigned hereby agree, upon the request and at the expense of said assignee, its successors and assigns, to execute any and all divisional, continuation and substitute applications for said invention or improvements, and any necessary oath, affidavit or declaration relating thereto, and any application for the reissue or extension of any Letters Patent that may be granted upon said application and any and all applications and other documents for Letters Patent in foreign countries on said invention or improvements, that said assignee, its successors or assigns may deem necessary or expedient, and for the said considerations the undersigned authorize said assignee to apply for patents for said invention or improvements in its own name in such countries where such procedure is proper and further agree, upon the request of said assignee, its successors and assigns, to cooperate to the best of the ability of the undersigned with said assignee, its successors and assigns, in any proceedings or transactions involving such applications or patents, including the preparation and execution of preliminary statements, giving and producing evidence, and performing any and all other acts necessary to obtain, maintain and enforce said Letters Patent, both United States and foreign, and vest all rights therein hereby conveyed in the assignee, its successors and assigns, whereby said Letters Patent will be held and enjoyed by the said assignee, its successors and assigns, to the full end of the term for which said Letters Patent will be granted, as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment had not been made.

This assignment is effective as of the filing date of the above-indicated application.

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Jeff Zablocki this 17th day of October 2007; and

Jeff Zablocki

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 17th, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Jeff Zablocki, personally known to me (~~or proved to me on the basis of satisfactory evidence~~) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/~~she~~ executed the same in his/~~her~~ authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

Marjory Jill Darrow (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771808
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Matthew Abelman this 18th day of October 2007; and

Matthew Abelman

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 18, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Matthew Abelman, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

(SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this _____ day of _____ 2007; and
Michael Organ

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Michael Organ, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0529

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Yaroslav Bilokin

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Yaroslav Bilokin, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0530

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Ivan Diamond this 22nd day of October 2007; and

Ivan Diamond

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 22, 2007, before me, Marjory Jill Darrow, a Notary Public

Marjory Jill Darrow

personally appeared, Ivan Diamond, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

Marjory Jill Darrow (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771808
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this 22 day of October 2007; and
Maria Pia Arolfo

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 22, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Maria Pia Arolfo, ~~personally known to me (or~~ proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that ~~he~~/she executed the same in ~~his~~/her authorized capacity, and that by ~~his~~/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

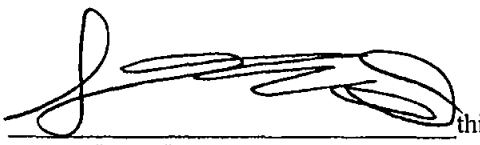
_____ (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771808
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011



PATENT
REEL: 020017 FRAME: 0532

IN TESTIMONY WHEREOF, I have hereunto set my hand.



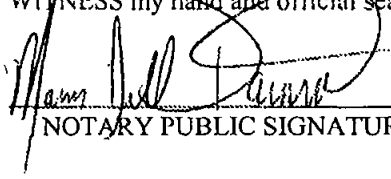
Lina Yao this 22nd day of October 2007; and

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

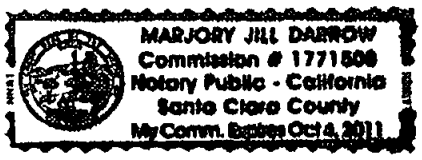
On October 22, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Lina Yao, ~~personally known to me (or~~ proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that ~~he~~/she executed the same in ~~his~~/her authorized capacity, and that by ~~his~~/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.



NOTARY PUBLIC SIGNATURE (SEAL)








560

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this 22nd day of October 2007; and
Peidong Fan

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 22, 2007, before me, Margory Jill Darrow, a Notary Public

personally appeared, Peidong Fan, ~~personally known to me~~ (~~or~~ proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/~~she~~ executed the same in his/~~her~~ authorized capacity, and that by his/~~her~~ signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771688
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Elfatih Elzein this 23 day of October 2007; and
Elfatih Elzein

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 23, 2007, before me, Marjory Jill Darrow,
a Notary Public

personally appeared, Elfatih Elzein, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/~~she~~ executed the same in his/~~her~~ authorized capacity, and that by his/~~her~~ signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771688
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this 22nd day of October 2007; and
Rao Kalla

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 22, 2007, before me, Marjory Jill Darrow,
a Notary Public

personally appeared, Rao Kalla, ~~personally known to me~~ (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/~~she~~ executed the same in his/~~her~~ authorized capacity, and that by his/~~her~~ signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm Expires Oct 4, 2011

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm Expires Oct 4, 2011

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Thao Perry this 18th day of October 2007; and

Thao Perry

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 18, 2007, before me, MARJORY JILL DARROW a Notary Public

personally appeared, Thao Perry, ~~personally known to me~~ (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that ~~he~~/she executed the same in ~~his~~/her authorized capacity, and that by ~~his~~/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

(SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

T Kobayashi this 10th day of October 2007; and
Tetsuya Kobayashi

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 10. , 2007, before me, Susan Ahearn ,
a Notary Public

personally appeared, Tetsuya Kobayashi, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

Susan Ahearn (SEAL)
NOTARY PUBLIC SIGNATURE

SUSAN AHEARN
Commission # 1526494
Notary Public - California
Santa Clara County
My Comm. Expires Nov 13, 2008

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Xiaofen Li this 18th day of October 2007; and

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 18, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Xiaofen Li, ~~personally known to me~~ (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that ~~he~~/she executed the same in ~~his~~/her authorized capacity, and that by ~~his~~/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

NOTARY PUBLIC SIGNATURE (SEAL)

MARJORY JILL DARROW
Commission # 1771608
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

IN TESTIMONY WHEREOF, I have hereunto set my hand.

RJG this 17th day of October 2007; and
Robert Jiang

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On October 17, 2007, before me, Marjory Jill Darrow, a Notary Public

personally appeared, Robert Jiang, ~~personally known to me~~ (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

Marjory Jill Darrow (SEAL)
NOTARY PUBLIC SIGNATURE

MARJORY JILL DARROW
Commission # 1771508
Notary Public - California
Santa Clara County
My Comm. Expires Oct 4, 2011

# ASSIGNMENT

Case No.: 06-0223-PRO, 06-0223-P2, & 06-0223
Filing Date: July 27, 2006, September 21, 2006, & July 27, 2007
Serial No.: 60/834,083, 60/846,428, & 11/829,836

Inventors: Jeff Zablocki
Matthew Abelman
Michael Organ
Yaroslav Bilokin
Ivan Diamond
Maria Pia Arolfo
Lina Yao
Peidong Fan
Elfatih Elzein
Rao Kalla
Thao Perry
Tetsuya Kobayashi
Xiaofen Li
Robert Jiang

In consideration of One Dollar ($1.00) and other good and valuable considerations in hand paid, the receipt and sufficiency whereof are hereby acknowledged, the undersigned hereby assign to:

**CV Therapeutics, Inc.**

its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in an application for Letters Patent of the United States, entitled:

**ALDH-2 INHIBITORS IN THE TREATMENT OF ADDICTION**

and identified as:

**Case Numbers:** **06-0223-PRO**
**06-0223-P2**
**06-0223**

in the offices of CV Therapeutics, Inc. and in said application and any and all other applications, both United States and foreign, which the undersigned may file, either solely or jointly with others, on said invention or improvements, and in any and all Letters Patent of the United States and foreign countries, which may be obtained on any of said applications, and in any reissue or extension of such patents, and further assigns to said assignee the priority right provided by the International Convention.

The undersigned hereby authorize and request the Commissioner of Patents and Trademarks to issue said Letters Patent to said assignee.

The undersigned hereby authorize and request the attorneys of record in said application to insert in this assignment the filing date and serial number of said application when officially known, and the date of execution of the application.



PATENT
REEL: 020017 FRAME: 0541

The undersigned warrant themselves to be the owners of the entire right, title and interest in said invention or improvements and to have the right to make this assignment, and further warrant that there are no outstanding prior assignments, licenses, or other encumbrances on the interest herein assigned.

For said considerations the undersigned hereby agree, upon the request and at the expense of said assignee, its successors and assigns, to execute any and all divisional, continuation and substitute applications for said invention or improvements, and any necessary oath, affidavit or declaration relating thereto, and any application for the reissue or extension of any Letters Patent that may be granted upon said application and any and all applications and other documents for Letters Patent in foreign countries on said invention or improvements, that said assignee, its successors or assigns may deem necessary or expedient, and for the said considerations the undersigned authorize said assignee to apply for patents for said invention or improvements in its own name in such countries where such procedure is proper and further agree, upon the request of said assignee, its successors and assigns, to cooperate to the best of the ability of the undersigned with said assignee, its successors and assigns, in any proceedings or transactions involving such applications or patents, including the preparation and execution of preliminary statements, giving and producing evidence, and performing any and all other acts necessary to obtain, maintain and enforce said Letters Patent, both United States and foreign, and vest all rights therein hereby conveyed in the assignee, its successors and assigns, whereby said Letters Patent will be held and enjoyed by the said assignee, its successors and assigns, to the full end of the term for which said Letters Patent will be granted, as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment had not been made.

This assignment is effective as of the filing date of the above-indicated application.

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Jeff Zablocki

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Jeff Zablocki, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Matthew Abelman

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Matthew Abelman, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0543

570

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Michael Organ

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Michael Organ, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0544

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Y. Bilokin this 18 day of October 2007; and
Yaroslav Bilokin

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Yaroslav Bilokin, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0545

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Ivan Diamond

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Ivan Diamond, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0546

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Maria Pia Arolfo

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Maria Pia Arolfo, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0547

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Lina Yao

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Lina Yao, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0548

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Peidong Fan

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Peidong Fan, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0549

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this _____ day of _____ 2007; and
Elfatih Elzein

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Elfatih Elzein, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0550

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Rao Kalla

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Rao Kalla, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Thao Perry

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Thao Perry, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0552

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Tetsuya Kobayashi

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Tetsuya Kobayashi, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0553

5780

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Xiaofen Li

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Xiaofen Li, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0554

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Robert Jiang

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Robert Jiang, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0555

J82

# ASSIGNMENT

Case No.: 06-0223-PRO, 06-0223-P2, & 06-0223
Filing Date: July 27, 2006, September 21, 2006, & July 27, 2007
Serial No.: 60/834,083, 60/846,428, & 11/829,836

Inventors: Jeff Zablocki
Matthew Abelman
Michael Organ
Yaroslav Bilokin
Ivan Diamond
Maria Pia Arolfo
Lina Yao
Peidong Fan
Elfatih Elzein
Rao Kalla
Thao Perry
Tetsuya Kobayashi
Xiaofen Li
Robert Jiang

In consideration of One Dollar ($1.00) and other good and valuable considerations in hand paid, the receipt and sufficiency whereof are hereby acknowledged, the undersigned hereby assign to:

**CV Therapeutics, Inc.**

its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in an application for Letters Patent of the United States, entitled:

**ALDH-2 INHIBITORS IN THE TREATMENT OF ADDICTION**

and identified as:

**Case Numbers:** **06-0223-PRO**
**06-0223-P2**
**06-0223**

in the offices of CV Therapeutics, Inc. and in said application and any and all other applications, both United States and foreign, which the undersigned may file, either solely or jointly with others, on said invention or improvements, and in any and all Letters Patent of the United States and foreign countries, which may be obtained on any of said applications, and in any reissue or extension of such patents, and further assigns to said assignee the priority right provided by the International Convention.

