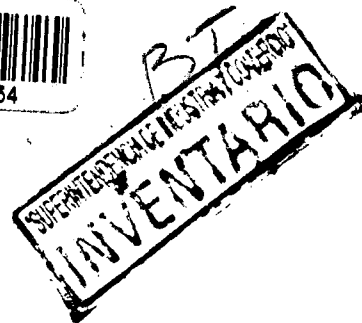
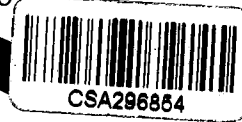




DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

SOLICITUD

PATENTE DE INVENCION

PCT

ENTRADA EN FASE NACIONAL

09-10531

21. EXPEDIENTE No.

54. TITULO COMPOSICIONES ANTI-COCAÍNA Y TRATAMIENTO

51. CLASIFICACION INTERNACIONAL

71. SOLICITANTE COLUMBIA UNIVERSITY

DOMICILIO NEW YORK, NY, ESTADOS UNIDOS DE AMÉRICA

74. APODERADO JUAN PABLO CADENA SARMIENTO

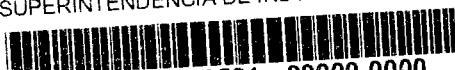
22. SANTAFE DE BOGOTA, D.C.

PETITORIO

AS: 109.391


Industria y Comercio
 SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-010531- -00000-0000

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 Tra. 11 PATENTEIYII
 Act. 411 PRESENTACION

 Dep. 2020 NUECREACION on
 Evs: 378 FASENACIONAL
 Folios: 446

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

10 Feb. 2009

1

SOLICITUD DE:

Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

2

SOLICITANTENombre: **COLUMBIA UNIVERSITY**

(Yd 510-535)

Dirección: 412 Low Memorial Library, 535 West 116th Street, Mc4308, New York, NY 10027, Estados Unidos de América

Nacionalidad o Domicilio: Estados Unidos de América

Lugar de Constitución: Estados Unidos de América

Teléfono:

Fax:

E-mail

IDENTIFICACIÓNC.C. ☐NIT ☐C.E. ☐Otro ☐

Cual _____

Número _____

3

**REPRESENTANTE
O APODERADO**

Nombre: JUAN PABLO CADENA SARMIENTO

Dirección: Cra. 7 No. 71-21 Torre B – Piso 4, Bogotá, Colombia.

Teléfono: 744-2200

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IDENTIFICACIÓNC.C. ☒NIT ☐C.E. ☐Otro ☐

Cual _____

Número 80.410.337 TP 57492

4

INVENTOR (ES)
(72)
Nombre: Donald LANDRY, Chang-Guo ZHAN, James H. WOODS, Roger SUNAHARA, Diwahr L. NARASIMHAN, Joanne MACDONALD, Victor YANG, Mei-Chuan Holden KO, Shi-Xian DENG, John J. TESMER, Tien-Yi LEE, Young-Min KWON, Daquan GAO.

Dirección: 29 Claremont Avenue, Apartment 2-South, New York, NY 10027, US; 2193 Allegheny Way, Lexington, KY 40523, US; Department Of Pharmacology, University Of Michigan, Medical School, 1301 MSRB III, Ann Arbor, MI 48109, US; 1130 Olivia Avenue, Ann Arbor, MI 48104, US; 1507 Pine Valley Boulevard #7, Ann Arbor, MI 48104, US; 605 W. 156th Street, Apt 40, New York, NY 10032, US; 3629 Tanglewood Drive, Ann Arbor, MI 48105, US; Department Of Pharmacology, University Of Michigan, Medical School, 1301 MSRB III, Ann Arbor, MI 48109-0632, US; 34 Odgen Avenue, White Plains, NY 10605, US; 2450 Adare Road, Ann Arbor, MI 48104, US; 2222 Fuller Court, #204A, Ann Arbor, MI 48105, US; 3660 Ranchero Dr., #203, Ann Arbor, MI 48108, US; 3929 Stonyrun Drive, Louisville, KY 40220, US (Respectivamente).
Nacionalidad o Domicilio: Estados Unidos de América5 **PCT (86)**

Solicitud Internacional No.

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10-07-2007

Publicación Internacional No

WO 2008/008358 A3

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17-01-20086 **Título (54):****COMPOSICIONES ANTI-COCAÍNA Y TRATAMIENTO**7 **Clasificación Internacional (51):**8 **Prioridad**SI ☒NO ☐

(33) País de Origen

US

(32) Fecha

10-07-2006

(31) Número de Solicitud

60/819,569

9 **Comprobante de pago No.**09-956; 09-4,843; 09-4,884**Fecha** 03-02-2009

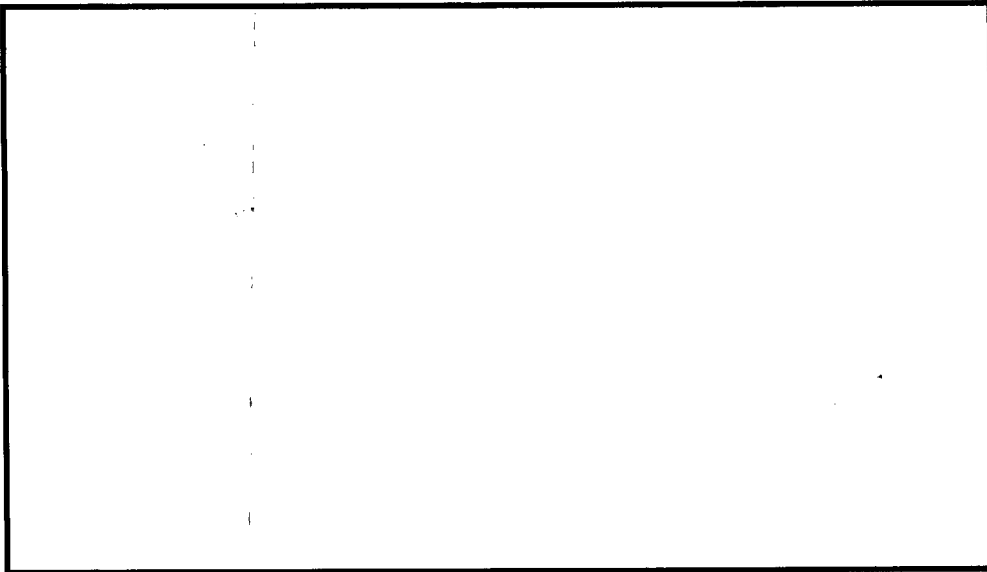
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

10

ANEXOS

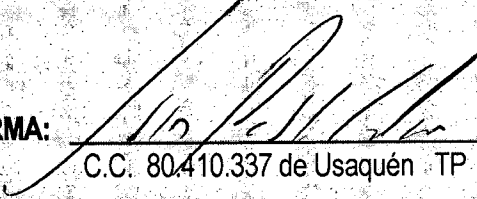
- ☒ Comprobante de pago de la tasa de presentación de la solicitud. \$ 540.000
- ☐ Documento que acredite la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☐ Poderes, si fuere el caso. **Fotocopia autenticada**
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen preliminar (IPEA)
- ☐ Traducción de examen preliminar internacional efectuado por la IPEA.
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12.
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☐ De ser el caso, modificaciones efectuadas a la solicitud internacional.
- ☒ Otros: Tarjeta de Propietarios
Documentos PCT
Listado de Secuencias
Comprobante(s) de pago de la tasa por concepto de Reivindicaciones adicionales. \$300.000
(12*\$25.000)

11 FIGURA CARACTERÍSTICA:



12 Solicito la concesión de la patente,

NOMBRE: JUAN PABLO CADENA SARMIENTO

FIRMA: 

C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

Instrucciones para la presentación de los anexos, ver reverso de esta página.

109391

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 09 - 956
FECHA : ENERO 6 DE 2009

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-010531- -00000-0000

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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 446

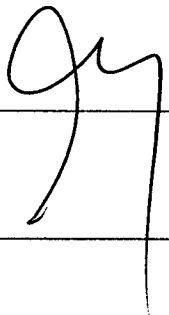
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
RI & CASTRO LTDA	CONSIGNACION	BANCO DE BOGOTA	062754387	92973716	06/01/2009	5,012,000.00
RD & CASTRO LTDA	CONSIGNACION	BANCO DE BOGOTA	062754387	92973720	06/01/2009	69,123,040.00

***** CONCEPTO *****

ANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	540,000.00
	TOTAL :	\$ 540,000.00

ON: QUINIENTOS CUARENTA MIL PESOS

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. 

Se activa folio 4
(creetido) tengo en archivo.

109391 4

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 09 - 4,843
FECHA : ENERO 21 DE 2009

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-010531- -00000-0000

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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 446

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
RD & CASTRO LTDA	CONSIGNACION	BANCO DE BOGOTA	062754387	105762016	20/01/2009	37,982,200.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		998 VALOR COMPLEMENTO TARIFA A#D ANTR	50,000.00
		TOTAL :	\$ 50,000.00

SON: CINCUENTA MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

10923915

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 09 - 4,884
FECHA : ENERO 21 DE 2009

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 446

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
GRIGARD & CASTRO LTDA	CONSIGNACION	BANCO DE BOGOTA	062754387	105762016	20/01/2009	37,982,200.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		998 VALOR COMPLEMENTO TARIFA A#0 ANTR	250,000.00
		TOTAL :	\$ 250,000.00

SON: DOSCIENTOS CINCUENTA MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 January 2008 (17.01.2008)

PCT

(10) International Publication Number
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- (51) International Patent Classification:
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- (21) International Application Number:
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- (22) International Filing Date: 10 July 2007 (10.07.2007)
- (25) Filing Language: English
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- (30) Priority Data:
60/819,569 10 July 2006 (10.07.2006) US
- (71) Applicant (for all designated States except US): COLUMBIA UNIVERSITY [US/US]; 412 Low Memorial Library, 535 West 116th Street, Mc4308, New York, NY 10027 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): LANDRY, Donald [US/US]; 29 Claremont Avenue, Apartment 2-South, New York, NY 10027 (US). ZHAN, Chang-Guo [CN/US]; 2193 Allegheny Way, Lexington, KY 40523 (US). WOODS, James, H. [US/US]; Department Of Pharmacology, University Of Michigan, Medical School, 1301 MSRB III, Ann Arbor, MI 48109 (US). SUNAHARA, Roger [CA/US]; 1130 Olivia Avenue, Ann Arbor, MI 48104 (US). NARASIMHAN, Diwahar, L. [IN/US]; 1507 Pine Valley Boulevard #7, Ann Arbor, MI 48104 (US). MACDONALD, Joanne [AU/US]; 605 W. 156th Street, Apt 40, New York, NY 10032 (US). YANG, Victor [US/US]; 3629 Tanglewood Drive, Ann Arbor, MI 48105 (US). KO, Mei-Chuan, Holden [—/US]; Department Of Pharmacology, University Of Michigan, Medical School, 1301 MSRB III, Ann Arbor, MI 48109-0632 (US). DENG, Shi-Xian [US/US]; 34 Odgen Avenue, White Plains, NY 10605 (US). TESMER, John, J. [US/US]; 2450 Adare Road, Ann Arbor, MI 48104 (US). LEE, Tien-Yi [—/US]; Ann Arbor, MI (US). KWON, Young, Min [KR/US]; Ann Arbor, MI (US). GAO, Daquan [CN/US]; 3929 Stonyrun Drive, Louisville, KY 40220 (US).
- (74) Agents: BLOSSER, G., Harley et al.; Sonnenschein Nath & Rosenthal LLP, P.O. Box 061080, Wacker Drive Station, Sears Tower, Chicago, IL 60606-1080 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
— with sequence listing part of description published separately in electronic form and available upon request from the International Bureau
- (88) Date of publication of the international search report:
24 December 2008

(54) Title: ANTI-COCAINE COMPOSITIONS AND TREATMENT

(57) Abstract: Embodiments of the invention disclosed herein generally relate to anti-cocaine therapeutics. Specifically, some embodiments of the invention relate to highly efficient, thermostable, and long-lasting cocaine esterase (CocE) mutants that can protect against the toxic and reinforcing effects of cocaine in subjects. Provided herein are mutant CocE polypeptides displaying thermostable esterase activity. Also provided are methods of treating cocaine-induced conditions in a subject in need via administration of mutant CocE as well as methods for high-throughput screening of candidate esterase polypeptides.

WO 2008/008358 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/15762

A. CLASSIFICATION OF SUBJECT MATTER

IPC: A61K 38/46(2006.01)

USPC: 514/183

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. : 514/183

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)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2,257,972 A (BRITT et al) 27 January 1993. (27.01.1993), abstract.	1-27
A	US 5,730,985 A (BARBER et al) 24 May 1998 (24.05.1998), abstract	1-27
A	US 5,977,314 A (LANDRY et al) 2 November 1999 (02.11.1999), abstract	1-27

☐ 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	"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
"E"	earlier application or patent published on or after the international filing date	"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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"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

28 August 2008 (28.08.2008)

Date of mailing of the international search report

29 SEP 2008

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (571) 273-3201

Authorized officer

ANNA PAGONAKIS

Telephone No. 571-270-3505

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/15762

Continuation of B. FIELDS SEARCHED Item 3:
EAST (USPat, USPGPub, Derwent, EPO)
STN

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 January 2008 (17.01.2008)

PCT

(10) International Publication Number
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- (51) International Patent Classification:
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- (30) Priority Data:
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- (71) Applicant (for all designated States except US): COLUMBIA UNIVERSITY [US/US]; 412 Low Memorial Library, 535 West 116th Street, Mc4308, New York, NY 10027 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): LANDRY, Donald [US/US]; 29 Claremont Avenue, Apartment 2-South, New York, NY 10027 (US). ZHAN, Chang-Guo [CN/US]; 2193 Allegheny Way, Lexington, KY 40523 (US). WOODS, James, H. [US/US]; Department Of Pharmacology, University Of Michigan, Medical School, 1301

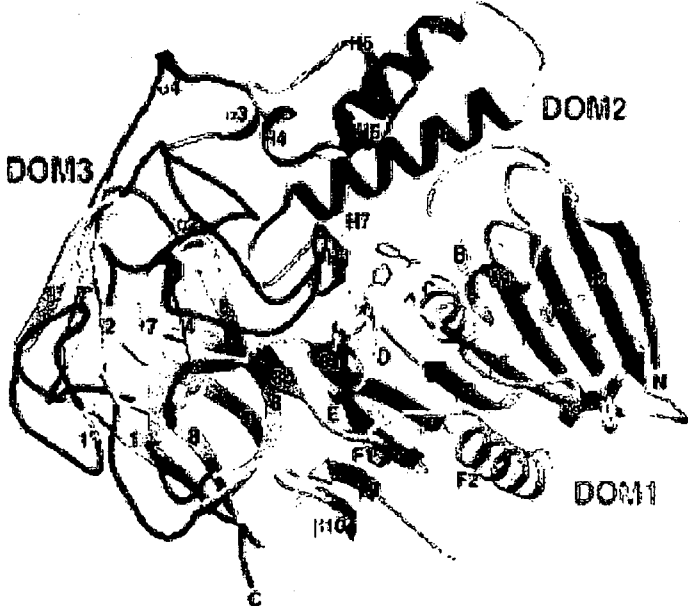
MSRB III, Ann Arbor, MI 48109 (US). SUNAHARA, Roger [CA/US]; 1130 Olivia Avenue, Ann Arbor, MI 48104 (US). NARASIMHAN, Diwahar, L. [IN/US]; 1507 Pine Valley Boulevard #7, Ann Arbor, MI 48104 (US). MACDONALD, Joanne [AU/US]; 605 W. 156th Street, Apt 40, New York, NY 10032 (US). YANG, Victor [US/US]; 3629 Tanglewood Drive, Ann Arbor, MI 48105 (US). KO, Mei-Chuan, Holden [—/US]; Department Of Pharmacology, University Of Michigan, Medical School, 1301 MSRB III, Ann Arbor, MI 48109-0632 (US). DENG, Shi-Xian [US/US]; 34 Odgen Avenue, White Plains, NY 10605 (US). TESMER, John, J. [US/US]; 2450 Adare Road, Ann Arbor, MI 48104 (US). LEE, Tien-Yi [—/US]; Ann Arbor, MI (US). KWON, Young, Min [KR/US]; Ann Arbor, MI (US). GAO, Daquan [CN/US]; 3929 Stonyrun Drive, Louisville, KY 40220 (US).