The undersigned hereby authorize and request the Commissioner of Patents and Trademarks to issue said Letters Patent to said assignee.

The undersigned hereby authorize and request the attorneys of record in said application to insert in this assignment the filing date and serial number of said application when officially known, and the date of execution of the application.



PATENT
REEL: 020017 FRAME: 0556

The undersigned warrant themselves to be the owners of the entire right, title and interest in said invention or improvements and to have the right to make this assignment, and further warrant that there are no outstanding prior assignments, licenses, or other encumbrances on the interest herein assigned.

For said considerations the undersigned hereby agree, upon the request and at the expense of said assignee, its successors and assigns, to execute any and all divisional, continuation and substitute applications for said invention or improvements, and any necessary oath, affidavit or declaration relating thereto, and any application for the reissue or extension of any Letters Patent that may be granted upon said application and any and all applications and other documents for Letters Patent in foreign countries on said invention or improvements, that said assignee, its successors or assigns may deem necessary or expedient, and for the said considerations the undersigned authorize said assignee to apply for patents for said invention or improvements in its own name in such countries where such procedure is proper and further agree, upon the request of said assignee, its successors and assigns, to cooperate to the best of the ability of the undersigned with said assignee, its successors and assigns, in any proceedings or transactions involving such applications or patents, including the preparation and execution of preliminary statements, giving and producing evidence, and performing any and all other acts necessary to obtain, maintain and enforce said Letters Patent, both United States and foreign, and vest all rights therein hereby conveyed in the assignee, its successors and assigns, whereby said Letters Patent will be held and enjoyed by the said assignee, its successors and assigns, to the full end of the term for which said Letters Patent will be granted, as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment had not been made.

This assignment is effective as of the filing date of the above-indicated application.

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Jeff Zablocki

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Jeff Zablocki, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Matthew Abelman

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Matthew Abelman, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE





385

IN TESTIMONY WHEREOF, I have hereunto set my hand.

Michael G. Organ this 17th day of October 2007; and
Michael Organ

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Michael Organ, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0559

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Yaroslav Bilokin

STATE OF )
COUNTY OF )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Yaroslav Bilokin, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0560

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Ivan Diamond

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Ivan Diamond, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0561

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Maria Pia Arolfo

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Maria Pia Arolfo, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0562

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Lina Yao

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Lina Yao, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0563

590

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Peidong Fan

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Peidong Fan, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0564

591

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Elfatih Elzein

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Elfatih Elzein, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0565

592

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Rao Kalla

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Rao Kalla, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0566

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this _____ day of _____ 2007; and
Thao Perry

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Thao Perry, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0567

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Tetsuya Kobayashi

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Tetsuya Kobayashi, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



PATENT
REEL: 020017 FRAME: 0568

595

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____ this _____ day of _____ 2007; and
Xiaofen Li

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Xiaofen Li, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE





596

IN TESTIMONY WHEREOF, I have hereunto set my hand.

_____this _____ day of _____ 2007; and
Robert Jiang

STATE OF CALIFORNIA )
COUNTY OF SANTA CLARA )

On _____, 2007, before me, _____,
a Notary Public

personally appeared, Robert Jiang, personally known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument and acknowledged to me that he/she executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or the entity upon behalf of which the person acted, executed the instrument.

WITNESS my hand and official seal.

_____ (SEAL)
NOTARY PUBLIC SIGNATURE



RECORDED: 10/25/2007

PATENT
REEL: 020017 FRAME: 0570

597

TRADUCCION OFICIAL No. 2009-07-16-423

DE UN DOCUMENTO ESCRITO EN INGLES

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Poder de Representación Legal, documento escrito en inglés y en español.

**Estado de California**

**Secretario de Estado**

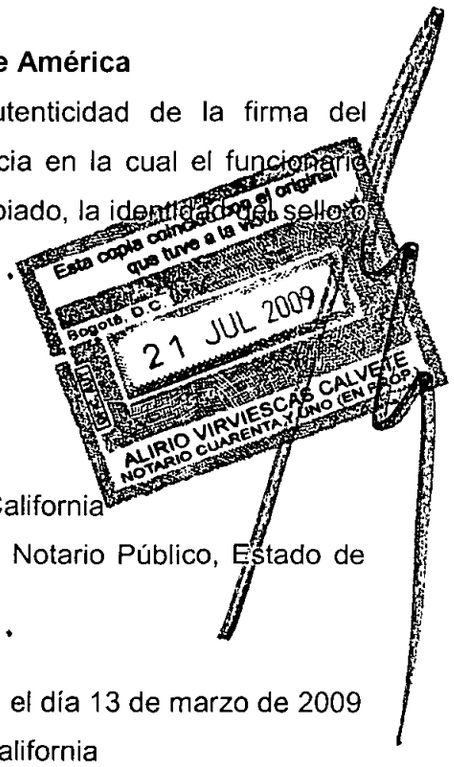
**APOSTILLA**

(Convención de La Haya del 5 de octubre de 1961)

**Solicitado para uso en Colombia.**

**No es para uso dentro de los Estados Unidos de América**

El propósito de la Apostilla es certificar la autenticidad de la firma del funcionario que firma el documento, la competencia en la cual el funcionario que firma el documento actuó, y, cuando sea apropiado, la identidad del sello o estampilla.




1. País: Estados Unidos de América
   Este documento público
2. ha sido firmado por Cathy M. Wong
3. actuando como Notario Público, Estado de California
4. lleva el sello/estampilla de Cathy M. Wong, Notario Público, Estado de California

**Certificado**

5. en Sacramento, California
6. el día 13 de marzo de 2009
7. por el Subsecretario de Estado, Estado de California
8. No. 653372
9. Sello/Estampilla
   El Gran Sello del Estado de California
10. Firma: (ilegible)
    Secretario de Estado
    Por: firma: ilegible)

TRADUCTORA OFICIAL
RES. 2177/89 MINJUSTICIA

ANITA ESCOBAR DE TAMAYO

**RECONOCIMIENTO**

Estado de California )
) ss.
Condado de Santa Clara )

El 21 de enero de 2009, ante mí, Cathy M. Wong, Notario Público, compareció personalmente Daniel W. Collins, quien me demostró con base en evidencia satisfactoria que es la persona cuyo nombre está suscrito en el instrumento adjunto y reconoció ante mí que él firmó el mismo como persona autorizada; y que por medio de su firma sobre el instrumento la persona, o entidad en nombre de quien la persona actúo, firmó el documento.

Certifico bajo PENA DE PERJURIO bajo las leyes del Estado de California que el párrafo anterior es fiel y correcto.

TESTIFICA mi firma y sello oficial.
Firma: (ilegible)

Cathy M. Wong
COMM. # 1672368
Notario Público – California
Condado de Santa Clara
Mi Comisión Expira el 4 de junio de 2010

(Sello)

Este Certificado se adjunta para:
Título o Tipo de Documento: Poder – Doctores Alicia Lloreda, Gustavo Tamayo, Ignacio Santamaría, Enrique Alvarez
Número de Páginas: 2
Fecha del Documento: 1 / 21 / 09
Signatario(s) Diferente al Nombrado Arriba: Ninguno
Número Telefónico del Notario: 650-290-3550

Esta copia coincide con el original que tuve a la vista
Bogotá, D.C.
21 JUL 2009
ALIRIO VIRVIESCAS CALVETE
NOTARIO CUARENTA Y UNO (EN PROP.)

Traducido según mi mejor saber y entender, este día 16 de julio de 2009, en la ciudad de Bogotá, D.C., por Anita Escobar de Tamayo, Traductora Oficial, Res. 2177 de 1989, Ministerio de Justicia, República de Colombia.

TRADUCTORA OFICIAL
RES. 2177/89 MINJUSTICIA
ANITA ESCOBAR DE TAMAYO

06-223-CO
10A

Los abajo firmados,

**CV THERAPEUTICS, INC.**

una sociedad organizada y existente bajo las leyes de Estado de Delaware, Estados Unidos de América
con domicilio en
3172 Porter Drive, Palo Alto, California 94304, Estados Unidos de América y 1013 Centre Road, Ciudad de Wilmington, Condado de New Castle, Delaware, Estados Unidos de América

nombramos por el presente a ALICIA LLOREDA y/o GUSTAVO TAMAYO y/o IGNACIO SANTAMARIA y/o ENRIQUE ALVAREZ, domiciliados en Bogotá, Colombia, con oficinas en dicha ciudad, Calle 72 No. 5-83, piso 6o., nuestros representantes legales y verdaderos en Bogotá, con facultades para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales, con el fin de dar cumplimiento al parágrafo 1o. del artículo 543 del decreto 410 de 1971.

Los doctores ALICIA LLOREDA y/o GUSTAVO TAMAYO y/o IGNACIO SANTAMARIA y/o ENRIQUE ALVAREZ, o los apoderados que cualquiera de ellos designe, quedan especialmente facultados para representarnos ante las autoridades colombianas, administrativas, judiciales o de policía, en todos los trámites relativos a la propiedad industrial, a cuyo efecto les facultamos para dar ante dichas autoridades todos los pasos necesarios al objeto indicado; elevar solicitudes, declaraciones, reclamos, recursos y presentar desistimientos; formular descripciones, enmiendas, limitar el alcance de las solicitudes, presentar oposiciones y apelaciones; instaurar cancelaciones, renunciar total o parcialmente a los registros, abonar todos los impuestos y cuotas y efectuar todo otro pago determinado por la ley; recibir todos los documentos y valores, dando el descargo respectivo; llenar cualesquiera otros requisitos y tomar, en fin, todas las medidas que creyeren conducentes al resguardo de nuestros intereses; y en caso de producirse oposición, pasando los antecedentes a los tribunales, quedan facultados para tomar intervención como demandantes o demandados ante los jueces que sean competentes, pudiendo transar, someter a árbitros, desistir, percibir y apelar, con todas las demás facultades que resulten necesarias, y por el presente declaramos desde ahora válido y bueno cuanto dichos representantes y/o los apoderados que cualquiera de ellos nombre hicieren en nuestro beneficio.

Asimismo, facultamos a los doctores ALICIA LLOREDA y/o GUSTAVO TAMAYO y/o IGNACIO SANTAMARIA y/o ENRIQUE ALVAREZ, para solicitar en la Oficina de Propiedad Industrial la protocolización de este documento y de cualquier poder que cualquiera de ellos otorgue a nombre nuestro.

Dado y firmado

Esta copia coincide con el original que tuvo a la vista
Bogotá, D.C.
21 JUL 2009
ALIRIO VIRVIESCAS CALVETE
NOTARIO CUARENTA Y UNO

The undersigned,

**CV THERAPEUTICS, INC.**

a corporation organized and existing under the laws of State of Delaware, United States of America

domiciled at
3172 Porter Drive, Palo Alto, California 94304, United States of America and 1013 Centre Road, City of Wilmington, County of New Castle, Delaware, United States of America

hereby appoint, ALICIA LLOREDA and/or GUSTAVO TAMAYO and/or IGNACIO SANTAMARIA and/or ENRIQUE ALVAREZ, of Bogota, Colombia, with offices in said town, Calle 72 No. 5-83, Sixth Floor, our true and legal representatives in Bogotá, with power to receive notifications and to appoint judicial or extrajudicial agents, so as to comply with paragraph 1o. of article 543 of decree 410 of 1971.

Doctors ALICIA LLOREDA and/or GUSTAVO TAMAYO and/or IGNACIO SANTAMARIA and/or ENRIQUE ALVAREZ, or the agents by any one of them appointed, are especially empowered to represent us before the administrative, judicial or police authorities of Colombia, in all proceedings pertaining to industrial property, for which purpose we authorize them to take before the said authorities all the necessary steps for the said end; to file petitions, declarations, claims, recourses and withdrawals, to draw up specifications; to file amendments, to limit the scope of applications, file oppositions; file cancellations total or partially waive registrations, to pay all installments and effect all other payments prescribed by law; to receive proper receipts, to comply with all other requirements and, in short, to take all those measures which they may deem fit for the protection of our interests; and in case of opposition, where the proceedings be passed over to the courts, we authorize said agents to act on our behalf, as complainants as well as defendants, before the competent judges, with full powers to make arrangements, to submit to arbitrators, to desist, to collect, to appeal and, in short, with all such further powers as may be required, and we hereby declare from now on as valid and good all that said representatives and/or the agents by any one of them appointed may do for our benefit.

We also authorize doctors ALICIA LLOREDA and/or GUSTAVO TAMAYO and/or IGNACIO SANTAMARIA and/or ENRIQUE ALVAREZ, to apply for protocolization of this document and of any power of attorney by any one of them conferred in our name, before the Office of Industrial Property.

Given and signed this 21 day of January of 2009

Signature

Name Daniel W. Collins
VP Legal, Intellectual Property

NOTARY SHOULD EXECUTE OTHER SIDE

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CERTIFICADO NOTARIAL (SOCIEDAD)

Estado de {ss
Condado de
Hoy de de 200 , compareció

, a quien conozco y de quien me consta que firmó el anterior documento, y quien debidamente juramentado declaró y dijo que él es

de

la compañía nombrada en el documento que antecede, una sociedad debidamente organizada y existente conforme a las leyes del Estado de , , domiciliada en la ciudad de , Condado de , Estado de , , legalmente constituida para un período de duración que aún no ha terminado, que ejerce su objeto social; conforme a las leyes del Estado de , ; que el declarante está autorizado por dicha Compañía para conferir este poder; que el sello estampado por él al pie de tal poder, en nombre y en representación de la expresada Compañía, es el sello oficial de ella; y que el objeto para el cual se confiere este instrumento está comprendido dentro del de la sociedad.
El suscrito Notario ha visto los Estatutos de incorporación firmados en , Estado de , con fecha , y certifica que de conformidad con la legislación de dicho Estado la anterior declaración juramentada es prueba suficiente de que los hechos sobre que ella versa son ciertos o verdaderos, de lo cual da fe.

Notary Public (Notario Público).

NOTARIAL CERTIFICATE (CORPORATION)

State of {ss
County of
On this day of , 200 , personally came

to me known, and known to me to be the person who signed the foregoing instrument, who being by me duly sworn did depose and say that he is the

of

, the company named within, a Corporation organized and existing under the laws of the State of , , domiciled at the City of , County of , State of , , legally constituted for a period of tenure not yet expired, which exercises its corporate purposes according to the laws of the State of , ; that the deponent is authorized by said Company to confer this power of attorney; that the seal affixed hereunto by the deponent in the name and on behalf of said Corporation is the corporate seal thereof; and that the purpose for which this instrument is granted is included within the corporate purposes of the Company.
The undersigned Notary Public has seen the articles of Incorporation of said Corporation executed at State of , under date of , and hereby certifies that under the laws of said State the foregoing acknowledgment is sufficient proof that the facts therein contained are true; which he attests.

Esta copia es fiel y coincide con el original que tuve a la vista
Bogotá, D.C.
21 JUL 2009
ALIRIO VIRVIESCAS CALVETE
NOTARIO CUARENTA

SEE ATTACHED ACKNOWLEDGMENT

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# State of California

## SECRETARY OF STATE

***Requested for use in Colombia.***
***Not for use within the United States of America.***
The purpose of the Apostille is to certify the authenticity of the signature of the official signing the document, the capacity in which the official signing the document has acted, and, where appropriate, the identity of the seal or stamp.

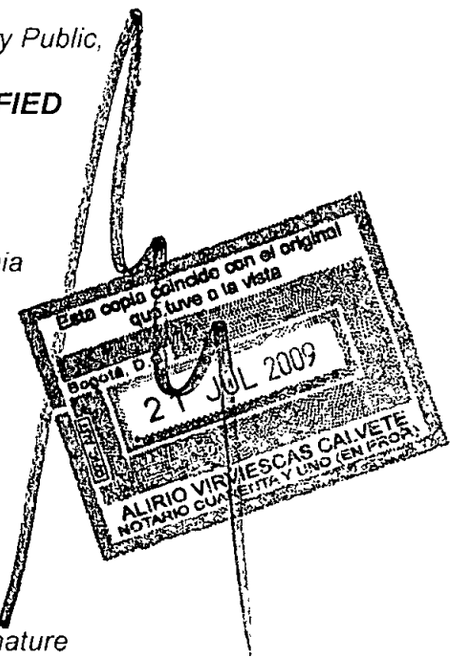
## *APOSTILLE*

*(Convention de La Haye du 5 octobre 1961)*

1. *Country: United States of America*
   *This public document*
2. *has been signed by Cathy M. Wong*
3. *acting in the capacity of Notary Public, State of California*
4. *bears the seal/stamp of Cathy M. Wong, Notary Public, State of California*

***CERTIFIED***

5. *At Sacramento, California*
6. *the 13th day of March 2009*
7. *by Deputy Secretary of State, State of California*
8. *No. 653372*
9. *Seal/Stamp:*




10. *Signature*

Jena Bowen
Secretary of State

*BY* _____

NP-24 A (REV. 1-07)

OSP 06 99602

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**ACKNOWLEDGMENT**

State of: California
County of: SANTA CLARA

On JANUARY 21, 2009, before me Cathy M. Wong, Notary Public
(here insert name and title of the officer)
personally appeared DANIEL W. COLLINS

who proved to me on the basis of satisfactory evidence to be the person ~~(s)~~ whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies~~)~~, and that by his/her/their signature(~~s~~) on the instrument the person(~~s~~), or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State of California that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

CATHY M. WONG
COMM. #1672368
Notary Public - California
Santa Clara County
My Comm. Expires Jun. 4, 2010

Signature _____

(Seal)

This Certificate is attached to:

Title or type of document: POWER OF ATTORNEY - DRS ALICIA LLORENTE AGUSTAVO TAMAYO IGNACIO SANDOVAL & ENRIQUE ALVAREZ

Number of pages: 2

Date of document: 1/21/09

Signer(s) other than named above: NONE

Notary Phone Number: 650-290-3550

Esta copia es fiel a la que tuve a la vista
Bogotá D.C. 21 JUL 2009
ALIRIVIESCAS CALVETE
NOTARIA CUARENTA Y UNO (E.P.)

TRADUCCION OFICIAL No. 2009-07-21-424
DE UN DOCUMENTO ESCRITO EN INGLES

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Delaware

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El Primer Estado 

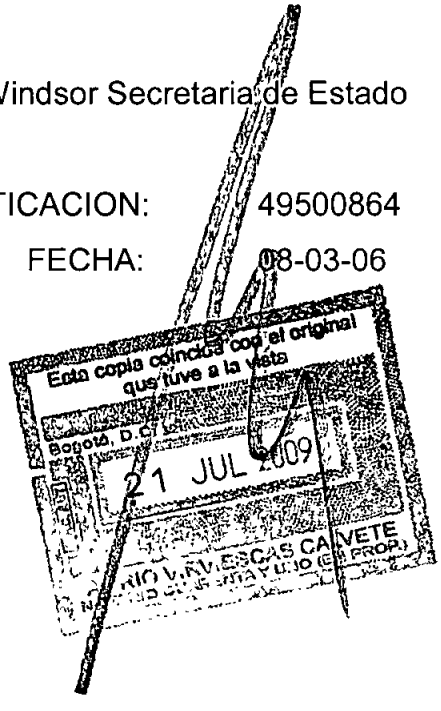
YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, POR EL PRESENTE CERTIFICO QUE LA COPIA ADJUNTA ES UNA COPIA FIEL Y CORRECTA DEL CERTIFICADO DE "CV THERAPEUTICS, INC." MODIFICADO, REGISTRADO EN ESTA OFICINA EL TERCER DIA DE AGOSTO, A.D. DE 2006, A LAS 7:03 P.M.

UNA COPIA REGISTRADA DE ESTE CERTIFICADO HA SIDO ENVIADA AL REGISTRADOR DE ESCRITURAS DEL CONDADO DE NEW CASTE.

(firma: ilegible)
Harriet Smith Windsor Secretaria de Estado

2248911 8100 AUTENTICACION: 49500864
060731642 FECHA: 08-03-06

Sello de la Oficina del Secretario de Delaware.