(74) Agents: BLOSSER, G., Harley et al.; Sonnenschein Nath & Rosenthal LLP, P.O. Box 061080, Wacker Drive Station, Sears Tower, Chicago, IL 60606-1080 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL,

[Continued on next page]

(54) Title: ANTI-COCAINE COMPOSITIONS AND TREATMENT



(57) Abstract: Embodiments of the invention disclosed herein generally relate to anti-cocaine therapeutics. Specifically, some embodiments of the invention relate to highly efficient, thermostable, and long-lasting cocaine esterase (CocE) mutants that can protect against the toxic and reinforcing effects of cocaine in subjects. Provided herein are mutant CocE polypeptides displaying thermostable esterase activity. Also provided are methods of treating cocaine-induced conditions in a subject in need via administration of mutant CocE as well as methods for high-throughput screening of candidate esterase polypeptides.

WO 2008/008358 A2



IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ANTI-COCAINE COMPOSITIONS AND TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application no. 60/819,569, filed July 10, 2006, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made in part with U.S. Government support under NIDA Grant No. DA021416. The U.S. Government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

[0003] The Sequence Listing, which is a part of the present disclosure, includes a computer readable form and a written sequence listing comprising nucleotide and/or amino acid sequences of the present invention. The sequence listing information recorded in computer readable form is identical to the written sequence listing. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0004] The invention disclosed herein generally relates to anti-cocaine therapeutics.

BACKGROUND

[0005] Abuse of cocaine is an intractable social and medical problem that is resistant to remediation through pharmacotherapy. Cocaine acts to block the reuptake of monoamines, dopamine, norepinephrine, and serotonin thus

prolonging and magnifying the effects of these neurotransmitters in the central nervous system (Benowitz NL (1993) *Pharmacol Toxicol* 72, 3-12). Cocaine toxicity is marked by both convulsions and cardiac dysfunction (e.g., myocardial infarction, cardiac arrhythmias, increased blood pressure, stroke, or dissecting aneurysm, and increased myocardial oxygen demand), due to effects on neurotransmitter systems and myocardial sodium channel blockade (Bauman JL and DiDomenico RJ (2002) *J Cardiovasc Pharmacol Ther* 7, 195-202; Wilson LD and Shelat C (2003) *J Toxicol Clin Toxicol* 41, 777-788; Knuepfer MM (2003) *Pharmacol Ther* 97, 181-222). Because of cocaine's ability to readily cross the blood brain barrier and its widespread effects on the central and peripheral nervous systems, overdose can result in sudden death (see Bauman JL and DiDomenico RJ (2002) *J Cardiovasc Pharmacol Ther* 7, 195-202, for review).

[0006] Although the mechanism of cocaine's action is well understood, this information has not yet resulted in the development of an effective antagonist of cocaine that could be used in abuse and overdose situations. The rapid and pleiotropic effects of cocaine present a complex problem for the treatment of acute cocaine toxicity (Carroll FI, Howell LL and Kuhar M.J (1999) *J Med Chem* 42, 2721-2736). The two types of therapies that are available for the treatment of opioid abuse, antagonism (e.g., naltrexone) and replacement (e.g., methadone), do not have parallels in the case of cocaine, although attempts at the latter are being considered (e.g., J. Grabowski et al. (2004) *Addictive Behaviors* 29, 1439-1464). One approach is to prevent or reduce the cocaine from reaching sites of action by administering either endogenous esterases, cocaine specific antibodies, or a catalytic antibody.

[0007] Naturally occurring cocaine is hydrolyzed at the benzoyl ester by serum butyrylcholinesterase (BChE) to nontoxic ecgonine methyl ester and benzoic acid. In the liver, carboxylesterase hCE-2 hydrolyzes the methyl ester to yield benzoylecgonine and methanol (see e.g., Figure 1). The elimination half-life of cocaine in the blood ranges from 0.5 to 1.5 hr (T. Inaba (1989)

Canadian Journal of Physiology & Pharmacology 67, 1154-1157). There have been a few attempts to use naturally occurring BChE or genetically engineered BChE to increase cocaine breakdown (see e.g., Carmona et al. (2000) Drug Metabolism & Disposition 28, 367-371; Xie et al. (1999) Molecular Pharmacology 55, 83-91; Sun et al. (2002a) Molecular Pharmacology; Sun et al. (2002b) Pharmacology & Experimental Therapeutics 302, 710-716; Duysen et al. (2002) Journal of Pharmacology & Experimental Therapeutics 302, 751-758; Gao Y and Brimijoin S (2004) Journal of Pharmacology & Experimental Therapeutics 310, 1046-1052; Gao et al. (2005) Molecular Pharmacology 67, 204-211). Other researchers have utilized a monoclonal antibody, Mab 15A10, as a catalytic antibody to cocaine (see e.g., Landry et al., 1993; Mets et al., 1998; Baird et al., 2000; Larsen et al., 2004), while others are exploring the use of cocaine vaccines (see e.g., Kosten et al. (2002) Vaccine 20, 1196-1204).

Table 1: Kinetics of several cocaine hydrolyzing enzymes against (-) cocaine.

Enzyme	Kcat (min ⁻¹)	Km (μM)	Efficiency (kcat/Km)	Reference
BChE	4.1	4.5	9.1×10^6	Sun et al., 2002a
Ala328W/Y332A	154	18	8.5×10^8	Sun et al., 2002a
Mab15A10	2.2	220	1×10^4	Larsen et al., 2004
AME 359	620	20	3.1×10^7	Gao et al., 2005
CocE	468	0.64	7.2×10^8	Turner et al., 2002

[0008] A bacterium, *Rhodococcus* sp. MB 1, indigenous to the soil surrounding the coca plant, has evolved the capacity to utilize cocaine as its sole carbon and nitrogen source. The bacterium expresses a cocaine esterase (CocE) that acts similarly to BChE to hydrolyze the benzoyl ester of cocaine, yielding ecgonine methyl ester and benzoic acid (see e.g., Figure 1) (Bresler et

al. (2000) *Appl Environ Microbiol* 66, 904-908; Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Struct Biol* 9, 17-21). The gene for CocE has been isolated and cloned (Bresler et al. (2000) *Appl Environ Microbiol* 66, 904-908), and the crystal structure of CocE has been determined (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Struct Biol* 9, 17-21). The structure of CocE (see e.g., Figure 2) reveals a classic serine esterase fold in addition to two other domains that combine to form a cocaine binding pocket. Altering any of three amino acids (Asp, His, or Ser) within the catalytic triad in the active site (for review, see Dodson G and Wlodawer A (1998) *Trends Biochem Sci* 23, 347-352) inactivates the esterase activity against cocaine. Furthermore, mutation of residues that make contact with the benzoate moiety of cocaine (e.g., Tyr44) also disrupts cocaine hydrolysis, presumably through impairing oxyanion stabilization in the transition state (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Structural Biology* 9, 17-21). The purified enzyme (MW ~65kDa) catalyzes cocaine very efficiently with Michaelis-Menten kinetics $k_{cat} = 7.2 \text{ s}^{-1}$ and $K_m = 640 \text{ nM}$ (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Structural Biology* 9, 17-21), nearly three orders of magnitude greater than endogenous esterases and, most likely, would act quickly enough to detoxify humans who have overdosed on cocaine (Landry et al. (1993) *Science* 259, 1899-1901; Mets et al. (1998) *National Academy of Sciences of the United States of America* 95, 10176-10181). Additionally, the esterase also metabolizes cocaethylene, a potent metabolite of cocaine and alcohol, almost as efficiently as it metabolizes cocaine ($k_{cat} = 9.4 \text{ s}^{-1}$ and $K_m = 1600 \text{ nM}$) (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Structural Biology* 9, 17-21).

[0009] Thus, it would be desirable to provide a stable CocE for anti-cocaine therapeutics.

SUMMARY OF THE INVENTION

[0010] Accordingly, the present inventors have succeeded in discovering highly efficient, thermostable, and long-lasting cocaine esterase mutants that can protect against the toxic and reinforcing effects of cocaine in subjects.

[0011] One aspect of the invention provides isolated mutant cocaine esterase (CocE) polypeptides of the wild-type CocE amino acid sequence (e.g., SEQ ID NO: 1) but with at least one amino acid residue substituted. The mutant CocE polypeptides have esterase activity with increased thermostability at 37°C as compared to wild-type CocE.

[0012] Various embodiments include mutant CocE polypeptides with at least two, three, four, five, or more substitutions of the wild-type CocE amino acid sequence. Examples of mutant CocE polypeptides within the scope of the invention include those with an amino acid sequence of SEQ ID NO: 3 (L163V); SEQ ID NO: 7 (V225I); SEQ ID NO: 8 (I218L); SEQ ID NO: 9 (A310D); SEQ ID NO: 10 (A149S); SEQ ID NO: 11 (S159A); SEQ ID NO: 12 (S265A); SEQ ID NO: 13 (S56G); SEQ ID NO: 14 (W220A); SEQ ID NO: 16 (S140A); SEQ ID NO: 17 (F189L); SEQ ID NO: 18 (A193D); SEQ ID NO: 19 (T254R); SEQ ID NO: 20 (N42V); SEQ ID NO: 21 (V262L); SEQ ID NO: 22 (L508G); SEQ ID NO: 23 (Y152H); SEQ ID NO: 24 (V160A); SEQ ID NO: 25 (T172R); SEQ ID NO: 26 (Y532F); SEQ ID NO: 27 (T74S); SEQ ID NO: 28 (W285T); SEQ ID NO: 29 (L146P); SEQ ID NO: 30 (D533S); SEQ ID NO: 31 (A194R); SEQ ID NO: 32 (G173Q); SEQ ID NO: 33 (C477T); SEQ ID NO: 34 (K531A); SEQ ID NO: 35 (R41I); SEQ ID NO: 36 (L119A); SEQ ID NO: 37 (K46A); SEQ ID NO: 38 (F84Y), T172R-G173Q (SEQ ID NO: 39); L169K (SEQ ID NO: 40); F189A (SEQ ID NO: 41), N197K (SEQ ID NO: 42), R182K (SEQ ID NO: 43), F189K (SEQ ID NO: 44), V190K (SEQ ID NO: 45), Q191K (SEQ ID NO: 46), and A194K (SEQ ID NO: 47), or a functional fragment(s) thereof. Additional exemplary mutant CocE polypeptides include, F189A/T172R, T172R/A193D, T172R/G173Q-I175-G-G-A186, T172R/G173Q-T176-G-G-D185, and the like. Given the naming

conventions and polypeptide sequences disclosed herein, one skilled in the art could determine the polypeptide sequences for the above-named mutant CocE polypeptides.

[0013] Another aspect of the invention provides pharmaceutical compositions that include among their components a mutant CocE polypeptide within the scope of the invention and a pharmaceutically acceptable carrier or excipient.

[0014] Another aspect of the invention provides isolated nucleic acids encoding the mutant CocE polypeptide described herein. In various embodiments, the nucleic acids include those with sequences that hybridize to the nucleic acid encoding wild-type CocE (e.g., SEQ ID NO: 2), or the complement thereto, under high stringency conditions. Such isolated nucleic acid encodes a mutant CocE polypeptide having esterase activity with increased thermostability at 37°C as compared to wild-type CocE. Various embodiments of the isolated nucleic acid sequence have at least about 85% sequence identity with the nucleic acid sequence of wild-type CocE (e.g., SEQ ID NO: 2). For example, the isolated nucleic acid sequence has at least about 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% sequence identity of wild-type CocE (e.g., SEQ ID NO: 2).

[0015] In various embodiments, mutant CocE polypeptides (or polypeptides encoded by nucleic acids within the scope of the invention) have increased melting temperatures as compared to wild-type CocE. In various embodiments, mutant CocE polypeptides (or polypeptides encoded by nucleic acids within the scope of the invention) increase mutant CocE polypeptide thermostability over wild-type CocE by at least about 2.0 kcal/mol. For example, the increased thermostability can be at least about 2.1 kcal/mol, at least about 2.2 kcal/mol, at least about 2.3 kcal/mol, at least about 2.4 kcal/mol, at least about 2.5 kcal/mol, at least about 2.6 kcal/mol, at least about 2.7 kcal/mol, at least about 2.8 kcal/mol, at least about 2.9 kcal/mol, at least about 3.0 kcal/mol, at least about 3.1 kcal/mol, at least about 3.2 kcal/mol, at least about 3.3

kcal/mol, at least about 3.4 kcal/mol, at least about 3.5 kcal/mol, at least about 3.6 kcal/mol, at least about 3.7 kcal/mol, at least about 3.8 kcal/mol, at least about 3.9 kcal/mol, at least about 4.0 kcal/mol, at least about 4.1 kcal/mol, at least about 4.2 kcal/mol, at least about 4.3 kcal/mol, at least about 4.4 kcal/mol, or at least about 4.5 kcal/mol.

[0016] In various embodiments, mutant CocE polypeptides (or polypeptides encoded by nucleic acids within the scope of the invention) have reduced immunogenicity as compared to wild-type CocE.

[0017] In some embodiments, the thermostable mutant CocE polypeptides have less esterase activity than wild-type CocE. For example, thermostable CocE mutants can have about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99% of the esterase activity of wild-type CocE. In other embodiments, the mutant CocE polypeptides have approximately the same, or greater, catalytic efficiency of wild-type CocE polypeptides. For example, thermostable CocE mutants can have about 100%, about 110%, about 120%, about 130%, about 140%, about 150%, or more of the esterase activity of wild-type CocE.

[0018] In various embodiments, the mutant CocE polypeptides are pegylated. In various embodiments, the mutant CocE polypeptide is encapsulated in a red blood cell. For example, a pegylated mutant CocE polypeptide(s) (or a pharmaceutical composition including a pegylated mutant CocE polypeptide(s)) can be encapsulated in a RBC.

[0019] In some embodiments, the mutant CocE polypeptides are stabilized by a substrate, product, and/or inhibitor.

[0020] Another aspect of the invention provides for methods of treating a cocaine-induced condition. In such methods, a therapeutically effective amount of a mutant CocE polypeptide(s) (or a pharmaceutical composition including a mutant CocE polypeptide(s)) within the scope of the invention is administered to

a subject in need thereof. In various embodiments, the cocaine-induced condition includes cocaine overdose, cocaine toxicity, cocaine addiction, cocaine dependence, and/or some combination thereof.

[0021] Yet another aspect of the invention provides for a high throughput screening method for identification of thermostable mutant CocE polypeptides. In such screening method, a cell is stably transformed with a nucleic acid encoding a mutant CocE polypeptide candidate. The mutant CocE polypeptide is expressed in the cell. The expressed mutant CocE polypeptide is isolated or displayed. The esterase activity of the isolated mutant CocE polypeptide is measured at one or more temperatures to determine thermostability of the isolated mutant CocE polypeptides. Such temperature(s) can be from about 30°C to about 50°C. Mutant CocE polypeptide with esterase activity at the predetermined temperature(s) are selected.

[0022] In some embodiments of the screening method, measuring esterase activity of the isolated mutant polypeptides can be accomplished by contacting the isolated mutant CocE polypeptide with (i) cocaine and a pH indicator or (ii) a thio-derivative of cocaine and a thiol indicator. Any change in the pH indicator or the thiol indicator is then detected. Such a change is correlated with the formation of benzoic acid from the hydrolysis of cocaine or cocaine derivative by the mutant CocE polypeptide.

[0023] Some embodiments of the screening method further include conducting several cycles of the screening procedure at increasing temperatures for measuring esterase activity. For example, the first cycle can employ a temperature for measuring esterase activity of about 30°C while a subsequent cycle can employ a temperature for measuring esterase activity of about 45°C.

[0024] In some embodiments of the screening method, expression of mutant CocE polypeptide occurs at a temperature at which wild type CocE substantially retains catalytic activity. In other embodiments of the screening method, expression of mutant CocE polypeptide occurs at a temperature at

which wild-type CocE polypeptide substantially partitions into inclusion bodies. For example, the expression temperature can be at least about 35°C, at least about 36°C, at least about 37°C, at least about 38°C, at least about 39°C, or at least about 40°C.

[0025] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0027] Figure 1 is a diagram illustrating the metabolism of cocaine by various enzymatic reactions.

[0028] Figure 2 is a ribbon diagram illustrating the CocE structure. Domain 1 (DOM1), domain 2 (DOM2), and domain 3 (DOM 3) are indicated, along with the active site located at the intersection of the three domains. A benzoic acid molecule is shown in the active site.

[0029] Figure 3 is a line plot depicting the elution profile for CocE by FPLC (Q-Sepharose), where UV absorbance and sodium chloride concentration is shown over time. For further methodology information, see Example 1.

[0030] Figure 4 is a line and scatter plot showing the in vitro degradation of cocaine in the presence of CocE in human plasma. For further methodology information, see Example 2.

[0031] Figure 5 is Michaelis-Menten enzyme kinetics plot for wild-type CocE, the T172R mutant CocE (SEQ ID NO: 26), and the S159A mutant CocE (SEQ ID NO: 11) for reactions carried out at 30°C and 37°C. For further methodology information, see Example 4.

[0032] Figure 6 is a set of photographs of the total protein staining after PAGE separation of wild-type and T172R mutant CocE using denaturing

(SDS+ β Me) or non-denaturing (Native) conditions after incubation at 37C for various time points. For more information see Example 4.

[0033] Figure 7 is a line plot depicting the circular dichroism determined melting temperatures of wild-type and T172R CocE. The full spectrum is shown in A, the near-UV spectrum is shown in B, and the estimated melting temperature for each mutant is shown in C. For more information see Example 4.

[0034] Figure 8 contains (A) a drawing of the hydrolysis of the benzoylthioester derivative of cocaine (thio-1) and the des-carbomethoxy cocaine (thio-2) followed by reaction of the released thiols (R-SH) with Ellman's reagent; and (B) a line plot showing the colorimetric reaction of control BL21 cells and BL21 cells containing CocE incubated with the benzoylthioester derivative (thio-1) and Ellman's reagent. For more information see Example 15.

[0035] Figure 9 is a line and scatter showing the effect of 1.0 mg CocE (closed circles) or PBS (vehicle, open circles) on cocaine-induced lethality when administered one minute after increasing doses of cocaine (n = 6-7). Data presented are expressed as percent and its standard error. For further methodology information, see Example 5.

[0036] Figure 10 is a bar graph showing the effect of increasing doses of CocE (CE) or human BChE (BChE), or PBS, on cocaine-induced lethality when administered one minute before 180 mg/kg cocaine (n = 6-7). Data presented are expressed as percent and its standard error mean. For further methodology information, see Example 5.

[0037] Figure 11 is a bar graph showing the effect of CocE mutants S117A and Y44F, or PMSF treated CocE on cocaine-induced convulsions and lethality when administered 1 minute before 180 mg/kg cocaine (n = 5-6). For further methodology information, see Example 5.

[0038] Figure 12 is a bar graph showing the effect of 1.0 mg CocE or PBS administered one minute after the 560 mg/kg WIN-35065-2, the determined

LD100 of the compound ($n = 6-8$). For further methodology information, see Example 5.

[0039] Figure 13 is a line and scatter plot showing time-dependent inactivation of CocE (125 ng/ml) in vitro. Exponential decay $t_{1/2}$ was calculated as 13.2. For further methodology information, see Example 6.

[0040] Figure 14 is a bar graph showing time-dependent protective effects of 1 mg CocE when administered at various times before and after 100 mg/kg cocaine ($n = 6-8$). For further methodology information, see Example 6.

[0041] Figure 15 is a bar graph showing the cocaine concentrations in human plasma treated with 0.8 μ M cocaine esterase or esterase vehicle at zero and one minute following esterase administration. For further methodology information, see Example 6.

[0042] Figure 16 is a series of line and scatter plots showing the effect of 0.32 mg CocE (closed circles), 0.32 mg of T172R mutant CocE (closed triangles), or PBS (vehicle, open circles) on cocaine-induced lethality when administered one minute after increasing doses of cocaine (left panel) and minutes to death after cocaine administration (right panel). For further methodology information, see Example 7.

[0043] Figure 17 is a line and scatter plot showing the effect of 0.32 mg/kg of CocE (closed circles) or T172R mutant CocE (closed triangles) on cocaine-induced lethality when administered at 1, 10, 30, and 60 minutes before administration of 320 mg/kg cocaine ($n=6$). For further methodology information, see Example 7.

[0044] Figure 18 is a series of line and scatter plots showing the effect of 1 mg of hBChE (closed triangle), 0.32 mg CocE (closed circle), or 1 mg of CocE (closed square) on cocaine-induced lethality. Figure 18A shows lethality as a function of cocaine concentration. Figure 18B shows minutes until death after cocaine administration as a function of cocaine concentration. For further methodology information, see Example 8.

[0045] Figure 19 is a trace of the MALDI-TOF mass spectrum of pegylated CocE. Mass difference between peaks corresponds to ~5500 Da; equivalent to the molecular weight of a single PEG chain. For further methodology information, see Example 11.

[0046] Figure 20 is an SEM image of glutaraldehyde fixed red blood cells. Figure 20A is normal RBC without treatment. Figure 20B is osmotic rupture-reseal RBC loaded with L-ASNase. Figure 20C is RBC loaded with LMWP-ASNase. For further methodology information, see Example 12.

[0047] Figure 21 is a scatter plot showing percent Asperaginase activity in blood as a function of time (days) for RBC/LMP-ASNase or RBC ghost/ASNase. For further methodology information, see Example 12.

[0048] Figure 22 is a line plot showing survival of DBA/2 mice bearing L5178Y lymphoma cells. Enzyme or saline were given on day 5, at which time symptoms were present. For further methodology information, see Example 12.

[0049] Figure 23 is a line and scatter plots demonstrating protective effects of CocE and its mutants against cocaine-induced toxicity. The figure depicts percentage of mice lethality exhibiting cocaine-induced lethality as a function of injected cocaine concentration in mice administered wild type CocE (0.3 mg), T172R (0.3 mg), T172R-G173Q (0.3 mg), or L169K (1 mg). CocE or mutants (mg) was administered intravenously 1 minute before cocaine administration (mg/kg, i.p.). Different symbols represent dose-response curves of cocaine-induced lethality in the absence or presence of CocE or mutants. Each data point represents the percentage of mice (n=8 for each dosing condition) exhibiting cocaine-induced lethality.

[0050] Figure 24 is a series of line and scatter plots demonstrating time course of protective effects of CocE against cocaine toxicity. CocE or mutants (0.1, 0.3, and 1 mg, i.v.) was administered at different time points before administration of i.p. cocaine 180 mg/kg. Figure 24A depicts percentage of mice exhibiting cocaine-induced lethality as a function of time of administration of wild

type CocE (0.1 mg), T172R (0.1 mg), L169K (0.1 mg), or T172R-G173Q (0.1 mg) before administration of i.p. cocaine 180 mg/kg. Figure 24B depicts percentage of mice exhibiting cocaine-induced lethality as a function of time of administration of wild type CocE (0.3 mg), T172R (0.3 mg), L169K (0.3 mg), or T172R-G173Q (0.1 mg) before administration of i.p. cocaine 180 mg/kg. Figure 24C depicts percentage of mice exhibiting cocaine-induced lethality as a function of time of administration of wild type CocE (1 mg), T172R (1 mg), L169K (1 mg), or T172R-G173Q (1 mg) before administration of i.p. cocaine 180 mg/kg. Each data point represents the percentage of mice (n=8 for each dosing condition) exhibiting cocaine-induced lethality.

[0051] Figure 25 is a line and scatter plot demonstrating estimated duration of protection for 50% lethality: The figure depicts the estimated duration (hours) of protection (50% lethality) of CocE mutants in mice as a function of dosage (mg, i.v.) of T172R-G173Q, L169K, T172R, and wild type CocE. The time required to reach 50% lethality was determined from Figure 24.

[0052] Figure 26 is a series of line and scatter plots demonstrating protective effects of CocE, T172R-G173Q, and their pegylated forms against cocaine-induced toxicity. Each enzyme (0.3 mg) was administered intravenously 1 min before cocaine administration (mg/kg, i.p.). Different symbols represent dose-response curves of cocaine-induced lethality in the absence or presence of the enzyme. Each data point represents the percentage of mice (n=8 for each dosing condition) exhibiting cocaine-induced lethality. Figure 26A depicts the percentage occurrence of lethality as a function of cocaine dosage (mg/kg, i.p.) for Vehicle/PBS, CocE wild type (0.3 mg), and PEG-CocE wild type (0.3 mg). Figure 26B depicts the percentage occurrence of lethality as a function of cocaine dosage (mg/kg, i.p.) for Vehicle/PBS, T172R-G173Q (0.3 mg), and PEG-T172R-G173Q (0.3 mg).

[0053] Figure 27 is a series of line and scatter plots showing the thermal stability of cocaine esterase and mutants: (A) wild-type CocE, (B)

T172R, (C) T272R/G173Q and L169K. For further methodology information, see Example 17.

[0054] Figure 28 is a series of chromatograms showing enzymes preincubated at 37°C for 0 minutes or 60 minutes and resolved by size exclusion chromatography (A) wild-type CocE, (B) T172R, (C) T172R/G173Q. Molecular weight standards, BSA (66Kda) and AD (150Kda) are included in A. For further methodology information, see Example 18.

[0055] Figure 29 is a series of smoothed CD spectra of wild-type CocE and four mutants showing temperature dependent melting observed between 200 and 250nm. For further methodology information, see Example 19.

[0056] Figure 30 is a series of spectra for wild-type CocE and four mutants deconvoluted to 3 curves via the CCA algorithm suggesting that the melting of CocE is at least a two-step process moving from the original curve (curve 1) to an intermediate unfolding step (curve 2) to a fully denatured protein (curve 3). For further methodology information, see Example 19.

[0057] Figure 31 is a series of line and scatter plots showing percentage contribution of each temperature in describing the 3 deconvoluted CCA curves shown in Figure 30. For further methodology information, see Example 19.

[0058] Figure 32 is a line and scatter plot showing melting and formation temperatures for (1) the initial spectra melts, (2) the formation and melt of the intermediate state, and (3) the accumulation of the fully melted protein. For further methodology information, see Example 19.

[0059] Figure 33 is a picture of a gel showing that Cocaine (mM range) prevented 37°C-induced formation of high molecular weight CocE aggregates (0.1mg/ml enzyme concentrations). For further methodology information, see Example 20.

[0060] Figure 34 is a line and scatter plot showing that Cocaine (μM quantities) stabilized 37°C -induced activity loss. For further methodology information, see Example 20.

[0061] Figure 35 is a picture of a gel showing that Benzoic acid (mM range) prevented 37°C -induced formation of high molecular weight CocE aggregates (0.1mg/ml enzyme concentrations). For further methodology information, see Example 20.

[0062] Figure 36 is a line and scatter plot showing that Cocaine (μM quantities) stabilized 37°C -induced activity loss. For further methodology information, see Example 20.

DETAILED DESCRIPTION OF THE INVENTION

[0063] Embodiments of the invention disclosed wherein provide compositions and methods for a catalytic degradation approach to anti-cocaine therapeutics. The technology described herein is based in part on the discovery of highly efficient, thermostable, and long-lasting cocaine esterase mutants that can protect against the toxic and reinforcing effects of cocaine in subjects. Such mutants provide treatment options for cocaine-induced conditions such as cocaine overdose and cocaine addiction.

MUTANT COCE POLYPEPTIDES

[0064] Despite the potency of wild-type CocE (see e.g., SEQ ID NO: 1, Accession No. AF173165) in metabolizing cocaine (see e.g., Example 2; Example 4; Example 5), application of wild-type CocE as a therapeutic agent in the treatment of cocaine overdose may be limited because of its low thermal stability at the physiological temperature (see e.g., Example 4; Example 6). Thermo-instability contributes to the short plasma half-life of wild-type CocE. Significant decay ($>50\%$) of CocE activity was observed following incubation of the enzyme in plasma at 37°C , or after its intravenous administration into a mouse. The $t_{1/2}$ of CocE at 37°C is approximately 15 minutes whereas at 4°C the

$t_{1/2}$ is greater than 6 months. Preliminary studies in rats demonstrated a relatively short duration of anti-cocaine effect of little more than 30 minutes for CocE.

[0065] One aspect of the invention thus provides purified mutant CocE polypeptides that exhibit increased thermal stability and plasma half-life as compared to wild-type CocE. The mutant CocE polypeptides of the invention hold significant clinical value because of their capability to efficiently hydrolyze cocaine, while also exhibiting increased thermostability and/or plasma half-life.

[0066] The invention provides mutant CocE polypeptides in which at least one amino acid residue of the wild-type CocE is substituted, where the mutant CocE has increased thermostability while retaining relatively high catalytic efficiency. In some embodiments, mutant CocE polypeptides substantially maintain the wild-type CocE polypeptide functional esterase activity (i.e., hydrolysis of cocaine). Mutant CocE polypeptides have a peptide sequence that differs from a native CocE polypeptide in one or more amino acids. The peptide sequence of such mutants can feature a substitution, deletion, or addition of one or more amino acids of a native CocE polypeptide. Amino acid insertions are preferably of about 1, 2, 3, and 4 to 5 contiguous amino acids, and deletions are preferably of about 1, 2, 3, 4, 5, 6, 7, 8, and 9 to 10 contiguous amino acids. In various embodiments, the mutant CocE polypeptide can contain at least one, two, three, four, or more amino acid substitutions, deletions, or additions, where the resulting mutant CocE polypeptide has increased thermostability.

[0067] The term amino acid, as used herein, is intended to include naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs and derivatives. The latter includes molecules containing an amino acid moiety. One skilled in the art will recognize that reference herein to an amino acid includes, for example, naturally occurring proteogenic L-amino acids; D-amino acids; chemically modified amino acids such as amino acid analogs and derivatives; naturally occurring non-proteogenic amino acids, and chemically synthesized compounds having properties known in

the art to be characteristic of amino acids. For all the amino acid sequences disclosed herein, it is understood that equivalent nucleotides and amino acids can be substituted into the sequences without affecting the function of the sequences. Such substitution is within the ability of a person of ordinary skill in the art.

[0068] The invention also provides purified mutant CocE polypeptides with the following substitutions: L163V (SEQ ID NO: 3); V121D (SEQ ID NO: 4); S167A (SEQ ID NO: 5); Q123E (SEQ ID NO: 6); V225I (SEQ ID NO: 7); I218L (SEQ ID NO: 8); A310D (SEQ ID NO: 9); A149S (SEQ ID NO: 10); S159A (SEQ ID NO: 11); S265A (SEQ ID NO: 12); S56G (SEQ ID NO: 13); W220A (SEQ ID NO: 14); T122A (SEQ ID NO: 15); S140A (SEQ ID NO: 16); F189L (SEQ ID NO: 17); A193D (SEQ ID NO: 18); T254R (SEQ ID NO: 19); N42V (SEQ ID NO: 20); V262L (SEQ ID NO: 21); L508G (SEQ ID NO: 22); Y152H (SEQ ID NO: 23); V160A (SEQ ID NO: 24); T172R (SEQ ID NO: 25); Y532F (SEQ ID NO: 26); T74S (SEQ ID NO: 27); W285T (SEQ ID NO: 28); L146P (SEQ ID NO: 29); D533S (SEQ ID NO: 30); A194R (SEQ ID NO: 31); G173Q (SEQ ID NO: 32); C477T (SEQ ID NO: 33); K531A (SEQ ID NO: 34); R41I (SEQ ID NO: 35); L119A (SEQ ID NO: 36); K46A (SEQ ID NO: 37); F84Y (SEQ ID NO: 38), T172R-G173Q (SEQ ID NO: 39); L169K (SEQ ID NO: 40); F189A (SEQ ID NO: 41), N197K (SEQ ID NO: 42), R182K (SEQ ID NO: 43), F189K (SEQ ID NO: 44), V190K (SEQ ID NO: 45), Q191K (SEQ ID NO: 46), and A194K (SEQ ID NO: 47). For example, the T172R mutant CocE polypeptide (SEQ ID NO: 25) has increased thermostability, increased Vmax and Km at 37°C, increased melting temperature (Tm), increased plasma half-life, greater reductions in lethality due to cocaine toxicity, and longer lasting anti-cocaine effects, as compared to wild-type CocE (see e.g., Example 4, Example 7).

[0069] The resulting increase in thermostability of the mutant CocE polypeptide is at least about 2 kcal/mol. Thermostability of a given polypeptide can be assessed by a variety of methods known to the art, including for example circular dichroism (CD) spectroscopy or differential scanning calorimeter. For

example, the resulting increase in thermostability can be at least about 2.1, at least about 2.2, at least about 2.3, at least about 2.4, at least about 2.5, at least about 2.6, at least about 2.7, at least about 2.8, at least about 2.9, at least about 3.0, at least about 3.1, at least about 3.2, at least about 3.3, at least about 3.4, at least about 3.5, at least about 3.6, at least about 3.7, at least about 3.8, at least about 3.9, at least about 4.0, at least about 4.1, at least about 4.2, at least about 4.3, at least about 4.4, or at least about 4.5 kcal/mol. Even greater thermostability increases are contemplated. It is thought that lowering the energy by about 2.1 to about 4.5 kcal/mol can extend the half-life time of the protein about 30 to about 1000 fold longer at room temperature.