605

Estado de Delaware
Secretario de Estado
División de Sociedades
ENTREGADO 07:05 PM 08/03/2006
REGISTRADO 07:03 PM 08/03/2006
SRV 060731643 -2248911 ARCHIVO

**CV Therapeutics, Inc.**

**CERTIFICADO DE INCORPORACION**

**MODIFICADO Y RESCRITO**
**(registrado según la sec. 242 & 245)**

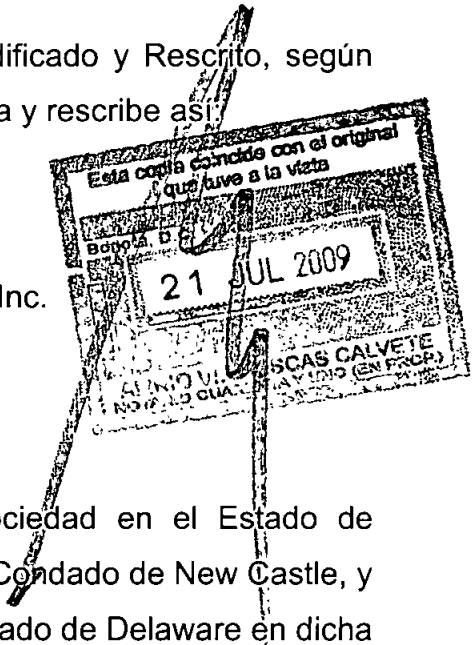
**DE**

**CV THERAPEUTICS, INC.**

Louis G. Lange M.D., Ph.D. por el presente certifica:

1. El es el Presidente de la Junta y Principal Funcionario Ejecutivo de CV Therapeutics, Inc., una sociedad organizada y existente bajo las leyes del Estado del Estado de Delaware. El Certificado de Incorporación original fue registrado ante el Secretario de Estado de Delaware el 11 de diciembre de 1990.

2. El Certificado de Incorporación Modificado y Rescrito, según se modificó, de esta Sociedad por el presente se modifica y rescribe así:




**I.**

El nombre de esta Sociedad es CV Therapeutics, Inc.

**II.**

La dirección de la oficina registrada de la Sociedad en el Estado de Delaware es 1013 Centre Road, Ciudad de Wilmington, Condado de New Castle, y el nombre del agente registrado de la Sociedad en el Estado de Delaware en dicha dirección es The Prentice-Hall Corporation System, Inc.



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**III.**

El propósito de la Sociedad es comprometerse en cualquier acto o actividad legal para el cual una sociedad puede organizarse bajo la Ley General de Sociedades del Estado de Delaware.

**IV.**

A. La Sociedad está autorizada para emitir dos clases de acciones designadas, respectivamente "Acciones Comunes" y "Acciones Preferentes". El número total de acciones que la Sociedad está autorizada para emitir es ciento ochenta y cinco millones (185.000.000) de acciones. Ciento ochenta millones (180.000.000) de acciones serán Acciones Comunes, cada una con un valor a la par de un décimo de un centavo ($.001). Cinco millones (5.000.000) de acciones serán Acciones Preferentes, cada una con un valor a la par de un décimo de un centavo ($.001).

B. Las Acciones Preferentes pueden ser emitidas de tiempo en tiempo en una o más series. La Junta Directiva por el presente está autorizada, registrando un certificado (una "Designación de Acciones Preferentes") según la Ley General de Sociedades de Delaware, para fijar o alterar de tiempo en tiempo la designación, facultades, preferencias y derechos de las acciones de dichas series y las salvedades, limitaciones o restricciones de cualquier serie no emitida completamente de Acciones Preferentes, y establecer de tiempo en tiempo el número de acciones que constituyen cualquiera de dichas series o cualquiera de ellas; aumentar o disminuir el número de acciones de cualquier serie subsiguiente a la emisión de acciones de esa serie, pero no inferior al número de acciones de dicha serie entonces en manos del público. En caso de que el número de acciones

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Bogotá, D.C. 21 JUL 2009

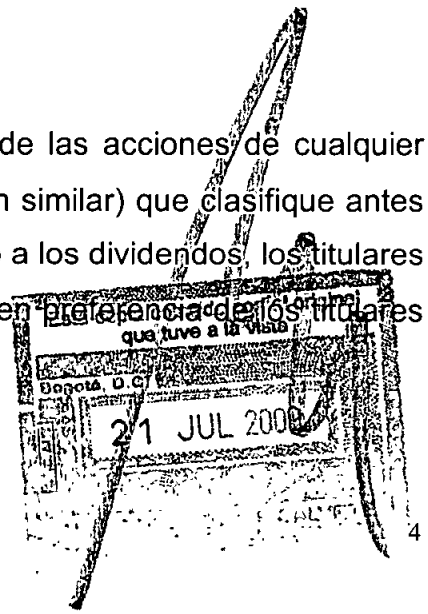
de una serie sea disminuido de conformidad con la oración anterior, las acciones que constituyen dicha disminución reasumirán el estado que tenían anterior a la adopción de la resolución que originalmente fijaba el número de acciones de dicha serie.

C. Los derechos, preferencias, privilegios y restricciones otorgados a e impuestos sobre la primera serie de dichas Acciones Preferentes, la Serie A Junior de las Acciones Preferentes que Participan, son como se indica a continuación en este Artículo Cuarto.

**Sección 1. Designación y Suma.** Ochocientos cincuenta mil (850.000) acciones de las Acciones Preferentes, valor a la par $.001, son designadas "Serie A Acciones Preferentes Junior que Participan" con las designaciones y los facultades, preferencias y derechos y las salvedades, limitaciones y restricciones en el presente especificados (las "Acciones Preferentes Junior"). Dicho número de acciones puede ser aumentado o disminuido por resolución de la Junta Directiva; *siempre y cuando,* que ninguna disminución reduzca el número de acciones de las Acciones Preferentes Junior a un número inferior al número de acciones entonces en manos del público más el número de acciones reservado para la emisión sobre el ejercicio de las opciones, derechos o garantías en manos del público o sobre la conversión de los valores en manos del público emitidos por la Sociedad convertibles en Acciones Preferentes Junior.

**Sección 2. Dividendos y Distribuciones.**

(A) Sujeto a los derechos de los titulares de las acciones de cualquier serie de las Acciones Preferentes (o cualquier acción similar) que clasifique antes y superior a las Acciones Preferentes Junior respecto a los dividendos, los titulares de las acciones de las Acciones Preferentes Junior, en preferencia de los titulares






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de las Acciones Ordinarias y de cualquier otra acción junior, estarán facultados para recibir, cuando, como y si hubiese sido declarado por la Junta Directiva de fondos legalmente disponibles para el propósito, dividendos trimestrales pagaderos en efectivo el primer día de abril, julio, octubre y enero en cada año (siendo dicha fecha referida en el presente como "Fecha de Pago de Dividendos Trimestralmente"), comenzando la primera Fecha de Pago de Dividendos Trimestralmente después de la primera emisión de una acción de fracción de una acción de las Acciones Preferentes Junior, en una suma por acción (redondeada al centavo más cercano) igual al mayor de (a) $1.00 o (b) sujeto a las disposiciones para ajuste posteriormente establecidas, 100 veces el agregado por suma de acción de todos los dividendos en efectivo, y 100 veces el agregado por suma de acción (pagadero en especie) de todos los dividendos no en efectivo u otra distribución, fuera de un dividendo pagadero en acciones de las Acciones Ordinarias o una subdivisión de las acciones en manos del público de las Acciones Ordinarias (por reclasificación o de lo contrario), declaradas sobre las Acciones Ordinarias desde la Fecha de Pago de Dividendos Trimestralmente inmediatamente anterior o, respecto a la primera Fecha de Pago de Dividendos Trimestralmente, desde la primera emisión de cualquier acción o fracción de una acción de las Acciones Preferentes Junior. En el evento en que la Sociedad en cualquier momento declare o pague un dividendo de las Acciones Ordinarias pagaderas en acciones de las Acciones Ordinarias, o efectúe una subdivisión o combinación o consolidación de las acciones en manos del público de las Acciones Ordinarias (por reclasificación o de lo contrario por pago de un dividendo en acciones de las Acciones Ordinarias) en un número mayor o menor de acciones de las Acciones Ordinarias, entonces en cada caso la suma a la cual los titulares de acciones de las Acciones Preferentes Junior estaban facultados inmediatamente antes de dicho evento bajo la cláusula (b) de la oración precedente se ajustará multiplicando dicha suma por una fracción cuyo numerador

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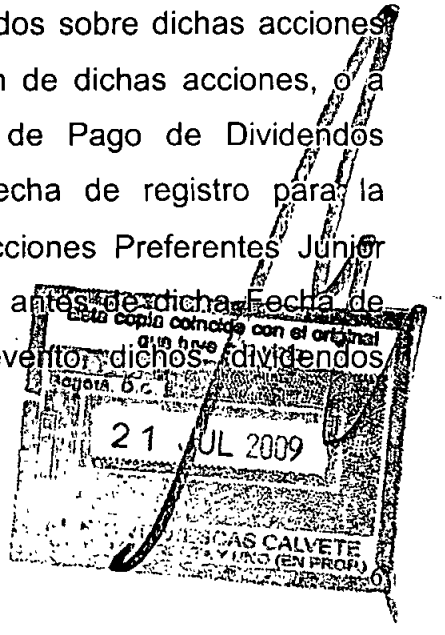


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del cual es el número de acciones de las Acciones Ordinarias en manos del público inmediatamente después de dicho evento y el denominador del cual es el número de acciones de las Acciones Ordinarias que estaban en manos del público inmediatamente antes de dicho evento.

(B) La Sociedad declarará un dividendo o distribución sobre las Acciones Preferentes Junior según se dispone en el párrafo (A) de esta Sección inmediatamente después de que declare un dividendo o distribución sobre las Acciones Ordinarias (fuera de un dividendo pagadero en acciones de las Acciones Ordinarias); *siempre y cuando,* que en el evento en que ningún dividendo o distribución haya sido declarado sobre las Acciones Ordinarias durante el período entre la Fecha de Pago de Dividendos Trimestralmente y la Fecha de Pago de Dividendos Trimestralmente subsiguiente, un dividendo de $1.00 por acción sobre las Acciones Preferentes Junior será sin embargo pagadero sobre dicha Fecha de Pago de Dividendos Trimestralmente subsiguiente.