[0070] Generally, the mutant CocE polypeptides have esterase activity with increased thermostability as compared to wild-type CocE. In some embodiments, the thermostable mutant CocE polypeptides can have less esterase activity than wild-type CocE. For example, thermostable CocE mutants can have about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99% of the esterase activity of wild-type CocE. In other embodiments, the mutant CocE polypeptides have approximately the same, or greater, catalytic efficiency of wild-type CocE polypeptides. For example, thermostable CocE mutants can have about 100%, about 110%, about 120%, about 130%, about 140%, about 150%, or more of the esterase activity of wild-type CocE.

[0071] Variants of the mutant CocE polypeptides such as fragments, analogs, and derivatives are also within the invention. CocE polypeptide fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 50, 75, 100, 125, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1150 and 1200 amino acids in length are intended to be within the scope of the invention disclosed herein. Isolated peptidyl portions of CocE

polypeptides can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a CocE polypeptide as described herein can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length.

[0072] Another aspect of the invention disclosed herein concerns recombinant forms of the CocE polypeptide. In some embodiments, isolated nucleic acid molecules of the invention include those polynucleotides encoding the above described CocE polypeptides. In other embodiments, the recombinant polypeptides of the invention disclosed herein are encoded by a nucleic acid that has at least 85% sequence identity (e.g., 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) with the nucleic acid sequence of SEQ ID NO: 2, where the expressed recombinant CocE polypeptide retains substantially the same or more catalytic efficiency of wild-type CocE polypeptide and has increased thermostability as compared to wild-type CocE.

[0073] Nucleic acids that hybridize under stringent conditions to the nucleic acids of SEQ ID NO: 2 or the complements of SEQ ID NO: 2 can also be used in the invention. For example, such nucleic acids that hybridize to SEQ ID NO: 2 or the complement of SEQ ID NO: 2 under low stringency conditions, moderate stringency conditions, or high stringency conditions and also encode a mutant CocE polypeptide that has esterase activity with increased thermostability as compared to wild-type CocE, are within the invention. Preferred nucleic acids are those having a nucleotide sequence that is the complement of all or a portion of SEQ ID NO: 2. Other variants of the native CocE gene within the invention are polynucleotides that share at least 65% (e.g., 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) sequence identity to SEQ ID NO: 2

or the complement of SEQ ID NO: 2. Nucleic acids that hybridize under stringent conditions to or share at least 65% sequence identity with SEQ ID NO: 2 or the complement of SEQ ID NO: 2 can be obtained by techniques known in the art such as by making mutations in the native CocE gene, or by isolation from an organism expressing such a nucleic acid (e.g., an allelic variant).

[0074] Nucleic acid molecules encoding mutant CocE fusion proteins are also within the invention. Such nucleic acids can be made by preparing a construct (e.g., an expression vector) that expresses mutant CocE fusion protein when introduced into a suitable host. For example, such a construct can be made by ligating a first polynucleotide encoding mutant CocE protein fused in frame with a second polynucleotide encoding another protein such that expression of the construct in a suitable expression system yields a fusion protein.

[0075] The nucleic acid molecules of the invention can be modified at a base moiety, sugar moiety, or the phosphate backbone, e.g., to improve stability of the molecule, hybridization, and the like. Nucleic acid molecules utilized in embodiments of the invention disclosed herein can be in the form of RNA or in the form of DNA (e.g., cDNA, genomic DNA, and synthetic DNA). The DNA can be double-stranded or single-stranded, and if single-stranded can be the coding (sense) strand or non-coding (anti-sense) strand. The coding sequence which encodes a mutant CocE polypeptide can be identical to the claimed nucleotide sequence, or it can also be a different coding sequence which, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide as the polynucleotides of SEQ ID NOS: 3-37.

DESIGN OF MUTANT COCE

[0076] CocE thermostabilizing mutations can be designed to either increase the thermodynamic stability of a polypeptide through lowering $\Delta G(\text{unfolded} \rightarrow \text{folded})$ or decrease rate of unfolding through increasing the activation free energy of the unfolding process. The $\Delta G(\text{unfolded} \rightarrow \text{folded})$ is the

free-energy difference between the unfolded and the folded state. For a thermodynamically stable polypeptide, $\Delta G(\text{unfolded} \rightarrow \text{folded})$ should be a negative value. Generally, the lower the $\Delta G(\text{unfolded} \rightarrow \text{folded})$ value, the more stable the folded state. The activation free energy of the unfolding process is the free energy difference between the folded state and the transition state of unfolding (Steipe, 1999).

[0077] Mutant CocE polypeptides with increased thermostability can be designed and generated by a variety of methods known to the art including, for example, rational design, directed evolution (e.g., random mutagenesis, mutagenesis of the CocE host organism), or a combination thereof. Directed evolution can be achieved through mutation and recombination followed by either screening for the desired trait or by applying a selective pressure to obtain the trait of interest (see e.g., Lehmann and Wyss, 2001). Mutagenesis can be performed either on the specific gene of interest or through mutagenesis and selection of a host organism such that the engineered property is conferred to the protein of interest. Preferably, the thermostability of CocE mutants are engineered through a three-prong approach of (i) rational design through computation-guided site-directed mutagenesis (see e.g., Example 3; Example 4), (ii) random mutagenesis of the CocE gene and high throughput screening (see e.g., Example 4; Example 15); and (iii) mutagenesis of the CocE host organism followed by genetic selection (see e.g., Example 14).

[0078] Various rational design concepts are known to those of skill in the art to accomplish lowering $\Delta G(\text{unfolded} \rightarrow \text{folded})$ and/or increasing the activation free energy of the unfolding process (see e.g., Lehmann, 2001). For example, one can: decrease the entropy of the unfolded state by introducing additional disulfide bridges or by X $\rightarrow$ Pro mutations; increase α -helix propensity by Gly $\rightarrow$ Ala substitutions or by stabilization of α -helix macrodipoles; improve electrostatic interactions between charged surface residues by introducing additional salt bridges or even salt-bridge networks, or by thermostabilizing mutations based on calculations of electrostatic potentials.

[0079] Molecular modeling, based on appropriate molecular dynamics (MD) simulations, can rationally guide site-directed mutagenesis so as to design mutant CocE polypeptides possessed of increased thermostability. Classical MD simulation enables the study of time evolution of a large system by taking many small successive time steps under atomic forces determined by a set of parameterized interaction functions (force field), including bonded interactions (bonds, angles, and dihedral angles), non-bonded van der Waals interactions, and electrostatic interactions based on net atomic charges. Due to the simple force field form, the MD simulation may be performed for a sufficiently long simulation time to give meaningful ensemble-averaged properties, even for a very large system involving over a hundred thousand atoms. So, for CocE and each proposed mutant, the MD simulation can lead to a reasonable, dynamically averaged 3D structure of the simulated polypeptide in water.

[0080] One approach used successfully herein focuses on the rational design of thermostabilizing mutations that lower the $\Delta G(\text{unfolded} \rightarrow \text{folded})$ value of the polypeptide (see e.g., Example 3). Such approach requires only the calculation of $\Delta G(\text{unfolded} \rightarrow \text{folded})$, without performing a more time-consuming computation on the structure and energetics of the transition state of the unfolding. Hence, to increase the thermostability of a polypeptide before pegylation, one can use a method implemented in a rational design program (e.g., RosettaDesign) that uses an energy function for evaluating the fitness of a particular sequence for a given fold and a Monte Carlo search algorithm for sampling sequence space. Such approach is known to produce increased thermostability of other enzymes with no reduction in catalytic efficiency (see e.g., Korkegian, 2005). For example, the fold used in the computation can correspond to that of the available CocE X-ray crystal structure.

[0081] The rational design program allows prediction of a set of modified amino acid sequences that potentially have lower energies (e.g., the $\Delta G(\text{unfolded} \rightarrow \text{folded})$ values) and, therefore, higher thermostability. Thus, one can use the computational design described herein to predict mutations in the

CocE polypeptide core that can lead to thermostabilization of the polypeptide without loss of catalytic efficiency. This approach minimizes experimental test time and greatly increases the success of experimental outcomes. The predicted thermostabilizing mutations can be tested individually by site-directed mutagenesis and then in combination, in an iterative process (*see e.g.*, Example 15).

[0082] Directed evolution can also be used to generate thermostable CocE mutants. Directed evolution encompasses a series of experimental techniques that produce accelerated diversity and adaptation through mutation and recombination followed by either screening for the desired trait or by applying a selective pressure to obtain the trait of interest (Lehmann & Wyss (2001) *Current Opinion in Biotechnology* 12, 371-375). Thus directed evolution involves both a process to generate diversity and an efficient screening or selection method for the detection or enrichment of the desired trait. Directed evolution has previously been successfully applied to the production of thermostable proteins, and the generation of diversity has been achieved through, for example, error prone PCR, saturation mutagenesis, DNA shuffling, chemical mutagenesis, and combinations thereof. Error prone PCR amplifies the gene of interest with non-proofreading polymerases and stressful conditions designed to randomly generate single base pair mutations. After each round the best mutants are selected and are used as parent sequences in the next round of mutagenesis. This technique has been used to generate a number of thermostable protein variants, including propyl endopeptidase (Uchiyama *ma.*, et al. 2000), betaglucuronidase (Flores, H. and A. D. Ellington (2002) *Journal of Molecular Biology* 315, 325-337) and family 10 xylanases (Andrews et al. (2004) *J Biol Chem* 279, 54369-79). Saturation mutagenesis also amplifies the gene of interest but incorporates universal bases during amplification to generate a much higher number of mutations. This technique has been used to generate thermostability in a psychrophilic enzyme (Miyazaki et al. (2000) *Journal of Molecular Biology* 297, 1015-1026). DNA shuffling involves one or more cycles

of recombination between a set of homologous sequences to obtain improved variants of a given enzyme. This technique can also be used in tandem with error-prone PCR, where the best mutants obtained by error-prone PCR are combined by DNA shuffling to generate a new subset of mutants. DNA shuffling has been implemented to generate thermostable variants of beta-glucuronidase (Flores, H. and A.D. Ellington (2002) *Journal of Molecular Biology* 315, 325-337). Chemical mutagenesis involves treating plasmid DNA with chemicals that introduce point mutations into the sequence such as hydroxylamine, nitrosamines or dimethyl sulfate. Plasmid treatment with hydroxylamine has been used to generate thermostable mutants of *Bacillus polymyxa* beta-glucosidase A (Lopez-Camacho et al. (1996) *Biochemistry Journal* 314, 833-838) and firefly luciferase (White et al. (1996) *Biochemistry Journal* 319 (Pt 2), 343-350).

[0083] Mutagenesis of the CocE host organism can also be used to generate thermostable CocE mutants. A simple and fast method for the production of thermostable CocE variants can be achieved by utilizing the enzyme's ability to confer metabolism of cocaine as a sole carbon source on its host organism. The CocE gene was originally sequenced from *Rhodococcus* MB1 by subcloning gene fragments into *Rhodococcus erythropolis* CW25, a bacterium unable to metabolize cocaine but able to grow on the cocaine esterase byproducts ecgonine methyl ester and benzoate (Bresler et al. (2000) *Applied & Environmental Microbiology* 66, 904-908). Another organism previously shown to metabolize cocaine byproducts is *Pseudomonas fluorescens* (MBER), which was able to grow in a symbiotic relationship with another bacterium able to metabolize cocaine via the esterase *Comamonas acidovorans* (MBLF).

[0084] While difficult to transform with plasmids at high efficiencies, these bacterial strains can be relatively easily transformed with the native CocE gene cloned into appropriate shuttle vectors and then traditional bacterial mutagenesis can be performed (see e.g., Example 14). Because these bacteria

would normally only grow at 25-30°C on cocaine hydrolysis products, selection for mutants able to metabolize cocaine efficiently at 37°C would be expected to select for highly active and CocE mutants that are stable at 37°C.

[0085] The exposure of bacteria to radiation or chemical agents for the production of mutants carrying new phenotypes is well known in the art (see e.g., Maron, D.M. and Ames, BN (1983) *Mutation Research* 113, 173-215). Mutagenesis by irradiation can involve both ionizing and non-ionizing radiation; however, non-ionization radiation is the widely used, and UV radiation at 260nm is most effective as a lethal agent. Mutagenesis is caused by the induction of pyrimidine dimers, increasing the likelihood of incorporating mismatches during replication. Cells are exposed to UV radiation at a dose predetermined to kill 90-95% of the cell population, and mutants are then sought among the survivors. Chemical mutagenesis includes the use of base analogs such as 5-bromouracil and 2-aminopurine which increase copy error during replication, or the use of agents that react directly with DNA such as hydroxylamine or nitrosoguanidine which induce mutations at a higher frequency than base analogs.

GENERATING MUTANT COCE POLYPEPTIDES

[0086] Embodiments of the invention further pertain to methods of producing the mutant CocE polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the mutant CocE polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The cells can be harvested, lysed, and the protein isolated. A mutant CocE polypeptide can be isolated from host cells using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such protein (see e.g., Example 1).

[0087] For example, after a mutant CocE polypeptide has been expressed in a cell, it can be isolated using any immuno-affinity chromatography.

More specifically, an anti-CocE antibody can be immobilized on a column chromatography matrix, and the matrix can be used for immuno-affinity chromatography to purify the mutant CocE polypeptide from cell lysates by standard methods (see, e.g., Sambrook and Russel (2006) *Condensed Protocols from Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, ISBN 0879697717;). After immuno-affinity chromatography, the mutant CocE polypeptide can be further purified by other standard techniques, e.g., high performance liquid chromatography. In another embodiment, a mutant CocE polypeptide is expressed as a fusion protein containing an affinity tag (e.g., His₆) that facilitates its purification (see e.g. Example 1).

PEGYLATION OF MUTANT COCE POLYPEPTIDES

[0088] The mutant CocE can be pegylated so as to increase the duration of action and heat stability, and decrease immunogenicity. Pegylation can further enhance the thermostability of the mutant CocE of the invention and increasing serum half life by decreasing renal clearance, proteolysis, macrophage uptake, and immunological response.

[0089] Pegylation is the process of attaching repeating units of ethylene glycol (i.e., polyethylene glycol, or PEG) to a polypeptide to reduce the polypeptide's immunogenicity and its rate of renal clearance (see generally (Veronese, FM and Harris, JM (2002b) *Advanced Drug Delivery Reviews* 54, 457-606, Veronese, FM and Harris, JM (2002c) *Advanced Drug Delivery Reviews* 55, 1259-1345, reviewing PEGylation technology). Each ethylene glycol unit can bind to two or three water molecules, which effectively increases the size of the peptide, and can protect the peptide from immune responses, enzymatic degradation, and/or rapid renal clearance. The polyethylene glycol can also stabilize against changes in temperature and pH. The net result is that the therapeutic polypeptide can be maintained longer in the blood, and induces a lesser immune response (Harris, JM and Chess, RB (2003) *Nature Reviews*.

Drug Discovery 2, 214-221). PEG possesses a unique set of properties, including absence of toxicity, antigenicity, and immunogenicity, a mass-dependent diminution of renal clearance, and a high flexibility and solubility in water. It imparts these characteristics to the proteins to which it is bound (Veronese, FM and Harris, JM (2002b) Advanced Drug Delivery Reviews 54, 457-606).

[0090] A PEG polymer can first be activated with a functional group that encourages covalent binding to an amino acid of the protein. The terminal hydroxyl group of the PEG can be modified by an active carbonate, active ester, aldehyde or tresylate derivative. The PEG can be attached to lysines or to introduced cysteine residues of the mutant CocE. Repeating units of ethylene oxide can be constructed in many configurations having different lengths, with or without branching, and with various molecular weights. Means of incorporation can include site-directed mutagenesis or use of maleimide derivatives of transglutaminase.

[0091] PEG is FDA approved for use as a vehicle or base in pharmaceuticals, including injectable, topical, rectal and nasal formulations (Harris and Chess, 2003). And PEGylated drugs have been approved for clinical use (see e.g., PEG-interferon alpha-2a (Hamidi, M and Tajerzadeh, H (2003) Drug Delivery 10, 9-20; PEG-interferon alpha-2b (Reddy et al. (2002) Advanced Drug Delivery Reviews 54, 571-586).