(C) Los dividendos comenzarán a acumularse y serán acumulativos sobre las acciones en manos del público de las Acciones Preferentes Junior desde la Fecha de Pago de Dividendos Trimestralmente siguiente precedente a la fecha de emisión de dichas acciones, a menos que la fecha de emisión de dichas acciones sea anterior a la fecha de registro para la primera Fecha de Pago de Dividendos Trimestralmente, en cuyo caso los dividendos sobre dichas acciones comenzarán a acumularse desde la fecha de emisión de dichas acciones, o a menos que la fecha de emisión sea una Fecha de Pago de Dividendos Trimestralmente o sea una fecha posterior a la fecha de registro para la determinación de los titulares de acciones de las Acciones Preferentes Junior facultadas para recibir un dividendo trimestralmente y antes de dicha Fecha de Pago de Dividendos Trimestralmente, en cuyo evento dichos dividendos



comenzarán a acumularse y serán acumulativos desde dicha Fecha de Pago de Dividendos Trimestralmente. Los dividendos acumulados pero sin pagar no devengarán intereses. Los dividendos pagados sobre las acciones de las Acciones Preferentes en una cantidad menor a la cantidad total de dichos dividendos al momento, acumulados y pagaderos en tales acciones serán colocadas proporcionalmente sobre una base de acción-por-acción entre tales acciones circulantes. La Junta Directiva puede fijar una fecha según registro para la determinación de titulares de Acciones Preferentes Subordinadas con derecho a recibir pago de un dividendo o distribución declarada sobre estos, cuya fecha según registro no será mayor de 60 días antes de la fecha fijada para el pago de los mismos.

**Sección 3. Derechos de Voto**. Los accionistas de la Acción Preferente Subordinada tendrán los siguientes derechos de voto:

(A) Sujeta a la provisión para ajuste indicada de aquí en adelante, cada Acción Preferente Subordinada le dará derecho al titular de la misma de 100 votos sobre todos los asuntos sometidos a un voto de los accionistas de la Sociedad. En caso que la sociedad, en cualquier momento, declare o pague cualquier dividendo sobre las Acciones Corrientes pagaderas en acciones corrientes, o efectúe una subdivisión o combinación o consolidación de las acciones circulantes de las Acciones Corrientes (mediante reclasificación o de otra forma diferente al pago de un dividendo en acciones corrientes) en un número mayor o menor de acciones corrientes, entonces en cada caso, el número de votos por acción al cual los titulares de Acciones Preferentes Subordinadas tenían derecho inmediatamente antes que tal evento, será ajustado multiplicando tal número por una fracción, cuyo numerador es el número de acciones corrientes circulantes

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inmediatamente después de tal evento, y cuyo denominador es el número de acciones ordinarias circulantes inmediatamente antes de tal evento.

(B) Excepto que se provea de otra forma en la presente, en cualquier Designación de Acción Preferente, o por ley, los titulares de Acciones Preferentes Subordinadas y los titulares de acciones corrientes y cualquier otra acción de capital de la sociedad que tienen derechos de voto general, votarán juntos como una clase sobre todos los asuntos sometidos a voto de los accionistas de la Sociedad.

(C) Excepto como se indique aquí, o esté de otra forma previsto en la ley, los titulares de Acción Preferente Subordinada no tendrán derechos de voto especiales y no se requerirá su consentimiento (excepto para la extensión a la que tienen derecho para votar con accionistas de acciones corrientes como se indicó en la presente) para tomar cualquier acción empresarial.

**Sección 4. Ciertas Restricciones**

(A) Siempre que dividendos trimestrales u otros dividendos o distribuciones pagaderas sobre la Acción Preferente Subordinada provista en la sección 2 estén en forma de arras, posteriormente y hasta que todos los dividendos y distribuciones acumuladas y no pagadas, declaradas o no, sobre acciones de Acción Preferente Subordinada circulante, hayan sido pagadas en su totalidad, la Sociedad:

(i) No declarará o pagará dividendos o hará cualquier otra distribución sobre cualquier acción con derecho de prelación inferior (en cuanto a

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Bogotá, D.C.
21 JUL 2009
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dividendos o a la liquidación, disolución o banca rota) a la Acción Preferente Subordinada;

(ii) No declarará o pagará dividendos o hará cualquier otra distribución sobre cualquier acción con derecho de prelación inferior sobre una paridad (en cuanto a dividendos o a la liquidación, disolución o banca rota) con Acción Preferente Subordinada, excepto dividendos pagados proporcionalmente sobre la Acción Preferente Subordinada y cualquier acción de paridad sobre la cual los dividendos son pagaderos o en arras en proporción a las cantidades totales sobre las cuales los accionistas de tales acciones tienen luego derecho;

(iii) No redimirá o comprará o adquirirá de otra forma para consideración de cualquier acción con derecho de prelación inferior (en cuanto a dividendos o a la liquidación, disolución o banca rota) a la Acción Preferente Subordinada, siempre y cuando la sociedad pueda en cualquier momento redimir, comprar o adquirir de otra forma acciones de cualquier acción subordinada a cambio de acciones de cualquier acción de derecho de prelación inferior de la Sociedad (en cuanto a dividendos o a la liquidación, disolución o banca rota) a la Acción Preferente Subordinada, o:

(iv) No redimirá o comprará o adquirirá de otra forma para consideración ninguna acción con derecho de prelación inferior, o cualquier acción con derecho de prelación inferior sobre una paridad (en cuanto a dividendos o a la liquidación, disolución o banca rota) con la Acción Preferente Subordinada, excepto de acuerdo con una oferta de compra hecha por escrito o mediante publicación (determinada por la Junta Directiva) a todos los accionistas de tales acciones en los términos que según la Junta Directiva, después de consideración de las tasas de dividendo anuales respectivas, u otros derechos relativos

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Bogotá, D.C.
21 JUL 2009
NOTARIA CALVETE
DOS Y UNO (E.P.)

preferencias de las respectivas series y clases, determinará de buena fe que producirán en un tratamiento justo y equitativo entre las respectivas series o clases.

(B) La Sociedad no permitirá que cualquier subsidiaria de la Sociedad compre o adquiera de otra forma para consideración, cualquier acción de las acciones de la Sociedad, a menos que la Sociedad pueda, bajo el parágrafo (A) de esta sección 4, comprar o adquirir de otra forma tales acciones en tal momento y de tal forma.

**Sección 5. Acciones Readquiridas**. Cualquier acción de las Acciones Preferentes Subordinadas comprada o de otra forma adquirida por la Sociedad de cualquier manera será retirada y cancelada oportunamente después de la adquisición de la misma. Todas estas acciones, a su cancelación, se volverán acciones autorizadas pero no emitidas de Acciones Preferentes y pueden ser reemitidas como parte de una nueva serie de acciones Preferentes sujetas a las condiciones y restricciones sobre emisión indicadas en la presente, o en cualquier Designación de Acciones Preferentes o como sea requerido de otra forma por la ley.

**Sección 6. Liquidación, Disolución o Banca Rota**. Ante cualquier liquidación, disolución o banca rota de la Sociedad, no se hará ninguna distribución (1) a los accionistas de acciones con derecho de prelación inferior (ya sea como dividendos o a la liquidación, disolución o banca rota) a las Acciones Preferentes Subordinadas a menos que, previamente a esto, los accionistas de Acciones Preferentes Subordinadas hayan recibido $100 por acción, más una cantidad igual a los dividendos acumulados y no pagados y las distribuciones de estos, declarados o no, a la fecha de tal pago, siempre y cuando los accionistas de

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acciones de Acciones Preferentes Subordinadas tengan derecho a recibir una cantidad adicional por acción, sujeta a las provisiones para ajuste indicadas de aquí en adelante, igual a 100 veces la cantidad adicional a ser distribuida por acción a los accionistas de Acciones Corrientes, o (2) a los accionistas de acciones con derecho a prelación inferior sobre una paridad (ya sea en cuanto a dividendos o a la liquidación, disolución o banca rota) con las Acciones Preferentes Subordinadas, excepto distribuciones hechas proporcionalmente sobre las Acciones Preferentes Subordinadas y toda acción de paridad en proporción a las cantidades totales a las cuales los accionistas de tales acciones tienen derecho al momento de tal liquidación, disolución o quiebra. En el evento que la Sociedad, a cualquier momento declare o pague cualquier dividendo sobre Acciones Corrientes pagaderas en acciones Corrientes, o efectúe una subdivisión o combinación o consolidación de las acciones corrientes circulantes (mediante reclasificación o mediante otra forma diferente a pago de un dividendo en acciones corrientes) en un número mayor o menor de acciones corrientes, entonces en cada caso la cantidad agregada a la cual los titulares de Acciones Preferentes Subordinadas tenían derecho inmediatamente antes de tal evento bajo la condición de la cláusula (1) de la oración anterior, será ajustada multiplicando tal cantidad por una fracción cuyo numerador es el número de acciones corrientes circulantes inmediatamente después de tal evento y cuyo denominador es el número de acciones corrientes que estaban circulando inmediatamente antes de tal evento.

**Sección 7. Consolidación, Fusión, etc.** En caso que la sociedad celebre cualquier consolidación, fusión, combinación u otra transacción en la cual las acciones Corrientes sean cambiadas por o cambiadas a otras acciones o valores, efectivo, y/o cualquier otra propiedad, entonces en cada caso cada acción de Acciones Preferentes Subordinadas, será al mismo tiempo igualmente

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intercambiada o cambiada a una cantidad por acción, sujeta a la provisión para ajuste indicada a continuación, igual a 100 veces la cantidad agregada de acción, valor, efectivo y/o cualquier otra propiedad (pagadera en especie) según sea el caso, en la cual o para la cual cada acción corriente sea cambiada o intercambiada. En el caso que la Sociedad en cualquier momento declare o pague cualquier dividendo sobre la Acción Corriente pagadera en acciones corrientes, o efectúe una subdivisión o combinación o consolidación de las acciones corrientes circulantes (mediante reclasificación o mediante otra forma diferente a pago de un dividendo en acciones corrientes) en un número mayor o menor de acciones corrientes, entonces en cada caso la cantidad indicada en la oración anterior con respecto al intercambio o cambio de Acciones Preferentes Subordinadas será ajustada multiplicando tal cantidad por una fracción cuyo numerador es el número de acciones corrientes circulantes inmediatamente después de tal evento y cuyo denominador es el número de acciones corrientes que estaban circulando inmediatamente antes de tal evento.

**Sección 8. No Redención**. Las Acciones Preferentes Subordinadas no serán redimibles.

**Sección 9. Clasificación**. Las Acciones Preferentes subordinadas se clasificarán, con respecto al pago de dividendos y la distribución de activos, como inferiores a todas las series de cualquier otra clase de Acciones Preferentes.

**Sección 10. Modificación**. Esta Acta de Constitución Modificada y Reformulada no será modificado de ninguna manera que altere o cambie materialmente los poderes, preferencias o derechos especiales de las Acciones Preferentes Subordinadas que las afecten adversamente sin el voto afirmativo de

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los titulares de por lo menos dos tercios de las Acciones Preferentes Subordinadas Circulantes, votando juntos como una sola clase.

**V.**

Para el manejo de los negocios y para la conducta de los asuntos de la Sociedad, y en la definición, limitación y regulación adicional de los poderes de la Sociedad, de sus directores y sus accionistas o cualquier clase de los mismos, según sea el caso, se prevé adicionalmente que:

A.