[0092] Owing to the unparallel spatial dynamic mobility of PEG, modification of the surface of a mutant CocE by the PEG polymer can shield the coated enzyme from actions by the proteolytic enzymes and recognition by the immune system; thereby reducing the immunogenicity and prolonging the circulating half-life of the pegylated mutant CocE. Success of pegylation strategies include PEG-modified liposomes (i.e., stealth liposomes, so termed due to their ability in dodging immune detection and renal clearance to yield a significantly extended circulating time (Lasic, DD (1997) Journal of Controlled Release 48, 203-222), as well as pegylated asparaginase whose in vivo half-life

has been drastically improved from 26 hours seen for free asparaginase to 15 days (Avramis et al. (2002) *Blood* 99, 1986-1994). Further, it is recognized in the art that pegylation can significantly enhance the thermal stability of an enzyme (Kazan, D. and Erarslan, A. (1997) *Applied Biochemistry & Biotechnology* 62, 1-13; Efremova et al. (1998) *Biochemistry (Moscow)* 63, 441-447) and reduce activation of the complement system (Chang et al. (2005) *Bioconjugate Chemistry* 16, 147-155). Thus, PEGylation technology is well suited to improve the pharmacological and pharmaceutical effects of the mutant CocE of the invention (see e.g., Example 11). Pegylation of mutant CocE can also be used in conjunction with RBC encapsulation.

ENCAPSULATING MUTANT CocE IN RBC

[0093] The mutant CocE can be encapsulated into red blood cells (RBC) so as to increase the duration of action and heat stability, and decrease immunogenicity. Cocaine, just as it rapidly crosses the blood-brain barrier, also rapidly transits the RBC plasma membrane, and this uptake has been demonstrated in a study of intravenous cocaine administration in man (Javaid et al. (1978) *Journal of Chromatography* 15, 105-113). Furthermore, cocaine concentrations in RBC exceed the concentrations in plasma (Javaid et al. (1978) *Journal of Chromatography* 15, 105-113). As such, RBC encapsulation can be utilized to shield thermostable forms of CocE from clearance.

[0094] Erythrocytes have been exploited extensively for their potential applications as drug carriers (Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). Being the most abundant cells of the human body, RBC offers unmatched advantages to function as a drug carrier. First, erythrocytes are completely biocompatible and biodegradable, particularly when the autologous cells are used for drug loading. In addition, the biconcave disk shape of erythrocytes provides them with the highest surface to volume ratio (1.9×10^4 cm/g) (Guyton, AG & Hall, JE (1996) *Textbook of Medical Physiology*, 425-433) usable for drug encapsulation. Encapsulation in RBC also prevents the loaded

drug from inactivation by endogenous factors as well as protects the organisms from toxic effects of the encapsulated drug (Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). Furthermore, RBC encapsulation enables avoidance of undesirable immune responses (i.e. antigenicity and immunogenicity) that may be triggered by foreign bodies (Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). And, erythrocytes possess longer circulation half-life in comparison to other synthetic carriers. For example, the normal life-span of an erythrocyte in systemic circulation is reported to be about 120 days (Guyton, AG & Hall, JE (1996) *Textbook of Medical Physiology*, 425-433).

[0095] Encapsulation of mutant CocE in erythrocytes can be according to several techniques known in the art, including electroporation, drug (e.g., primaquine)-induced endocytosis, and osmosis (see e.g., Green, R. and Widder, KJ (1987) *Methods In Enzymology*, Vol. 149). These methods can include swelling and disrupting the cell membrane, releasing the interior content including primarily hemoglobin and also cytoskeleton, entrapping the drug, and then resealing the membrane to yield the cup-shaped, spherical, pink or white-colored RBC (generally termed "RBC-Ghost").

[0096] Alternatively, membrane-penetrating peptides can be employed to encapsulate mutant CocE in erythrocytes (see e.g., Example 12). Membrane-penetrating peptides, or protein transduction domain (PTD) peptides are a family of small (normally consists of 10-15 amino acid residues) peptides including TAT, LMWP, and other arginine-rich cationic peptides (see generally, Dietz, GPH and Bahr, M (2004) *Molecular Cell Neurosciences* 27, 85-131). It is known to the art that, by covalently linking PTD to almost any type of molecular species including proteins (MW>150 kDa; more than 60 different proteins have already been tested (Dietz, GPH and Bahr, M (2004) *Molecular Cell Neurosciences* 27, 85-131) and nano-carriers (e.g. liposomes), PTD can ferry the attached species across cell membrane of all organ types including the brain (Schwarze et al. (1999) *Science* 285, 1569-1572). PTD is neither toxic nor immunogenic

(Schwarze et al. (1999) Science 285, 1569-1572), and the PTD-mediated cell internalization does not induce perturbation or alteration of the erythrocytes cell membrane (Suzuki et al. (2002) Journal of Biological Chemistry 25, 2437-2443). PTD has been used successfully in protein loading RBC, and results in erythrocytes with unaltered physical and chemical attributes (see e.g., Li et al. (2003) American Pharmaceutical Review 6, 22-26). Thus, PTD peptide conjugation to mutant CocE can facilitate RBC encapsulation. RBC encapsulation can also be carried out by using pegylated mutant CocE.

STABILIZING MUTANT CocE

[0097] Another aspect of the invention is directed toward stabilization of mutant CocE polypeptides using substrates, products, and/or inhibitors of cocaine. Substrates and products useful in embodiments disclosed herein include, for example, but are not limited to, cocaine; cocaine derivatives, such as, for example, (-)-cocaine, (+)-cocaine, tropococaine, and the like; thio-cocaine derivatives, such as, for example, Thiol-1, Thiol-2, and the like; amide-cocaine derivatives; provitamin-cocaine derivatives, such as PABA cocaine, Niacin cocaine, and the like; benzoic acid; 4-nitrophenyl acetate (4NPA); 4-nitrophenol (4NP); and the like. Exemplary inhibitors include, but are not limited to, substrate analogues, such as, phospho-fluorococaine, O-Phospho-cocaine, O-methylphosphococaine, S-Methylphosphococaine, and the like; product analogues, such as, Ecgonine and Ecgonine derivatives, such as, boronic acid ecgonine methylester analog; phenylboronic acid; benzoic acid derivatives, such as, 4-tert-Butyl benzoic acid, 1-Naphthoic acid, 2,3,4-trimethyl-benzoic acid methyl ester, and the like. Additional chemicals include, for example, but are not limited to, SDS, glycerol, PEG, and the like.

[0098] Preferably, the substrates, products, and/or inhibitors stabilize thermal denaturation of the polypeptides disclosed herein. In some embodiments, the substrates, products and/or inhibitors also prevent thermally-induced aggregation in gel electrophoresis. Generally, use of a substrate,

product, and/or inhibitor results in at least about a 10% increase in stability and/or inhibition, respectively. For example the increase can be about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 120%, 150%, 200%, 300%, or even 500% or greater. Thus, substrates, products, and/or inhibitors are well suited to stabilize the mutant CocE polypeptides disclosed herein (see e.g., Example 20).

[0099] In one embodiment, small molecules are used to thermostabilize the mutant CocE polypeptides disclosed herein. In a preferred embodiment, such molecules do not occupy the active site of the polypeptide.

[0100] In some embodiments, the polypeptides disclosed herein can be co-infused with a stabilizing molecule. In other embodiments, the stabilizing molecules can be used to stabilize the polypeptide during manufacturing. In still other embodiments, the stabilizing molecules can be used to stabilize the mutant CocE polypeptides until ready for use.

TREATMENT METHODS

[0101] Another aspect of the invention is directed toward catalytic degradation approach to anti-cocaine therapeutics. Provided are treatments, both prophylactic and therapeutic, of cocaine-induced conditions through the administration of thermostable, esterase active, mutant CocE polypeptides to a subject in need thereof. The cocaine esterase variants of the invention hold significant clinical value because of their increased thermostability and longer plasma half-life than known naturally occurring CocE. It is this increase in thermostability and plasma half-life that enables a much more rapid response to the life-threatening symptoms of cocaine toxicity that sets the CocE variants of the invention apart from other treatment options.

[0102] A determination of the need for treatment will typically be assessed by a history and physical exam consistent with the cocaine-induced condition. Cocaine-induced conditions include, but are not limited to, cocaine

overdose, cocaine toxicity, and cocaine dependence and/or addiction. For example, the diagnosis of cocaine toxicity can include convulsions, grand-mal seizures, cardiac arrest, myocardial infarction, cardiac arrhythmias, increased blood pressure, stroke, drug-induced psychosis, dissecting aneurysm, and increased myocardial oxygen demand. As another example, in the case of cocaine dependence and/or addiction, withdrawal symptoms include subjective sensations of mild to severe dysphoria, depression, anxiety, or irritability. Subjects with an identified need of therapy include those with a diagnosed cocaine-induced condition, an indication of a cocaine-induced condition, and subjects who have been treated, are being treated, or will be treated for a cocaine-induced condition. The subject is preferably an animal, including, but not limited to, mammals, reptiles, and avians, more preferably horses, cows, dogs, cats, sheep, pigs, and chickens, and most preferably human.

[0103] An effective amount of the mutant CocE polypeptides described herein is generally that which can reduce the cocaine-toxicity or the severity of a cocaine-induced condition. Reduction in severity includes, for example, an arrest or a decrease in symptoms, physiological indicators, biochemical markers, or metabolic indicators. When used in the methods of the invention, a therapeutically effective amount of mutant CocE polypeptide described herein can be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt form and with or without a pharmaceutically acceptable excipient. For example, the mutant CocE polypeptides of the invention can be administered at a reasonable benefit/risk ratio applicable to any medical treatment, in an amount sufficient to substantially reduce the cocaine concentration in the blood and/or tissues of the subject.

[0104] Toxicity and therapeutic efficacy of mutant CocE polypeptides can be determined by standard pharmaceutical procedures in cell cultures and/or experimental animals for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀, (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the

therapeutic index that can be expressed as the ratio LD_{50}/ED_{50} , where large therapeutic indices are preferred.

[0105] The amount of mutated CocE polypeptide that can be combined with a pharmaceutically acceptable carrier to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. It will be appreciated by those skilled in the art that the unit content of agent contained in an individual dose of each dosage form need not in itself constitute a therapeutically effective amount, as the necessary therapeutically effective amount could be reached by administration of a number of individual doses. Agent administration can occur as a single event or over a time course of treatment. For example, an agent can be administered daily, weekly, bi-weekly, or monthly. For some conditions, treatment could extend from several weeks to several months or even a year or more.

[0106] The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the cocaine-induced condition being treated and the severity of the cocaine-induced condition; activity of the mutant CocE polypeptide employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the plasma half-life of the mutant CocE polypeptide; the rate of excretion of the mutant CocE polypeptide employed; the duration of the treatment; drugs used in combination or coincidental with the mutant CocE polypeptide employed; and like factors well known in the medical arts (see e.g., Koda-Kimble et al. (2004) Applied Therapeutics: The Clinical Use of Drugs, Lippincott Williams & Wilkins, ISBN 0781748453; Winter (2003) Basic Clinical Pharmacokinetics, 4th ed., Lippincott Williams & Wilkins, ISBN 0781741475; Sharqel (2004) Applied Biopharmaceutics & Pharmacokinetics, McGraw-Hill/Appleton & Lange, ISBN 0071375503). It will be understood by a skilled practitioner that the total daily usage of the mutant CocE polypeptide for use in embodiments of the invention

disclosed herein will be decided by the attending physician within the scope of sound medical judgment.

[0107] Mutant CocE polypeptides described herein can also be used in combination with other therapeutic modalities. Thus, in addition to the therapies described herein, one can also provide to the subject other therapies known to be efficacious for particular cocaine-induced conditions.

[0108] The mutant CocE polypeptides described herein can be formulated by any conventional manner using one or more pharmaceutically acceptable carriers and/or excipients (see e.g., Gennaro (2005) Remington the Science and Practice of Pharmacy 21st ed. Lippincott Williams & Wilkins, ISBN 0781746736). Such formulations will contain a therapeutically effective amount of the mutant CocE polypeptide, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration. Mutant CocE polypeptides of use with the current invention can be formulated by known methods for administration to a subject using several routes which include, but are not limited to, parenteral, pulmonary, oral, topical, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, ophthalmic, buccal, and rectal. The mutant CocE polypeptide can also be administered in combination with one or more additional agents disclosed herein and/or together with other biologically active or biologically inert agents. Such biologically active or inert agents can be in fluid or mechanical communication with the agent(s) or attached to the agent(s) by ionic, covalent, Van der Waals, hydrophobic, hydrophilic or other physical forces.

[0109] Mutant CocE polypeptides described herein can be administered parenterally, including intravenous, intramuscular, subcutaneous, and intraperitoneal injections. Excipients, commonly used in the parenteral delivery of small drug molecules, including solubility enhancers, osmotic agents, buffers, and preservatives, can also be included in biomolecule formulations. Inclusion of antiaggregation and antiadsorption agents, such as surfactants and

albumin, when formulating and delivering biomolecules can add increased stability and decrease the risk of the active biomolecule interacting with an interface, which can lead to unfolding, aggregation, and/or precipitation. The mutant CocE polypeptide can be lyophilized for added stability during storage, and re-processed before parenteral administration.

[0110] Preferably, the mutant CocE polypeptide is pegylated, thus providing increased stability and lessened immunogenicity (see above).

[0111] Pulmonary delivery of macromolecules, such as mutant CocE polypeptides, provide for relatively easy, non-invasive administration to the circulatory system for systemic distribution (see e.g., Cryan (2004) AAPS J. 7(1) article 4, E20-41, providing a review of pulmonary delivery technology).

Advantages of pulmonary delivery include noninvasiveness, large surface area for absorption ($\sim 75 \text{ m}^2$), thin (~ 0.1 to $0.5 \text{ }\mu\text{m}$) alveolar epithelium permitting rapid absorption, absence of first pass metabolism, decreased proteolytic activity, rapid onset of action, and high bioavailability. Various inhalation delivery devices, such as metered-dose inhalers, nebulizers, and dry-powder inhalers, that can be used to deliver the biomolecules described herein are known to the art (e.g., AERx (Aradigm, CA); Respimat (Boehringer, Germany); AeroDose (Aerogen Inc., CA)). Dry powder inhalation devices are particularly preferred for pulmonary delivery of protein-based agents (e.g., Spinhaler (Fisons Pharmaceuticals, NY); Rotohaler (GSK, NC); Diskhaler (GSK, NC); Spiros (Dura Pharmaceuticals, CA); Nektar (Nektar Pharmaceuticals, CA)).

[0112] Controlled-release (or sustained-release) preparations can be formulated to extend the activity of the mutant CocE polypeptide and reduce dosage frequency. Controlled-release preparations can also be used to effect the time of onset of action or other characteristics, such as blood levels of the agent, and consequently affect the occurrence of side effects. Controlled-release preparations can be designed to initially release an amount of mutant CocE polypeptide that produces the desired therapeutic effect, and gradually and continually release other amounts to maintain the level of therapeutic effect over

an extended period of time. In order to maintain a near-constant level of mutant CocE polypeptide in the body, the agent can be released from the dosage form at a rate that will replace the amount of agent being metabolized and/or excreted from the body. The controlled-release of an agent can be stimulated by various inducers, e.g., change in pH, change in temperature, enzymes, water, or other physiological conditions or molecules.

[0113] Controlled-release systems can include, for example, an infusion pump which can be used to administer the agent in a manner similar to that used for delivering insulin or chemotherapy to specific organs or tumors. The agents of the invention can be administered by other controlled-release means or delivery devices that are well known to those of ordinary skill in the art, including, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, depots, multilayer coatings, microparticles, liposomes, microspheres, or the like, or a combination of any of the above to provide the desired release profile in varying proportions. Other methods of controlled-release delivery of agents will be known to the skilled artisan and are within the scope of the invention.

[0114] Mutant CocE polypeptides can be encapsulated and administered in a variety of carrier delivery systems. Carrier-based systems for biomolecular agent delivery can: provide for intracellular delivery; tailor biomolecule/agent release rates; increase the proportion of biomolecule that reaches its site of action; improve the transport of the drug to its site of action; allow colocalized deposition with other agents or excipients; improve the stability of the agent *in vivo*; prolong the residence time of the agent at its site of action by reducing clearance; decrease the nonspecific delivery of the agent to nontarget tissues; decrease irritation caused by the agent; decrease toxicity due to high initial doses of the agent; alter the immunogenicity of the agent; decrease dosage frequency, improve taste of the product; and/or improve shelf life of the product.

[0115] Examples of carrier delivery systems for with mutant CocE polypeptides described herein include microspheres (*see e.g.*, Varde & Pack (2004) Expert Opin. Biol. 4(1) 35-51), hydrogels (*see generally*, Sakiyama et al. (2001) FASEB J. 15, 1300-1302), polymeric implants (*see generally*, Teng et al (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 3024-3029), smart polymeric carriers (*see generally*, Stayton et al. (2005) Orthod Craniofacial Res 8, 219-225; Wu et al. (2005) Nature Biotech (2005) 23(9), 1137-1146), and liposomes (*see e.g.*, Galovic et al. (2002) Eur. J. Pharm. Sci. 15, 441-448; Wagner et al. (2002) J. Liposome Res. 12, 259-270). Preferably, the mutant CocE polypeptide is encapsulated in RBC (*see above*; Example 12).

SCREENING METHODS

[0116] Another aspect of the invention is directed toward screening methods for the generation, identification, and purification of thermostable mutant CocE polypeptides. Generally, mutant CocE polypeptides can be initially designed according to the approaches described above. Such designed polypeptides can then be screened for preferred characteristics, such as retention of hydrolytic efficiency, increased thermostability, increased plasma-half-life, and/or reduced antigenicity. Also, random mutant CocE polypeptides can be screened for the desired characteristics.

[0117] Detection methods to screen for thermostable mutants encompass a wide variety of techniques. The following is an exemplary summary of a generic protocol. Nucleic acid encoding the mutant CocE polypeptides (generated, for example, through rational design, random mutagenesis, or host mutagenesis) is transformed into an appropriate expression host (for example, E. coli cells such as E. coli BL21 Gold (Stratagene)), and expression of the mutant polypeptide is induced according to standard protocols (e.g. by IPTG). Expression is performed at temperatures to produce optimal protein expression (e.g., 16°C for CocE, *see e.g.*, Example 1) for a pre-determined period of time (e.g., anywhere from 30 minutes to 24 hours or

longer). Alternatively, expression is performed at an elevated temperature (for example, the elevated temperature can be at least about 35°C, at least about 36°C, at least about 37°C, at least about 38°C, at least about 39°C, at least about 40°C, or even higher). Preferably, the elevated temperature at which mutated CocE polypeptide is expressed is 37°C. Around this temperature, wild-type CocE polypeptide partitions almost exclusively into inclusion bodies. Cells containing the expressed mutant polypeptides are screened for the presence of thermostable variants of CocE.

[0118] Screening for the presence of thermostable variants of CocE generally involves either direct measurement on cultured cells, on cell lysates, or following cellular disruption and isolation of the mutant CocE polypeptide. Cellular disruption can include osmotic shock, chemical lysis, sonication, and/or homogenization, and isolation of the mutant polypeptide can be obtained through numerous methods including either direct absorption to a matrix or affinity absorption through the use of anti-cocaine antibodies or fusion-protein specific capture systems. Suitable matrix for absorption includes nitrocellulose paper, filters, untreated or affinity-treated microtiter plates, agarose or sepharose resins, and/or affinity-coated tips.

[0119] The esterase activity of the cultured cells or isolated mutant polypeptide can be subsequently measured at one or more temperatures to determine the thermostability of the mutants. The temperature at which the activity assay is performed determines the degree of thermostability detection. Thus, while the final mutants will preferably have a melting temperature of 45°C or higher (as determined by, for example, circular dichroism), often, the initial screening at 45 °C will not find active enzymes. Rather, several cycles of mutagenesis and screening at subsequently increasing temperatures can be performed to achieve thermostable mutants. Thus, initial screening can be performed at 30°C, and after further cycles of mutagenesis, screening can be performed with incrementally increasing temperatures (for example, 34 °C, 37°C, 40°C, 42.5°C, 45°C, etc.), until a mutant of suitable thermostability is achieved.

The incremental temperature increases are determined empirically during the procedure, and are affected by the number of hits at particular temperatures and the determined T_m of the generated mutants.

[0120] While under no obligation to do so, and while not wishing to be bound by theory, herein follows what is believed to be a mechanistic explanation of melting of various embodiments of mutant CocE polypeptides described herein. CD spectra data illustrate that the CD melting of cocaine esterase and mutants is irreversible, as cooling to 0 degrees does not reform the original spectra (see, e.g., Example 19). While thermodynamic parameters cannot be ascertained, CD spectra can be used to comparatively determine whether mutants are more or less stable, whether they have different secondary structures or aggregation properties. It is believed that the CocE polypeptides described herein melt via an intermediate step, that is, the polypeptides undergo a 2-step melting process.

[0121] Detection of esterase activity can be performed using a variety of methods, where substrates are coupled to a specific detection system. Appropriate substrates for use in determining esterase activity can include cocaine, tritiated (3H) cocaine, cocaine substrate derivatives such as a thio-cocaine derivative (see e.g., Figure 6), and/or substrates that report general esterase activity such as 4-nitrophenyl acetate. The detection system can be directly coupled to the specifics of the substrate, for example: cleavage of unmodified cocaine can be detected by monitoring changes in cocaine absorbance at 240nm (see e.g., Example 4), or by monitoring pH changes that result from the accumulation of the acidic benzoic acid product (see e.g., Example 15), or through the use of cocaine aptamers (see e.g., Stojanovic, M. N., de Prada, P. & Landry, D. W. (2001) J Am Chem Soc 123, 4928-4931; Stojanovic, M. N. & Landry, D. W. (2002) J Am Chem Soc 124, 9678-9679) by monitoring changes in fluorescence upon degradation of cocaine (see example 15); cleavage of tritiated (3H) cocaine can be detected by acidification and detection of tritiated benzoic acid product through separation by chromatography

(see example 1 and 15); cleavage of cocaine derivatives such as thio-cocaine can be monitored by the detection of reactive sulfhydryl groups, through the addition of Ellman's reagent and determination of absorbance changes at 412nm (see e.g., Example 15), or by the addition and visualization of precipitating sulfhydryl reacting heavy metals; cleavage of 4-nitrophenyl acetate can be detected by monitoring changes in absorbance at 420nm (see e.g., Halgasova, N. et al. (1994) *Biochem J* 298 Pt 3, 751-755; O'Conner, C.J. & Manuel, R.D. (1993) *J Dairy Sci.* 76, 3674-3682).

[0122] Mutant CocE polypeptides identified through the above procedures, or a similar high throughput assay, can be further evaluated using in vitro procedures described herein (e.g., Kcat and Km values, stability at 37°, melting temperature (Tm), endotoxin levels, ability to degrade cocaine in plasma). Mutant CocE polypeptides with thermostable esterase activity and/or reduced immunogenicity, can be further evaluated using in vivo procedures described herein (e.g., potency, duration of action, effects with repeated dosing, and/or immunological evaluation). Preferably, mutant CocE polypeptides magnitudinal decrease of cocaine toxicity is examined first (see e.g., Examples 5, 7, and 8), and those mutants that reduce toxicity by at least about 5-10 fold can be further evaluated for time course of action (see e.g., Example 6). Candidate mutant CocE polypeptides can be further stabilized by, for example, pegylation and/or encapsulation in RBC and re-evaluated in the above described procedures.

[0123] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0124] The following non-limiting examples are provided to further illustrate various embodiments of the invention disclosed herein. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1 : COCE EXPRESSION

[0125] A method for expression and purification of CocE was established, where CocE is expressed in *E. Coli* as a fusion protein with a carboxyl terminus Hisx6-tag. The cocaine esterase gene was subcloned into the *E. coli* expression vector pET-22b(+) and high levels of cocaine esterase enzyme containing a C-terminal histidine tag were induced upon the addition of IPTG at 23C. The recombinant protein accumulated to approximately 10-15% of total protein. CocE was enriched on either a cobalt-chelate column (TalonTM Clontech) or a nickel-chelated agarose column (Pierce) by virtue of the Hisx6 tag. The eluted protein was approximately 95% pure by SDS-PAGE and Coomassie blue staining, and was subsequently resolved by ion-exchange chromatography FPLC column (Q-Sepharose) using an NaCl gradient. The protein eluted as a single peak was approximately 99% pure, as indexed by SDS-PAGE and Coomassie blue staining (see e.g., Figure 3).

[0126] Enzyme activity was determined through two assays: a radio-ligand activity assay in which tritiated cocaine was hydrolyzed and then after acidification, the tritiated benzoic acid product was separated from tritiated cocaine hydrochloride by chromatography; and a spectrophotometric assay under similar conditions as described by Turner et al. (2002) *Biochemistry* 41, 12297-

12307. The unique absorption spectra of cocaine (extinction coefficient 6.7 L/mmol/cm, at 240 nm) allows for the observation of remaining cocaine following enzymatic cleavage. The initial linear rates of decay of cocaine, representing velocity, were determined in a SpectraMax 190 plate reader (Molecular Devices) using SOFTmax Pro software (v1.13). The reaction was initiated by adding 150 μ L of a 2x enzyme solution to the 150 μ L of a 2x cocaine solution. Final CocE concentrations ranged from 100 ng/mL to 20 ng/mL. Final cocaine concentrations were as follows: 250, 126, 62.5, 31.25, 15.63, 7.81, 3.91, and 1.95 μ M. For the kinetics of all enzymes, the buffer was phosphate buffered saline, pH 7.4. Initial rates were fitted to the Michaelis-Menten equation, with k_{cat} and K_m as adjustable parameters (GraphPad; PRISM, v4).

[0127] As determined using the cocaine spectrophotometric assay, the purified wild-type CocE polypeptide hydrolyzes cocaine with a K_{cat} of approx. 500 min^{-1} and a K_m of approx. 2 μ M, which is consistent with previously reported values (see e.g., Turner et al. 2000).

[0128] Such expression procedures can be utilized for the mutant CocE polypeptides described herein.

EXAMPLE 2: EX VIVO COCE PLASMA ACTIVITY

[0129] Ex vivo determination of cocaine levels following cocaine esterase was examined in human plasma (University of Michigan Hospital blood bank). Cocaine was obtained from The National Institute of Drug Abuse (Bethesda, MD, USA). Cocaine was dissolved in sterile water. Aliquots (3 ml) of human plasma were maintained at 37° C in a water bath for 10 minutes prior to the start and for the duration of the experiment. After equilibrating plasma in water bath, cocaine was added to a final concentration of 300 μ M and vortexed for 30 seconds. Plasma samples were removed and placed in a microcentrifuge tube containing the internal standard and a saturated sodium fluoride solution to prevent further cocaine metabolism. Immediately after taking the first plasma sample (cocaine alone), 0.05 mg/ml CocE or vehicle CocE was added and

vortexed. Plasma samples were collected at 1, 2, 4, 6, 8, 10, 15, 30, 45, 60, and 120 minutes after adding CocE. Levels of cocaine were measured using high performance liquid chromatography tandem mass spectrometry.

[0130] Liquid chromatography was performed using a Surveyor HPLC system (ThermoElectron Corp., Franklin, MA) with a quaternary pump and autosampler configured with a 10 μ l injection loop. Separation was achieved using a Phenomex C18 3 μ m 30 x 4.6 mm column with corresponding guard column (Waters Corp., Milford, MA) at a flow rate of 600 μ l/min. Solvent A consisted of a 0.1% formic acid solution, and solvent B was 0.1% formic acid in acetonitrile (high purity grade; Burdick and Jackson, Muskegon, MI). A 3 minute ballistic gradient was used with cocaine and the internal standard co-eluting at 2.3 min.

[0131] For detection and quantification by mass spectrometry, a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer equipped with an IonMax electrospray ionization source (ThermoElectron Corp., Franklin, MA) was used in positive ion, selected reaction monitoring mode. Nitrogen served as the nebulizing gas and argon as the collision gas. Gas flow rates, spray voltages, and collision energies were optimized. Calibration curves were determined for cocaine with 50 nM deuterated cocaine (cocaine D₃) as the internal standard in untreated plasma samples. Unknown samples were also spiked with cocaine D₃. All samples were evaluated in triplicate. Standard curves and unknowns were analyzed by Quan Browser program in Xcalibur version 1.4 (ThermoElectron Corp., Franklin, MA) software. Calibration curves were constructed using linear regression of cocaine peak area /internal standard area ratio as a function of standard concentration with a weighting factor of 1/x. Standard curve fit values were accepted at a value is greater than 0.99, and RSD values for replicate samples are between 0-10%.

[0132] Results demonstrate that CocE is able to degrade cocaine very rapidly when the two are mixed briefly in human plasma (see e.g., Figure 4). The first time point indicates the cocaine concentration prior to the addition of

cocaine esterase or esterase vehicle. Prior to esterase treatment, cocaine levels were similar, but within 1 min of cocaine esterase administration, cocaine levels were decreased at least 100-fold to approximately 2 μ M as compared with the vehicle-treated plasma sample. Cocaine levels continued to decrease in the esterase-treated plasma samples, falling below 1 microM by the 2-minute timepoint.

[0133] The therapeutic efficiency of the enzyme was demonstrated by the increasing dose of cocaine required to produce toxic effects after a single intravenous injection of CocE. The wild-type enzyme demonstrated rapid kinetics for cocaine degradation ex vivo in rat and human serum. Two inactive mutants of CocE failed to protect the rats from the toxic effects of cocaine, confirming the protective effects are due to hydrolytic activity. Furthermore, CocE did not change the lethality of WIN-35065-2, a cocaine analog that lacks the benzoyl ester moiety targeted by CocE. The in vivo and ex vivo characterization of CocE supports the role of the enzyme as a suitable antidote to toxicity in humans.

EXAMPLE 3: PREDICTED THERMOSTABLE MUTANT COCE

[0134] Rational design of mutant CocE described herein was based upon molecular dynamics (MD) simulations. A computational model of CocE was constructed using the published crystal structure of wild-type CocE (see e.g., Figure 2). Such models can be used to identify certain amino acid modifications that increase the theoretical melting temperature of the protein without disrupting the structure at the active site. Classical MD simulation enables the study of time evolution of a large system by taking many small successive time steps under atomic forces determined by a set of parameterized interaction functions (force field), including bonded interactions (bonds, angles, and dihedral angles), non-bonded van der Waals interactions, and electrostatic interactions based on net atomic charges. Due to the simple force field form the MD simulation may be performed for a sufficiently long simulation time to give meaningful ensemble-averaged properties, even for a very large system involving over a hundred

thousand atoms. So, for CocE and each proposed mutant, the MD simulation will lead to a reasonable, dynamically averaged 3D structure of the simulated polypeptide in water.

[0135] Based on the X-ray crystal structure (PDB code 1JU3) of the bacterial cocaine esterase (CocE) (Larson et al. (2002) Nature 9, 17), a complete 3D model of CocE binding with (-)-cocaine suitable for computational modeling its thermodynamic stability was built. To increase the thermostability of CocE, a computational method was implemented in RosettaDesign program (Kuhlman and Baker (2000) PNAS 97, 10383) capable of predicting thermostabilizing mutations within a given fold while minimizing any shift in the backbone that might structurally disrupt the active site structure or quench its flexibility. The method implemented in the RosettaDesign program uses an energy function for evaluating the fitness of a particular sequence for a given fold and a Monte Carlo search algorithm for sampling sequence space. A similar method has been successfully used by other researchers to increase thermostability of an enzyme with no reduction in catalytic efficiency (Korkegian et al. (2005) Science 308, 857). The partial atomic charges for the non-standard residue atoms were calculated by using the standard RESP protocol implemented in the Antechamber module of the Amber7 (or 8) program package (Case, 2002). The computational modeling using the RosettaDesign program allowed prediction of a set of CocE mutations calculated as having lower energy and, therefore, increased thermostability (see e.g., Table 2). For this example, computation considered only possible mutations on the amino acid residues having a distance of between 6-25 Å from the cocaine substrate molecule.