(1) El manejo de los negocios y la conducta de los asuntos de la Sociedad recaerá en su Junta Directiva. El número de Directores que constituirían la Junta Directiva completa será fijado exclusivamente por una o más resoluciones aprobadas de tanto en tanto por la Junta Directiva.

(2) Someter a los derechos de los accionistas de cualquier serie de Acciones Preferentes la elección de directores adicionales bajo circunstancias específicas, después de la fecha en la cual la Sociedad no está sometida a la Sección 2115 del Código de Sociedades de California (la "Fecha de Registro de Calificación"), los directores estarán divididos en tres clases designadas como Clase I, Clase II y Clase III, respectivamente. Los directores serán asignados a cada clase de acuerdo con una resolución o resoluciones aprobadas por la Junta Directiva. En la primera reunión anual de accionistas después de la fecha de "Registro de Calificación", el período de los directores Clase I expirará y los directores Clase I serán elegidos por un período total de tres años. En la segunda reunión anual de accionistas, después de la Fecha de Registro de Calificación, el período de los Directores Clase II expirará y estos serán elegidos por un período

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total de tres años. En la tercera reunión anual de accionistas, después de la Fecha de Registro de Calificación", el período de los Directores Clase III expirará y estos serán elegidos por un período total de tres años. En cada reunión anual sucesiva de accionistas, los directores serán elegidos por un período total de tres años para suceder a los directores de la clase cuyo período expira en dicha reunión anual.

No obstante las provisiones anteriores de este Artículo, cada director servirá hasta que su sucesor(a) sea debidamente elegido y calificado o hasta su muerte, renuncia o destitución. La no disminución del número de directores que constituye la Junta Directiva acortará el periodo de cualquier director titular.

(3) Someter a los derechos de los titulares de cualquier serie de Acciones Preferentes, que la Junta Directiva o cualquier director individual pueda ser removido de su cargo en cualquier momento (i) con causa por el voto afirmativo de los titulares de una mayoría del poder votante de todas las acciones circulantes actuales con derecho a voto de la Sociedad, con derecho a votar en una elección de directores (las "Acciones con derecho a voto") o (ii) sin causa por el voto afirmativo de los accionistas de al menos sesenta y seis y dos tercios por ciento (66 $^{2}/_{3}$ %) del poder votante de todas las acciones circulantes actuales con derecho a voto.

(4) Someter a los derechos de los titulares de cualquier serie de Acciones Preferentes, cualquier vacante de la Junta Directiva resultante de la muerte, renuncia, descalificación, destitución u otra causa y cualquier dirección recién creada resultante de cualquier aumento en el número de directores, será, a menos que la Junta Directiva determine mediante resolución que cualquier vacante o dirección creada recientemente será ocupada por los accionistas

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excepto sea previsto de otra forma por la ley, será ocupada únicamente por el voto afirmativo de una mayoría de los directores actualmente a cargo, a pesar de menos del quórum de la Junta Directiva, y no por los accionistas. Cualquier director elegido de acuerdo con la frase anterior permanecerá en su cargo por el resto del período total del director para el cual se creó u ocurrió la vacante y hasta que el sucesor de dicho director sea elegido y calificado.

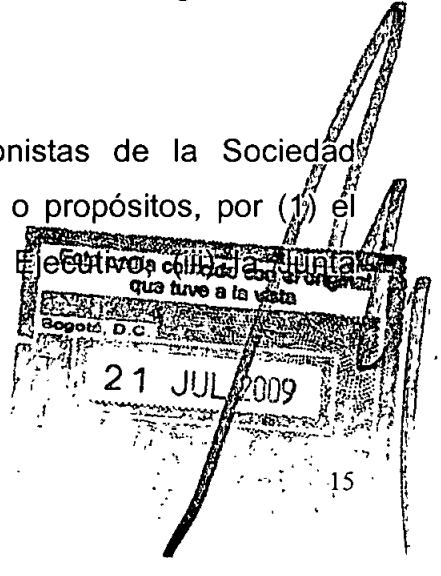
B.

(1) Sujeto al párrafo (h) de la sección 43 de los Estatutos, los estatutos pueden ser alterados o modificados o se pueden aprobar nuevos Estatutos mediante el voto afirmativo de al menos sesenta y seis y dos tercios por ciento (66 $^{2}/_{3}$%) del poder votante de todas las acciones actualmente circulantes de Acciones con Derecho a Voto. La Junta Directiva también tendrá el poder de aprobar, modificar o revocar los Estatutos.

(2) Los directores de la Sociedad no necesitan ser elegidos mediante votación por escrito a menos que los Estatutos lo prevean de esta forma.

(3) Loa accionistas de la Sociedad no tomarán ninguna acción excepto en una reunión anual o especial de accionistas convocada de acuerdo con los Estatutos y los accionistas no tomarán ninguna acción mediante consentimiento escrito.

(4) Las reuniones especiales de accionistas de la Sociedad pueden ser convocadas, para cualquier propósito o propósitos, por (1) el Presidente de la Junta Directiva, (ii) el Director Ejecutivo, o la Junta






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Directiva en cuanto a una resolución aprobada por una mayoría del número total de directores autorizados (ya sea que existan o no vacantes en direcciones previamente autorizadas al momento que tal resolución sea presentada a la Junta Directiva para aprobación), o (iv) por los accionistas de las acciones con derecho a emitir no menos del porcentaje (10%) de los votos en la reunión, y será llevada a cabo en un lugar y en una fecha tal que la Junta Directiva fijará.

(5) El aviso por adelantado de las nominaciones de accionistas para la elección de directores y de negocios a ser llevado por los accionistas antes de cualquier reunión de accionistas de la Sociedad será entregado de la manera prevista en los Estatutos de la Sociedad.

VI.

A. A la mayor extensión posible permitida por la Ley de Sociedades General de Delaware, según esta exista o pueda ser de aquí en adelante modificada, un director de la Sociedad no será personalmente responsable de la Sociedad o sus accionistas por daños monetarios de violación del deber fiduciario como un director.

B. La Sociedad indemnizará en la total extensión permitida por la ley a cualquier persona hecha parte o amenazada de ser parte de una acción o procedimiento, ya sea criminal o civil, administrativo o investigativo, por razón del hecho que el o ella, o su intestato, sea o era un director o funcionario de la Sociedad o cualquier predecesor de la Sociedad, o que sirva o sirvió en cualquier otra empresa como director o funcionario a solicitud de la Sociedad o cualquier predecesor de la Sociedad.

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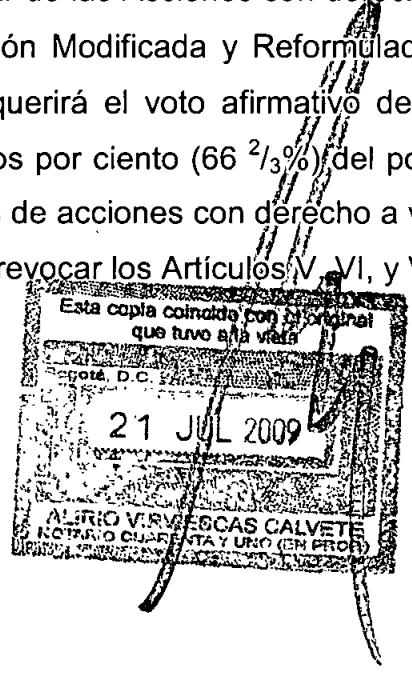
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C. Ninguna modificación ni revocatoria de este Artículo VI, ni la aprobación de ninguna provisión de los certificados de constitución de la Sociedad inconsistente con este Artículo VI, eliminará o reducirá el efecto de este Artículo VI con respecto a cualquier asunto que ocurra, o cualquier acción o procedimiento que se acumule, o surja sobre el mismo, pero para este Artículo VI, que se acumulara o surgiera, antes de tal modificación, revocatoria o aprobación de una posición inconsistente.

VII.

A. La Sociedad se reserva el derecho de modificar, alterar, cambiar o revocar cualquier provisión contenida en esta Acta de Constitución Modificada y Reformulada, de la manera prescrita ahora y de aquí en adelante mediante estatuto, excepto lo previsto en la sección 10 del párrafo C del Artículo IV y párrafo B de este Artículo VII, y todos los derechos conferidos a los accionistas en la presente son concedidos sujetos a esta reserva.

B. No obstante cualquier otra provisión de esta Acta de Constitución Modificada y Reformulada o cualquier provisión de ley que pueda permitir de otra forma un menor voto o no voto, pero adicionalmente a cualquier voto afirmativo de los accionistas de cualquier clase o serie particular de las Acciones con derecho a Voto requeridas por ley, esta Acta de Constitución Modificada y Reformulada o cualquier Designación de Acción Preferente, requerirá el voto afirmativo de los accionistas en al menos sesenta y seis dos tercios por ciento (66 $^{2}/_{3}$%) del poder votante de todas las acciones circulantes actuales de acciones con derecho a voto junto con una sola clase, para alterar, modificar o revocar los Artículos V, VI, y VII.






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La anterior Acta de Constitución Modificada y Reformulada ha sido debidamente aprobada por la Junta Directiva.

La anterior Acta de Constitución Modificada y Reformulada ha sido debidamente aprobada por el voto de los accionistas de acuerdo con las Secciones 242 y 245 de la Ley de Sociedades General de Delaware. El número total de acciones circulantes de la Sociedad con derecho a voto sobre la modificación fue de 45,025,027 Acciones Corrientes desde Abril 12 de 2006. El número de acciones que votaron a favor de la modificación igualó o excedió el voto requerido. El porcentaje de voto requerido era de más del 50% de Acciones Corrientes.

Adicionalmente, declaro bajo penalidad o perjurio bajo las leyes del Estado de Delaware que los asuntos indicados en esta Acta de Constitución Modificada y Reformulada son verdaderos y correctos.

(Firma ilegible)
Louis G. Lange, M.D, PhD.
Presidente de la Junta y Director Ejecutivo

Traducido según mi mejor saber y entender, este día 21 de julio de 2009, en la ciudad de Bogotá, D.C., por Anita Escobar de Tamayo, Traductora Oficial, Res. 2177 de 1989, Ministerio de Justicia, República de Colombia.

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NOTARIO CINCUENTA Y UNO (E.P.)

TRADUCTORA OFICIAL
RES. 2177/89 MINJUSTICIA
ANITA ESCOBAR DE TAMAYO



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# Delaware



*The First State*

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE RESTATED CERTIFICATE OF "CV THERAPEUTICS, INC.", FILED IN THIS OFFICE ON THE THIRD DAY OF AUGUST, A.D. 2006, AT 7:03 O'CLOCK P.M.

A FILED COPY OF THIS CERTIFICATE HAS BEEN FORWARDED TO THE NEW CASTLE COUNTY RECORDER OF DEEDS.







Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

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AUTHENTICATION: 4950864

DATE: 08-03-06

State of Delaware
Secretary of State
Division of Corporations
Delivered 07:05 PM 08/03/2006
FILED 07:03 PM 08/03/2006
SRV 060731643 - 2248911 FILE

**CV Therapeutics, Inc.**

**AMENDED AND RESTATED**
**CERTIFICATE OF INCORPORATION**
**(filed pursuant to sec. 242 & 245)**
**OF**

**CV THERAPEUTICS, INC.**

Louis G. Lange, M.D., Ph.D. does hereby certify:

1. He is the Chairman of the Board and Chief Executive Officer of CV Therapeutics, Inc., a corporation organized and existing under the laws of the state of Delaware. The original Certificate of Incorporation was filed with the Secretary of State of the State of Delaware on December 11, 1990.

2. The Amended and Restated Certificate of Incorporation, as amended, of this Corporation is hereby amended and restated as follows:

**I.**

The name of this Corporation is CV Therapeutics, Inc.

**II.**

The address of the registered office of the Corporation in the State of Delaware is 1013 Centre Road, City of Wilmington, County of New Castle, and the name of the registered agent of the Corporation in the State of Delaware at such address is The Prentice-Hall Corporation System, Inc.

**III.**

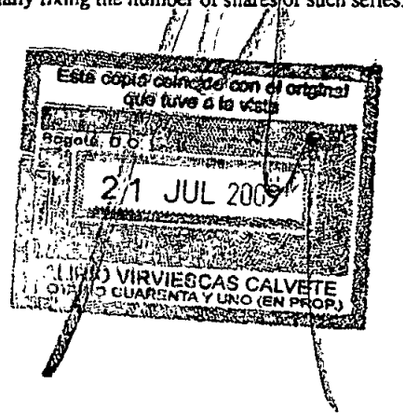
The purpose of the Corporation is to engage in any lawful act or activity for which a corporation may be organized under the General Corporation Law of the State of Delaware.

**IV.**

A. The Corporation is authorized to issue two classes of stock to be designated, respectively, "Common Stock" and "Preferred Stock." The total number of shares which the Corporation is authorized to issue is one hundred eighty five million (185,000,000) shares. One hundred eighty million (180,000,000) shares shall be Common Stock each having a par value of one tenth of one cent ($.001). Five million (5,000,000) shares shall be Preferred Stock, each having a par value of one tenth of one cent ($.001).

B. The Preferred Stock may be issued from time to time in one or more series. The Board of Directors is hereby authorized, by filing a certificate (a "Preferred Stock Designation") pursuant to the Delaware General Corporation Law, to fix or alter from time to time the designation, powers, preferences and rights of the shares of each such series and the qualifications, limitations or restrictions of any wholly unissued series of Preferred Stock, and to establish from time to time the number of shares constituting any such series or any of them; and to increase or decrease the number of shares of any series subsequent to the issuance of shares of that series, but not below the number of shares of such series then outstanding. In case the number of shares of any series shall be decreased in accordance with the foregoing sentence, the shares constituting such decrease shall resume the status that they had prior to the adoption of the resolution originally fixing the number of shares of such series.





C. The rights, preferences, privileges and restrictions granted to and imposed on the first series of such Preferred Stock, the Series A Junior Participating Preferred Stock, are as set forth below in this Fourth Article.

**Section 1. Designation and Amount.** Eight hundred fifty thousand (850,000) shares of Preferred Stock, $.001 par value, are designated "Series A Junior Participating Preferred Stock" with the designations and the powers, preferences and rights, and the qualifications, limitations and restrictions specified herein (the "Junior Preferred Stock"). Such number of shares may be increased or decreased by resolution of the Board of Directors; *provided*, that no decrease shall reduce the number of shares of Junior Preferred Stock to a number less than the number of shares then outstanding plus the number of shares reserved for issuance upon the exercise of outstanding options, rights or warrants or upon the conversion of any outstanding securities issued by the Corporation convertible into Junior Preferred Stock.

**Section 2. Dividends and Distributions.**

(A) Subject to the rights of the holders of any shares of any series of Preferred Stock (or any similar stock) ranking prior and superior to the Junior Preferred Stock with respect to dividends, the holders of shares of Junior Preferred Stock, in preference to the holders of Common Stock and of any other junior stock, shall be entitled to receive, when, as and if declared by the Board of Directors out of funds legally available for the purpose, quarterly dividends payable in cash on the first day of April, July, October and January in each year (each such date being referred to herein as a "Quarterly Dividend Payment Date"), commencing on the first Quarterly Dividend Payment Date after the first issuance of a share of fraction of a share of Junior Preferred Stock, in an amount per share (rounded to the nearest cent) equal to the greater of (a) $1.00 or (b) subject to the provision for adjustment hereinafter set forth, 100 times the aggregate per share amount of all cash dividends, and 100 times the aggregate per share amount (payable in kind) of all non-cash dividends or other distributions, other than a dividend payable in shares of Common Stock or a subdivision of the outstanding shares of Common Stock (by reclassification or otherwise), declared on the Common Stock since the immediately preceding Quarterly Dividend Payment Date or, with respect to the first Quarterly Dividend Payment Date, since the first issuance of any share or fraction of a share of Junior Preferred Stock. In the event the Corporation shall at any time declare or pay any dividend on the Common Stock payable in shares of Common Stock, or effect a subdivision or combination or consolidation of the outstanding shares of Common Stock (by reclassification or otherwise than by payment of a dividend in shares of Common Stock) into a greater or lesser number of shares of Common Stock, then in each such case the amount to which holders of shares of Junior Preferred Stock were entitled immediately prior to such event under clause (b) of the preceding sentence shall be adjusted by multiplying such amount by a fraction, the numerator of which is the number of shares of Common Stock outstanding immediately after such event and the denominator of which is the number of shares of Common Stock that were outstanding immediately prior to such event.

(B) The Corporation shall declare a dividend or distribution on the Junior Preferred Stock as provided in paragraph (A) of this Section immediately after it declares a dividend or distribution on the Common Stock (other than a dividend payable in shares of Common Stock); *provided*, that in the event no dividend or distribution shall have been declared on the Common Stock during the period between any Quarterly Dividend Payment Date and the next subsequent Quarterly Dividend Payment Date, a dividend of $1.00 per share on the Junior Preferred Stock shall nevertheless be payable on such subsequent Quarterly Dividend Payment Date.

(C) Dividends shall begin to accrue and be cumulative on outstanding shares of Junior Preferred Stock from the Quarterly Dividend Payment Date next preceding the date of issue of such shares, unless the date of issue of such shares is prior to the record date for the first Quarterly Dividend Payment Date, in which case dividends on such shares shall begin to accrue from the date of issue of such shares, or unless the date of issue is a Quarterly Dividend Payment Date or is a date after the record date for the determination of holders of shares of Junior Preferred Stock entitled to receive a quarterly dividend and before such Quarterly Dividend Payment Date, in either of which events such dividends shall begin to accrue and be cumulative from such Quarterly Dividend Payment Date. Accrued but unpaid dividends shall not bear interest. Dividends paid on the shares of Junior



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Preferred Stock in an amount less than the total amount of such dividends at the time accrued and payable on such shares shall be allocated pro rata on a share-by-share basis among all such shares at the time outstanding. The Board of Directors may fix a record date for the determination of holders of shares of Junior Preferred Stock entitled to receive payment of a dividend or distribution declared thereon, which record date shall be not more than 60 days prior to the date fixed for the payment thereof.

**Section 3. Voting Rights.** The holders of the Junior Preferred Stock shall have the following voting rights:

(A) Subject to the provision for adjustment hereinafter set forth, each share of Junior Preferred Stock shall entitle the holder thereof to 100 votes on all matters submitted to a vote of the stockholders of the Corporation. In the event the Corporation shall at any time declare or pay any dividend on the Common Stock payable in shares of Common Stock, or effect a subdivision or combination or consolidation of the outstanding shares of Common Stock (by reclassification or otherwise than by payment of a dividend in shares of Common Stock) into a greater or lesser number of shares of Common Stock, then in each such case the number of votes per share to which the holders of shares of Junior Preferred Stock were entitled immediately prior to such event shall be adjusted by multiplying such number by a fraction, the numerator of which is the number of shares of Common Stock outstanding immediately after such event and the denominator of which is the number of shares of Common Stock that were outstanding immediately prior to such event.

(B) Except as otherwise provided herein, in any Preferred Stock Designation or by law, the holders of shares of Junior Preferred Stock and the holders of shares of Common Stock and any other capital stock of the Corporation having general voting rights shall vote together as one class on all matters submitted to a vote of stockholders of the Corporation.

(C) Except as set forth herein, or as otherwise provided by law, holders of Junior Preferred Stock shall have not special voting rights and their consent shall not be required (except to the extent they are entitled to vote with holders of Common Stock as set forth herein) for taking any corporate action.

**Section 4. Certain Restrictions.**

(A) Whenever quarterly dividends or other dividends or distributions payable on the Junior Preferred Stock as provided in Section 2 are in arrears, thereafter and until all accrued and unpaid dividends and distributions, whether or not declared, on shares of Junior Preferred Stock outstanding shall have been paid in full, the Corporation shall not:

(i) declare or pay dividends, or make any other distributions, on any shares of stock ranking junior (either as to dividends or upon liquidation, dissolution or winding up) to the Junior Preferred Stock;

(ii) declare or pay dividends, or make any other distributions, on any shares of stock ranking on a parity (either as to dividends or upon liquidation, dissolution or winding up) with the Junior Preferred Stock, except dividends paid ratably on the Junior Preferred Stock and all such parity stock on which dividends are payable or in arrears in proportion to the total amounts to which the holders of all such shares are then entitled;

(iii) redeem or purchase or otherwise acquire for consideration shares of any stock ranking junior (either as to dividends or upon liquidation, dissolution or winding up) to the Junior Preferred Stock; provided that the Corporation may at any time redeem, purchase or otherwise acquire shares of any such junior stock in exchange for shares of any stock of the Corporation ranking junior (either as to dividends or upon dissolution, liquidation or winding up) to the Junior Preferred Stock; or

(iv) redeem or purchase or otherwise acquire for consideration any shares of Junior Preferred Stock, or any shares of stock ranking on a parity (either as to dividends or upon liquidation, dissolution or winding up) with the Junior Preferred Stock, except in accordance with a purchase offer made in writing or by



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publication (as determined by the Board of Directors) to all holders of such shares upon such terms as the Board of Directors, after consideration of the respective annual dividend rates and other relative rights and preferences of the respective series and classes, shall determine in good faith will result in fair and equitable treatment among the respective series or classes.