[0136] Identified single mutation CocE polypeptides calculated to stabilize CocE by about 2.1 to about 4.5 kcal/mol included: L163V (SEQ ID NO: 3); V121D (SEQ ID NO: 4); S167A (SEQ ID NO: 5); Q123E (SEQ ID NO: 6); V225I (SEQ ID NO: 7); I218L (SEQ ID NO: 8); A310D (SEQ ID NO: 9); A149S (SEQ ID NO: 10); S159A (SEQ ID NO: 11); S265A (SEQ ID NO: 12); S56G (SEQ ID NO: 13); W220A (SEQ ID NO: 14); T122A (SEQ ID NO: 15); S140A

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(SEQ ID NO: 16); F189L (SEQ ID NO: 17); A193D (SEQ ID NO: 18); T254R (SEQ ID NO: 19); N42V (SEQ ID NO: 20); V262L (SEQ ID NO: 21); L508G (SEQ ID NO: 22); Y152H (SEQ ID NO: 23); V160A (SEQ ID NO: 24); T172R (SEQ ID NO: 25); Y532F (SEQ ID NO: 26); T74S (SEQ ID NO: 27); W285T (SEQ ID NO: 28); L146P (SEQ ID NO: 29); D533S (SEQ ID NO: 30); A194R (SEQ ID NO: 31); G173Q (SEQ ID NO: 32); C477T (SEQ ID NO: 33); K531A (SEQ ID NO: 34); R41I (SEQ ID NO: 35); L119A (SEQ ID NO: 36); K46A (SEQ ID NO: 37); and F84Y (SEQ ID NO: 38)

Table 2: Summary of computational modeling using the RosettaDesign program with consensus approach

R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Consensus mutation	Energy Change (kcal/mol)
163	163	163	163	163	163	163	163	163	163	L163V	-4.5
121	121	121	121	121	121	121	121	121	121	V121D	-3.9
167	167	167	167	167	167	167	167	167	167	S167A	-3.9
123	123	123	123	123	123	123	123	123	123	Q123E	-3.8
225	310	310	225	225	225	225	310	225	218	V225I	-3.6
218	225	218	218	218	218	218	225	218	225	I218L	-3.5
310	218	225	310	310	310	310	218	310	152	A310D	-3.4
149	149	152	149	149	149	149	149	149	310	A149S	-3.3
159	159	140	159	159	159	159	159	159	149	S159A	-3.3
189	265	149	265	265	265	265	265	189	265	S265A	-3.3
265	140	265	56	56	56	56	220	265	159	S56G	-3.2
56	220	159	220	220	220	220	122	56	220	W220A	-3.2

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220	122	220	122	122	122	122	140	220	56	T122A	-3.1
122	189	122	140	140	140	140	189	122	122	S140A	-3.1
140	193	189	189	189	189	189	193	140	140	F189L	-3.1
254	42	193	193	193	193	193	42	254	189	A193D	-3.1
262	262	42	254	254	254	254	254	42	193	T254R	-3.1
508	508	262	42	42	42	42	262	262	262	N42V	-3.0
152	152	508	262	262	262	262	508	508	508	V262L	-3.0
160	160	198	508	508	508	508	152	152	198	L508G	-2.9
74	198	160	152	152	152	152	160	160	254	Y152H	-2.9
172	74	74	160	160	160	160	198	198	74	V160A	-2.8
193	172	172	198	198	74	198	74	172	160	T172R	-2.8
532	532	532	532	74	172	74	172	74	172	Y532F	-2.7
146	146	56	74	172	532	172	532	193	532	T74S	-2.7
285	285	285	172	532	146	532	146	532	285	W285T	-2.6
533	290	146	285	146	285	146	285	285	290	L146P	-2.6
173	254	533	146	285	533	285	533	146	146		
194	533	173	533	533	173	533	173	533	533	D533S	-2.5
477	56	194	173	194	194	173	194	173	173	A194R	-2.4
531	173	200	194	200	290	194	200	194	194	G173Q	-2.4
42	194	477	200	290	477	200	290	200	200	C477T	-2.4
119	200	531	290	477	531	477	477	290	477	K531A	-2.4
200	477	305	477	531	200	531	531	477	531		
41	531	41	41	41	41	41	41	531	42	R41I	-2.2

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46	41	119	119	119	119	119	119	41	119	L119A	-2.2
84	119	46	46	173	46	46	46	119	41	K46A	-2.1
305	46	84	84	46	84	84	56	46	46	F84Y	-2.1
478	57	158	478	84	478	305	84	84	57		
57	84	307	57	478	57	478	307	478	84		
87	478	478	142	57	87	57	478	57	158		
142	87	57	263	87	142	87	57	142	478		
263	142	142	307	142	263	142	142	263	142		
307	263	263	78	263	307	263	263	307	263		
78	307	78	257	307	78	307	78	78	78		
257	78	257	531	48	257	78	257	257	257		
290	257	290	49	78	49	257	49	49	49		
291	201	49	201	257	305	201	201	201	201		
49	49	201	305	45	291	290	412	305	307		
176	291	291	412	49	176	291	291	412	291		
45	305	412	291	201	45	49	176	291	176		
54	176	176	176	305	54	176	45	45	305		
406	45	254	45	291	406	45	413	176	45		
50	54	45	413	176	50	54	54	413	54		
	406	54	54	54		406	305	54	406		
	50	413	50	406		50	406	406	50		
		406	406	50			50	50			
		50									

EXAMPLE 4: KINETIC PARAMETERS OF T172R AND S159A MUTANT CocE

[0137] Wild-type CocE and the T172R and S159A mutant CocE polypeptides were tested for catalytic efficiency.

[0138] Site directed mutagenesis (QuickChange™, Invitrogen) of CocE was performed to generate the S159A (SEQ ID NO: 11) mutant CocE polypeptide. Cloning and expression techniques used to produce the S159A were the same as in Example 1, except as indicated otherwise. The CocE gene was amplified through Polymerase Chain Reaction (PCR) in the presence of primers that contain the specific mutation required (Integrated DNA Technologies, Inc.). The specific mutation was subcloned back into the expression plasmid and the nucleotide sequence of these plasmids determined to verify the presence of the mutation.

[0139] Mutant T172R was generated by overlapping PCR using 5' and 3' primers containing the specific T172R mutation, as well as an additional Sac II restriction enzyme site for easy detection of the mutated gene. Primer pairs CocE 20-5'F-Nde I (5' GATATACATATGGTGGACGGGAATTAC 3') and T172R-3'R (5' CAGACCTCGACGTGATGAGCCCGCGGCCTATGAGAGCTGACCAGC 3') as well as CocE-1800-3'R (5' GTGGTGCTCGAGTCGCTTGATAATCG 3') and T172R-5'F (5' GCTGGTCAGCTCTCATAGGCCGCGGGCTCATCACGTCGAGGTCTG 3') were PCR-amplified using the high-fidelity Pfu enzyme (Stratagene) with an annealing temperature of 55°C. Resultant PCR products were combined and re-amplified, generating a full-length CocE gene encoding the T172R mutation. The gene was digested with Nde I and Xho I; subcloned into the expression vector, and sequenced in its entirety to verify both the presence of the mutation and the absence of additional PCR-copy error mutations.

[0140] Plasmids containing the mutations were transformed into *E. coli* BL21 cells and IPTG-induced enzyme was purified on Ni-Agarose. Expressed proteins were then tested for enzymatic activity and for thermostability at 37°C. Enzyme activity was measured using the spectrophotometric assay as described in Example 1. Thermostability was also tested via the spectrophotometric assay by pre-incubation of both wild-type and mutants at 37° C for various times. Additionally, the nature of the thermo-instability of both the wild-type and T172R mutant was analyzed by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions. Briefly, mutant and wild-type enzyme at 0.1mg/ml were incubated at 37°C for various time-points, cooled to 4°C, mixed with SDS-loading dye containing β -mercaptoethanol and run on 10% SDS-PAGE gels (denaturing conditions) or run on native 10% polyacrylamide gels (non-denaturing conditions) at 4°C. Gels were fixed with 10% methanol, 7% acetic acid for 30 minutes, and then stained with Sypro-Ruby protein gel stain (Molecular Probes, Invitrogen) for 3 hours. Protein staining was visualized under UV-light using an Alphamager™ 3400 (Alpha Innotech). Finally, the exact melting temperature of both the wild-type and T172R mutant were determined by circular dichroism using a JASCO-810 spectropolarimeter driven by a JASCO V500/FP-750 analysis program for Windows. The CD spectra were measured in millidegrees and normalized against PBS buffer.

[0141] Results from the spectrophotometric assay showed that T172R had an increased V_{max} and K_m at room temperature and a greatly increased V_{max} and K_m at 37° C as compared to wild-type CocE (see e.g., Table 3; Figure 5). In fact, the T172R V_{max} and K_m at 37° C was comparable to the V_{max} and K_m of wild-type CocE at room temperature. The S159A mutant CocE showed a slightly increased V_{max} and K_m at 37° C, but a decreased V_{max} and K_m at room temperature, as compared to wild-type CocE. Electrophoresis under denaturing conditions (Figure 6a) indicated a single protein band for both the wild-type and T172R of approximately 65000 Da, regardless of incubation temperature, indicating the proteolytic degradation does not adequately describe

the mechanism for thermoinstability. The non-denaturing gels (Figure 6b) showed a single protein band for the wild-type enzyme prior to incubation at 37°C, however upon incubation at 37°C, higher molecular weight species are seen to appear as the original band fades. These putative protein aggregates can also be observed for the T172R mutant, however the time to aggregation upon 37°C incubation is longer, and in this procedure the T172R mutant was approximated to have a 8x longer half-life at 37°C than the wildtype. Analysis of the protein melting temperature by circular dichroism (Figure 7) indicated temperature sensitive changes in protein tertiary structure were occurring in the near-UV range of the spectrum (between 260nm and 320nm). Curve-fitting of temperature-sensitive changes in this region of the spectrum indicated the wild-type CocE has a melting temperature of 36.15°C, with detectable denaturation beginning at approximately 30°C. The T172R mutant was determined to have a melting temperature of 41.43°C, with detectable denaturation beginning at approximately 28°C. Thus the single amino acid change from Tyrosine to Arginine at Amino acid 172, with an estimated 2.8kCa/mol increase in thermostability, was determined to have a full 5 degrees increased melting temperature compared to the wild-type CocE.

TABLE 3: Kinetic parameters for T172R, S159A, and wt CocE.

	S159A	S159A (40min @ 37°C)	T172R	T172R (40 min@ 37°C)	wt CocE	wt CocE (40 min@ 37°C)
Vmax	876.6	130.7	1466	1267	1264	94.06
Km	43.65	15.69	88.20	78.80	71.81	12.30

EXAMPLE 5: IN VIVO WILD-TYPE CocE PREVENTION OF COCAINE LETHALITY IN RATS

[0142] To determine CocE's esteratic activity in vivo, a rodent model of acute cocaine toxicity was implemented. When treated with high doses of cocaine, rats first exhibit convulsions followed by cessation of respiration and movement. The lowest toxic dose of cocaine, when administered intraperitoneally, will produce fatality within 15 minutes of treatment.

[0143] Protection against cocaine-induced lethality by wild-type CocE was determined and compared to the protective effects of human BChE. The esteratic activity of CocE was established by assessing the activity of two mutant enzymes, each lacking one of three amino acids in the active site. Additionally, activity of a modified wild-type enzyme by a covalent modification of Ser17 within the active site, by phenylmethyl sulphonate fluoride (PMSF) was determined. Esteratic degradation of cocaine was shown to be the mechanism of CocE's protective effects by verifying if the enzyme protected against toxicity induced by WIN 35065-2 (Madras et al. (1989) J Pharmacol Exp Ther 251, 13 -141), a cocaine analog which lacks the ester bridge at the proposed site of enzymatic hydrolysis.

[0144] Male Sprague-Dawley rats (300 grams) (Harlan Sprague Dawley, Indianapolis, IN) were housed three animals per cage. Following surgical implantation of a jugular catheter, all rats were individually housed until termination of the experiment. Rats were maintained on a 12-h light/dark cycle, with lights turned on at 7:30 a.m. and food and water were available ad libitum. After rats were anesthetized with ketamine hydrochloride (100mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), intravenous catheters (Micro-renethane tubing, 15 cm, MRE-040, Braintree Scientific Inc., Braintree, MA) were implanted into the right jugular vein. Approximately 3 cm of the catheter was inserted into the vein; the remaining tubing was passed subcutaneously to the back, where it exited from an incision made between the shoulder blades. The exposed tubing was capped with a 1 cm piece of stainless steel (0.28 diameter, Small Parts Inc., Miami, FL).

Catheters were flushed daily with 0.5 ml of heparinized saline (50 U/ml) to maintain catheter patency. Following surgery, rats were allowed one week to recover. Each rat was used for a single experiment, and all experimental groups consisted of 6-8 rats.

[0145] To determine the lowest effective dose of CocE that blocked cocaine-induced convulsions and death, 0.1, 0.32, or 1.0 mg CocE or vehicle (phosphate buffered saline, PBS) was administered intravenously one minute after 180 mg/kg cocaine (i.p.). To determine the catalytic limits of CocE, increasing doses of cocaine were administered (100, 560, 1000 mg/kg, i.p.) one minute prior to 1.0 mg CocE (i.v.). Mutants and PMSF-blocked CocE were administered (1mg, i.v.) one minute before 180 mg/kg cocaine (i.p.). CocE (1.0 mg, i.v.) was also administered one minute after the lowest dose of WIN-35065-2 (560 mg/kg, i.p.). CocE (1.0 mg, i.v.) was given before and after cocaine (100 mg/kg, i.p.) to determine the in vivo half-life of the esterase. All intravenous injections were followed by a heparinized saline flush (0.5 ml). After treatment, rats were observed for convulsion; and death. Number of convulsant episodes, duration of each episode, and type of convulsion were recorded. Death was defined as cessation of observed movement and respiration. Percent of animals in each experimental group exhibiting convulsions and lethality were calculated. Percent standard error mean was then calculated for each data point.

[0146] Results showed that, in the rodent model of acute toxicity, cocaine dose-dependently induced convulsions and death in rats; death was observed in less than 15 minutes after administration in 100% of animals given 100 mg/kg cocaine (see e.g., Figure 9). CocE (1.0 mg) infused after cocaine administration produced a ten-fold shift in the cocaine-toxicity dose effect curve (see e.g., Figure 9), such that 1000 mg/kg cocaine was required to surmount the protective, catalytic properties of CocE. This treatment regimen closely resembles human toxicity situations, where the antidote to overdose is given only after cocaine has been ingested, inhaled, or injected.

[0147] Wild-type CocE showed superior catalytic efficiency over human BChE. Given one minute before 180 mg/kg cocaine, 1 mg CocE offered 100% protection against cocaine-induced lethality (see e.g., Figure 10), while a 10-times molar equivalent dose of human BChE (13 mg) offered no protection, similar to a ten-fold lower dose of CocE (0.1 mg) (see e.g., Figure 10).

[0148] Both mutations of CocE (Ser117Ala or Tyr44Phe) lacked in vivo activity and, therefore, had no protective effects (Figure 11b). Furthermore, PMSF-treated enzyme also eliminated CocE's protective effect against cocaine (Figure 11b). Additionally, the lethal effect of the nonhydrolyzable cocaine analog, WIN 35065-2, was not overcome by treatment with CocE (Figure 12). Based upon in vivo protection studies performed with catalytically inactivated preparations of the enzyme (PMSF-treated and CocE inactive mutants), it is clear that the protective effects of the enzyme are due to its ability to hydrolyze cocaine. Taken together, these data comply with in vitro assessments of CocE's esteratic activity and confirm the enzyme's mechanism of protection against cocaine-induced lethality in vivo.

EXAMPLE 6: TIME-DEPENDENT EFFECTS OF WILD-TYPE COCE

[0149] The effects of administering wild-type CocE prior to cocaine dosage was examined. The rat toxicity model was as described in Example 5. Wild-type CocE was administered 100, 30, 10, 3, and 1 minute before and 1 and 6 minutes after cocaine administration. Compound extractions from human plasma samples were performed in 100% acetonitrile (3x volume), incubated for approximately 15 min, centrifuged at 13,000 rpm for 4.5 min, and the resulting supernatant was collected. The extracts were concentrated on a Savant Speed Vac (ThermoElectron Corp., Franklin, MA) to remove the acetonitrile. Extracted samples were reconstituted in water and further diluted 10-1000 times.

[0150] Human plasma samples were spiked with 300 μ M cocaine and maintained at 37°C. One aliquot of plasma was sampled prior to the addition of

cocaine esterase or esterase vehicle, and another aliquots was collected 1 min following esterase administration. Plasma aliquots were mixed immediately with the internal standard (cocaine-D₃) and a saturated sodium fluoride solution to prevent further cocaine metabolism. Tissue extractions were performed and levels of cocaine and internal standard were quantified by HPLC with tandem mass spectrometry.

[0151] For time-dependent inactivation of CocE in vitro, purified CocE enzyme (at 250 ng/ml) was incubated in assay buffer in the absence of cocaine at 37° C for various times. Following incubation at 37° C the samples were placed on ice. To assess the effect of temperature on CocE activity, samples were incubated with (-) cocaine at various concentrations as indicated at a final enzyme concentration of 125 ng/ml. The rate of decay of (-)-cocaine at A240 was measured on a multiplate reader. The data were fitted to a single exponential decay using Kaleidagraph™ (Synergy software) yielding a $t_{1/2}$ of 13.2.

[0152] Wild-type CocE was found to have time-dependent protective effects; 100% of rats were saved when treated with CocE (1 mg) 1 minute before cocaine, while only 66% and 32% of rats survived when treated with CocE 30 and 1 minutes before cocaine, respectively (see e.g., Figure 14). CocE's protective effects were eliminated when rats were treated 100 min prior to cocaine. This time-dependent effect is most likely due to the thermal deactivation of the enzyme in vivo. In rat plasma, CocE was found to have a remarkably short half-life (about 10 minutes, Figure 13), most likely due to sensitivity to changes in pH and temperature. Additionally, preliminary in vitro data with the purified enzyme suggests that CocE undergoes a temperature-dependent inactivation with a $t_{1/2}$ of approximately 15 min at 37° C.