(B) The Corporation shall not permit any subsidiary of the Corporation to purchase or otherwise acquire for consideration any shares of stock of the Corporation unless the Corporation could, under paragraph (A) of this Section 4, purchase or otherwise acquire such shares at such time and in such manner.

**Section 5. Reacquired Shares.** Any shares of Junior Preferred Stock purchased or otherwise acquired by the Corporation in any manner whatsoever shall be retired and cancelled promptly after the acquisition thereof. All such shares shall upon their cancellation become authorized but unissued shares of Preferred Stock and may be reissued as part of a new series of Preferred stock subject to the conditions and restrictions on issuance set forth herein, or in any Preferred Stock Designation or as otherwise required by law.

**Section 6. Liquidation, Dissolution or Winding Up.** Upon any liquidation, dissolution or winding up of the Corporation, no distribution shall be made (1) to the holders of shares of stock ranking junior (either as to dividends or upon liquidation, dissolution or winding up) to the Junior Preferred Stock unless, prior thereto, the holders of shares of Junior Preferred Stock shall have received $100 per share, plus an amount equal to accrued and unpaid dividends and distributions thereon, whether or not declared, to the date of such payment, provided that the holders of shares of Junior Preferred Stock shall be entitled to receive an aggregate amount per share, subject to the provisions for adjustment hereinafter set forth, equal to 100 times the aggregate amount to be distributed per share to holders of shares of Common Stock, or (2) to the holders of shares of stock ranking on a parity (either as to dividends or upon liquidation, dissolution or winding up) with the Junior Preferred Stock, except distributions made ratably on the Junior Preferred Stock and all such parity stock in proportion to the total amounts to which the holders of all such shares are entitled upon such liquidation, dissolution or winding up. In the event the Corporation shall at any time declare or pay any dividend on the Common Stock payable in shares of Common Stock or effect a subdivision or combination or consolidation of the outstanding shares of Common Stock (by reclassification or otherwise than by payment of a dividend in shares of Common Stock) into a greater or lesser number of shares of Common Stock, then in each such case the aggregate amount to which holders of shares of Junior Preferred Stock were entitled immediately prior to such event under the proviso in clause (1) of the preceding sentence shall be adjusted by multiplying such amount by a fraction the numerator of which is the number of shares of Common Stock outstanding immediately after such event and the denominator of which is the number of shares of Common Stock that were outstanding immediately prior to such event.

**Section 7. Consolidation, Merger, etc.** In case the Corporation shall enter into any consolidation, merger, combination or other transactions in which the shares of Common Stock are exchanged for or changed into other stock or securities, cash and/or any other property, then in any such case each share of Junior Preferred Stock shall at the same time be similarly exchanged or changes into an amount per share, subject to the provision for adjustment hereinafter set forth, equal to 100 times the aggregate amount of stock, securities, cash and/or any other property (payable in kind), as the case may be, into which or for which each share of Common Stock is changes or exchanged. In the event the Corporation shall at any time declare or pay any dividend on the Common Stock payable in shares of Common Stock, or effect a subdivision or combination or consolidation of the outstanding shares of Common Stock (by reclassification or otherwise than by payment of a dividend in shares of Common Stock) into a greater or lesser number of shares of Common Stock, then in each such case the amount set forth in the preceding sentence with respect to the exchange or change of shares of Junior Preferred Stock shall be adjusted by multiplying such amount by a fraction, the numerator of which is the number of shares of Common Stock outstanding immediately after such event and the denominator of which is the number of shares of Common Stock that were outstanding immediately prior to such event.

**Section 8. No Redemption.** The shares of Junior Preferred Stock shall not be redeemable.

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**Section 9. Rank.** The Junior Preferred Stock shall rank, with respect to the payment of dividends and the distribution of assets, junior to all series of any other class of Preferred Stock.

**Section 10. Amendment.** This Amended and Restated Certificate of Incorporation shall not be amended in any manner which would materially alter or change the powers, preferences or special rights of the Junior Preferred Stock so as to affect them adversely without the affirmative vote of the holders of at least two-thirds of the outstanding shares of Junior Preferred Stock, voting together as a single class.

V.

For the management of the business and for the conduct of the affairs of the Corporation, and in further definition, limitation and regulation of the powers of the Corporation, of its directors and of its stockholders or any class thereof, as the case may be, it is further provided that:

A.

(1) The management of the business and the conduct of the affairs of the Corporation shall be vested in its Board of Directors. The number of directors which shall constitute the whole Board of Directors shall be fixed exclusively by one or more resolutions adopted from time to time by the Board of Directors.

(2) Subject to the rights of the holders of any series of Preferred Stock to elect additional directors under specified circumstances, following the date on which the Corporation is no longer subject to Section 2115 of the California Corporations Code (the "Qualifying Record Date"), the directors shall be divided into three classes designated as Class I, Class II and Class III, respectively. Directors shall be assigned to each class in accordance with a resolution or resolutions adopted by the Board of Directors. At the first annual meeting of stockholders following the Qualifying Record Date, the term of office of the Class I directors shall expire and Class I directors shall be elected for a full term of three years. At the second annual meeting of stockholders, following the Qualifying Record Date, the term of office of the Class II directors shall expire and Class II directors shall be elected for a full term of three years. At the third annual meeting of stockholders following the Qualifying Record Date, the term of office of the Class III directors shall expire and Class III directors shall be elected for a full term of three years. At each succeeding annual meeting of stockholders, directors shall be elected for a full term of three years to succeed the directors of the class whose terms expire at such annual meeting.

Notwithstanding the foregoing provisions of this Article, each director shall serve until his or her successor is duly elected and qualified or until his or her death, resignation or removal. No decrease in the number of directors constituting the Board of Directors shall shorten the term of any incumbent director.

(3) Subject to the rights of the holders of any series of Preferred Stock, the Board of Directors or any individual director may be removed from office at any time (i) with cause by the affirmative vote of the holders of a majority of the voting power of all the then outstanding shares of voting stock of the Corporation, entitled to vote at an election of directors (the "Voting Stock") or (ii) without cause by the affirmative vote of the holders of at least sixty-six and two-thirds percent (66⅔%) of the voting power of all the then-outstanding shares of the Voting Stock.

(4) Subject to the rights of the holders of any series of Preferred Stock, any vacancies on the Board of Directors resulting from death, resignation, disqualification, removal or other causes and any newly created directorships resulting from any increase in the number of directors, shall, unless the Board of Directors determines by resolution that any such vacancies or newly created directorships shall be filled by the stockholders, except as otherwise provided by law, be filled only by the affirmative vote of a majority of the directors then in office, even though less than a quorum of the Board of Directors, and not by the stockholders. Any director elected in accordance with the preceding sentence shall hold office for the remainder of the full term of the director for which the vacancy was created or occurred and until such director's successor shall have been elected and qualified.

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B.

(1) Subject to paragraph (h) of Section 43 of the Bylaws, the Bylaws may be altered or amended or new Bylaws adopted by the affirmative vote of at least sixty-six and two-thirds percent (66⅔%) of the voting power of all of the then-outstanding shares of the Voting Stock. The Board of Directors shall also have the power to adopt, amend, or repeal Bylaws.

(2) The directors of the Corporation need not be elected by written ballot unless the Bylaws so provide.

(3) No action shall be taken by the stockholders of the Corporation except at an annual or special meeting of stockholders called in accordance with the Bylaws and no action shall be taken by the stockholders by written consent.

(4) Special meetings of the stockholders of the Corporation may be called, for any purpose or purposes, by (i) the Chairman of the Board of Directors, (ii) the Chief Executive Officer, (iii) the Board of Directors pursuant to a resolution adopted by a majority of the total number of authorized directors (whether or not there exist any vacancies in previously authorized directorships at the time any such resolution is presented to the Board of Directors for adoption) or (iv) by the holders of the shares entitled to cast not less than percent (10%) of the votes at the meeting, and shall be held at such place, on such date, and at such time as the Board of Directors shall fix.

(5) Advance notice of stockholder nominations for the election of directors and of business to be brought by stockholders before any meeting of the stockholders of the Corporation shall be given in the manner provided in the Bylaws of the Corporation.

VI.

A. To the maximum extent permitted by the Delaware General Corporation Law, as the same exists or as may hereafter be amended, a director of the Corporation shall not be personally liable to the Corporation or its stockholders for monetary damages for breach of fiduciary duty as a director.

B. The Corporation shall indemnify to the fullest extent permitted by law any person made or threatened to be made a party to an action or proceeding, whether criminal or civil, administrative or investigative, by reason of the fact that he or she, or his or her testator or intestate is or was a director or officer of the Corporation or any predecessor of the Corporation, or serves or served at any other enterprise as a director or officer at the request of the Corporation or any predecessor to the Corporation.

C. Neither any amendment nor repeal of this Article VI, nor the adoption of any provision of the Corporation's certificate of incorporation inconsistent with this Article VI, shall eliminate or reduce the effect of this Article VI in respect of any matter occurring, or any action or proceeding accruing or arising or that, but for this Article VI, would accrue or arise, prior to such amendment, repeal or adoption of an inconsistent position.

VII.

A. The Corporation reserves the right to amend, alter, change or repeal any provision contained in this Amended and Restated Certificate of Incorporation, in the manner now or hereafter prescribed by statute, except as provided in Section 10 of paragraph C of Article IV and paragraph B of this Article VII, and all rights conferred upon the stockholders herein are granted subject to this reservation.

B. Notwithstanding any other provisions of this Amended and Restated Certificate of Incorporation or any provision of law which might otherwise permit a lesser vote or no vote, but in addition to any affirmative vote of the holders of any particular class or series of the Voting Stock required by law, this Amended and Restated Certificate of Incorporation or any Preferred Stock Designation, the affirmative vote of the holders of at least sixty-six and two-thirds percent (66⅔%) of the voting power of all of the then-outstanding shares of the Voting Stock, voting together as a single class, shall be required to alter, amend or repeal Articles V, VI and VII.



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The foregoing Amended and Restated Certificate of Incorporation has been duly approved by the Board of Directors.

The foregoing Amended and Restated Certificate of Incorporation has been duly approved by the vote of the stockholders in accordance with Sections 242 and 245 of the Delaware General Corporation Law. The total number of outstanding shares of the Corporation entitled to vote on the amendment was 45,052,027 shares of Common Stock as of April 12, 2006. The number of shares voting in favor of the amendment equaled or exceeded the vote required. The percentage vote required was more than 50% of the Common Stock.

I further declare under penalty of perjury under the laws of the state of Delaware that the matters set forth in this Amended and Restated Certificate of Incorporation are true and correct.

Louis G. Lange, M.D., Ph.D.
Chairman of the Board and
Chief Executive Officer