[0153] Given these data, it can be approximated that a 1 mg of CocE dose administered 30 minutes prior to cocaine, will decay approximately 3 half-lives, leaving 0.25 mg of wild-type enzyme in the general circulation when cocaine is administered.

[0154] Previous studies have suggested that lethal blood concentrations of cocaine in the rat vary between 50-128 μM (Mets B and Virag L (1995) *Anesth Analg* 81, 1033-1038; Mets et al. (1999) *Life Sci* 65, 1317-1328), and peak plasma levels of cocaine occur about 13 minutes after an intraperitoneal injection (Sun et al. (2002) *J Pharmacol Exp Ther* 302, 710-716). Based on reported kinetics of intraperitoneal cocaine administration (Sun et al. (2002) *J Pharmacol Exp Ther* 302, 710-716), it is estimated that 100 and 320 mg/kg cocaine yield peak cocaine blood concentration of 35 μM and 113 μM , respectively. Lethal concentrations of cocaine are of a similar magnitude in humans (20-200 μM) (Finkle BS and McCloskey KL (1978) *J Forensic Sci* 23, 173-189; Wetli and Wright (1979) *J Am Med Assoc* 241, 2519-2522). As 1 mg of wild-type CocE saved rats treated with these doses of cocaine (Figure 9), it can be justifiably predicted that the enzyme would protect against cocaine-toxicity in the human. Furthermore, cocaine levels measured using high performance liquid chromatography tandem mass spectrometry in human plasma spiked with 300 μM cocaine, a concentration that exceeds reported toxic levels of cocaine, and then treated with CocE (a molar equivalent of our in vivo 1.0 mg dose), reduced the cocaine concentration to approximately 2 μM in less than a minute (Figure 10).

[0155] Protection against an LD50 dose of cocaine in rats requires a 10 mg/kg treatment of BChE (Lynch et al. (1997) *Toxicol Appl Pharmacol* 145, 363-371), assuming that the enzyme is distributed similarly in the human, a 70 kg individual would require a 700 mg dose of exogenous BChE to protect against an overdose. Furthermore, there is no evidence that BChE can act to reverse cocaine-toxicity when administered after cocaine, a necessary characteristic of an antidote for cocaine toxicity.

[0156] It is thus demonstrated that a dose of 1 mg wild-type CocE in a 300 gram rat is sufficient to protect against a dose of cocaine that exceeds the LD100 (Figure 9). Additionally, the enzyme given both before, and more importantly, up to 6 minutes after the LD100 dose of cocaine, provided

protection from toxicity (Figure 14). CocE metabolized cocaine concentrations in serum by 150-fold in less than one minute (Figure 15).

[0157] Given these data, it is predicted that a 250 mg of CocE administered to a 70 kg human after toxic cocaine ingestion would rescue the individual from certain death. The above demonstrates that wild-type CocE is an efficient anti-cocaine molecule but that the short activity time of the enzyme under physiological conditions limits its therapeutic value. Such results point to the importance of extending the thermostability of the wild-type CocE.

EXAMPLE 7: IN VIVO T172R MUTANT COCE PREVENTION OF COCAINE LETHALITY IN RATS

[0158] The hydrolytic activity of wild-type CocE and T172R mutant CocE was characterized and confirmed in vivo by assessing its ability to prevent cocaine-induced lethality in rats.

[0159] Animal treatment was as described in Example 5. Increasing doses of cocaine were administered (i.p.) to rats, and one minute after, wild-type CocE (0.32 mg), mutant CocE T172R (0.32 mg), or vehicle was administered intravenously. All intravenous injections were followed by a heparinized flush (0.5 ml). After treatment, rats were observed for death. Time until death was recorded for the 1g/kg cocaine dosage. Wild-type and T172R CocE (0.32 mg) was administered at various times preceding (1, 10, 30, and 60 minutes) administration of 320 mg/kg i.p. of cocaine, with rats then monitored for death.

[0160] Results demonstrate the ability of intravenous CocE and, especially, mutant CocE to reverse or prevent the lethal effects produced by cocaine. Wild-type CocE administered one minute after cocaine administration prevented lethality at cocaine dosages that killed control rats and 100% lethality did not occur until cocaine dosages of 1g/kg (see e.g., Figure 16). The T172R mutant CocE treated rats were able to tolerate even higher dosages, with the 1g/kg of cocaine resulting in only about 70% lethality. And the

prophylactic effects of T172R mutant CocE was longer lasting than the wild-type CocE (see e.g., Figure 17).

EXAMPLE 8: IN VIVO REPEATED CocE DOSE IN MICE

[0161] Additional studies were done on the effect of CocE on toxicity in the mouse, particularly with respect to repeated doses.

[0162] The animal toxicity model was similar to that described previously, except as described. Male NIH Swiss mice were used. For tail intravenous injection, mice are placed in a small restraint chamber that exposes their tail. A heat lamp with an infrared, 250w bulb is placed about 4 inches from the tail, and left for a couple of minutes. The tail is then cleansed with an alcohol wipe and a 30G 1/2 precision glide needle (Fisher Scientific), is inserted into one of the side veins for infusion. To verify whether the needle is in the vein a small amount of drug is infused, if in the correct location the solution should infuse easily without any indication of an incorrect subcutaneous location, which appears white at the site of injection. For intravenous catheterization, Male NIH Swiss mice are anesthetized with ketamine 100 mg/kg and xylazine 10 mg/kg co-administered i.p. When mice are no longer responsive to paw pressure, the neck is shaved and prepped by alternating Betadine and alcohol wipes. Under clean conditions, a right transverse neck incision is made and the external jugular vein is isolated. A catheter is inserted into the vein with the aid of a dissecting microscope to the level of the right atrium, and is secured in the vein with nylon, 4-0 sutures and tissue adhesive, 3M Vetbond (3M Animal Care Products, St. Paul, MN). The catheters are a short length of Tygon tubing with an inner diameter of 0.010 in. and an outer diameter of 0.030 in. (Small Parts, Inc., Miami Lakes, FL). A small incision is made in the middle of the animal's back, and a trocar inserted subcutaneously to exit at the ventral incision site. The catheter is then pulled through the trocar and brought out the animal's back where it is held in place with nylon suture material and the tissue adhesive. A short piece of steel wire with a diameter of 0.011 in. (Small Parts, Inc., Miami

Lakes, FL) is inserted into the end of the catheter. The ventral incision is closed with 4-0 Vicryl suture material and the mouse placed under a heat lamp for recovery. Approximately an hour later, the mouse is returned to its home cage.

[0163] Mice that survived a first administration of the combination of 0.32 mg CocE and 320 mg/kg cocaine were given this combination again, 14 days following the initial administration. All of the mice survived this second administration. All mice also survived a third administration 21 days following the first dose combination. The effectiveness of repeated administration suggests that a strong immune response is not being mounted to CocE in this preparation, perhaps because of its rapid clearance.

[0164] Results showed that a dramatic shift to the right in the potency of cocaine in producing death was afforded by both 0.32 and 1.0 mg/kg cocaine (see e.g., Figure 18). The latency to death was markedly extended by CocE: following 180 mg/kg i.p. cocaine, mice typically died in about 3 min. After a dose of 1.0 gm/kg cocaine plus 1.0 mg of CocE, death occurred at 28 minutes on average. Data was also examined for the ability of hBChE to block the toxicity of cocaine. At a dose of 1 mg, hBChE produced a slight increase in the dose of cocaine necessary to kill the mice (see e.g., Figure 18). This enzyme is markedly less effective than CocE. Although larger doses of cocaine were necessary to kill the mice following administration of hBChE, the time to death was not modified by this enzyme.

EXAMPLE 9: IN VIVO HALF-LIFE AND BIODISTRIBUTION TESTS

[0165] The in vivo half-life and biodistribution of the mutant CocE polypeptides are examined in BALB/c mice. In brief, ¹²⁵I is labeled to the tyrosine residues of mutant CocE by using the well-established chloramine-T iodination method (Hunter, WM and Greenwood, FC (1962) Nature 194, 495-496). BALB/c mice are injected via their tail veins with 0.1 mL of either 0.5 µCi ¹²⁵I-labeled mutant CocE or the mutant CocE-PEG conjugates. Each experimental group consists of 24 mice. Three mice are sacrificed by cervical dislocation at the 15-,

30-, 90-minute, 3-, 12-, 24-, 48-, and 72-hour time intervals following the drug injection. Blood samples as well as tissue samples of liver, lung, heart, kidney, and spleen are collected, weighed, and measured for radioactivity using a gamma counter. The blood samples are also centrifuged, and the supernatants will be collected and counted for estimation of plasma-associated radiolabels. The ratio of the peak to that of the internal standard is used as the assay parameter. PK parameters are calculated by using the KINFIT (Kaltenbach, ML and Vistelle, R (1994) Anticancer Research 14, 2375-2377) nonlinear least-squares computer program by fitting the plasma radioactivity data to a biexponential equation (Gibaldi, M. and Perrier, D. (1982) Pharmacokinetics):

$$A(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

[0166] Where $A(t) = \%ID/mL$ plasma and $ID =$ injected dose. k_2 will be used to calculate the first-order elimination time $t_{1/2}$. The area under the curve (AUC) of the plasma concentration-time curve, the steady-state volume of distribution (V_{ss}), total plasma clearance (Cl), and the mean residence time (MRT) are calculated from A_1 , A_2 , k_1 , k_2 , and the body weight (kg) of the mouse as described by Gibaldi and Perrier, 1982). The organ permeability-surface area (PS) product is calculated as:

$$PS = [V_d \cdot V_o] C_{p(60min)} / AUC_{(0-60min)}$$

[0167] Where $C_{p(60min)}$ is the terminal plasma concentration (dpm/ μ L) at 60 min after injection, V_d is the tissue volume of distribution determined from the ratio of disintegrations per minute per gram of tissue to $C_{p(60min)}$, and V_o is the organ plasma volume. The organ delivery of the samples is determined as:

$$\%ID/g = PS \times AUC_{(0-60min)}$$

[0168] Where $\%ID/g$ is the percent injected dose taken up by gram of organ.

EXAMPLE 10: IMMUNOLOGY

[0169] CocE can be used in incomplete Freund's adjuvant (IFA) to immunize mice (see Table 4). A direct ELISA specific for CocE antibodies was set up by a standard protocol. CocE was used (1 ug/ml) to coat a 96-well micro-titer plate using a borate buffered saline (1.5 M NaCl, 0.5 M H₃BO₃, 1.0 M NaOH) to resuspend the cocaine esterase (50 uL/well). The coating plates was left overnight at 4 C. The coating buffer was removed the following morning and the plates blocked with 2% normal goat serum in PBS for 1 hr at 37C and washed 3 times. Serum from the various groups of mice was serially diluted in 50 µL of PBS in the wells in a range of 10² to 10⁷ and run in duplicate. The plates were covered and incubated for 30 minutes at 37C. Subsequently, the plates were washed 3 times and 50 µL/well of Goat anti-mouse IgG peroxidase labeled antibody diluted 1:400. The plates were then washed 3 times and 100 µL peroxidase substrate solution (OPD dissolved in citratelphosphate buffer) was added to each well. After a 5-10 minute incubation (based upon color development in the positive controls) the reaction was stopped using 3M H₂SO₄ (50 µL/well). The plates were read at 490 nm and titer determined by the highest dilution that showed increases over background absorbance. Positive controls were derived by immunizing Balb/c mice with 100 µg in 100 µL of Cocaine esterase emulsified in incomplete Freund's adjuvant (IPA) by intraperitoneal (IP) injection. In Positive 1 group the serum was isolated from 2 week immunized mice. In Positive 2 group immunized mice were boosted using 100 µg in 100 µL by IP injection at 2 weeks post-primary immunization and the serum collected after an additional week (3 week post-primary).

[0170] High titers were derived from the two positive control groups immunized with CocE, 10⁵ and 10⁶, respectively. The antibody titers from animals given CocE i.v. during cocaine challenges demonstrated detectable but relatively low titers compared to the positive control animals immunized by CocE plus IFA. Serum collected from animals immunized once with CocE/IFA demonstrated high titer of 10⁵, while serum from animals given an additional

boost were had a higher titer of 10⁶ (3 mice/group). The serum collected from these animals will serve as positive controls for all subsequent titering experiments.

TABLE 4: CocE titers of immunized mice

Group	Protocol	Titer (log10 dilution +/- SE
Positive 1 (n=3) 2 weeks	Immunized (IFA/IP)	5
Positive 2 (n=3) 3weeks	Immunized / Boost (IFA/IP)	6
Challenged and Treated (3x)	CocE given with cocaine	3.33 +/- 0.333
Challenged and Treated (4x)	CocE given with cocaine	3.5 +/- 0.5

EXAMPLE 11: COCE PEGYLATION

[0171] Conjugation of one to two PEG polymers per enzyme molecule is generally sufficient to yield the desired protective effects (Avramis et al. (2002) Blood 99, 1986-1994). Because each wild type CocE molecule is reported to contain 8 lysine residues, with none in the active site but only 2 being close to the active site (Turner et al. (2002) Biochemistry 41, 12297-12307), targeting lysine for pegylation is unlikely to inactivate the enzyme. The mutant CocE is mixed with various monomethoxy-PEG (m-PEG) polymers (MW ranging from 3-12 KDa); all contain an activated N-end functional hydroxysuccinyl ester group (mPEG-NHS; from Shear Water Inc., Birmingham, AL). PEG with a molecular weight of 5.5 KDa will be first attempted, because the inventors' results and those of other investigators (see e.g., Veronese, FM and Harris, JM (2002) Advanced Drug Delivery Reviews 54, 453-456; Avramis et al. (2002) Blood 99, 1986-1994) demonstrated that this molecular weight yields the beneficial protection. Different molar ratios of [NH₂]:[mPEG] (the former is calculated based on the total moles of lysine residues of mutant CocE) ranging from 1:2 to 1:10

are tested to obtain optimal conditions. Conjugation proceeds for about 40 minutes at 4°C with gentle agitation. The reaction products are then purified by ultrafiltration (MWCO 10,000) at 4°C. Activities of the pegylated mutant CecE products are determined by measuring the initial rates of cocaine hydrolysis using the previously established procedure (Turner et al. (2002) *Biochemistry* 41, 12297-12307). In addition, MALDI-TOF mass spectrophotometry is performed on these products to analyze the degree of pegylation and their molecular weight. The pegylated products are stored at -40°C and thawed immediately prior to their uses.

[0172] Standard characterization, including determination of the optimal pH, temperature, ionic strength, as well as kinetic parameters (e.g. K_m , V_m), of the mutant CocE-PEG conjugates are conducted in PBS. Furthermore, thermal stability of the pegylated products and their stability against proteolytic degradation are examined in the presence of human plasma or blood. In vivo functional tests of the mutant CocE-PEG products are conducted as described above. The In vivo half-life and biodistribution of the mutant CocE-PEG conjugates, as compared to free CocE are conducted as described above (see Example 9).

[0173] Statistical analysis is performed on the results obtained from the pegylation experiments. Random block two-way ANOVA with Dennett's post-test is performed on data sets with two variables using GraphPad (San Diego, CA) Prism for Windows and GraphPad Software. Paired t-test is performed for experiments with two conditions.

[0174] Conjugation of mutant CocE to high molecular weight (e.g., up to 60 KDa) branched PEG can also be performed (see e.g., Reddy et al. (2002) *Advanced Drug Delivery Reviews* 54, 571-586).

[0175] Additionally, site specific PEGylation can be a viable alternative to reduce functional and structural heterogeneity. The removal of cysteine residues near the active site or incorporation of cysteine residues on

the protein surface can serve as better PEGylation substrates (through maleimide coupling). Similarly, amine-coupling of PEG to CocE can be employed through, for example, the conservative substitution of arginine residues for any of mutant CocE's (nine lysines total in wild-type CocE, seven of which are surface lysines).

[0176] Preliminary findings have shown that wild-type (WT) CocE was successfully linked with monomethoxy-PEG (m-PEG) polymers (MW: 5.5KDa) containing an activated succinimidyl functional group at one end, using the procedures described above. A high yield of the initial CocE activity (>70%) was recovered after the pegylation reaction. MALDI-TOF mass spectra revealed four primary peaks for the PEG-CocE products, indicating the presence of a heterogeneous mixture of the conjugates containing different numbers of the PEG chains (ranging from 1 to 4, respectively) (see e.g., Figure 19). It has been suggested in the literature that conjugation of merely 1-2 PEG chains per protein molecule would be able to yield PEG-induced protective effects (Veronese, FM and Harris, JM (2002b) *Advanced Drug Delivery Reviews* 54, 457-606; Avramis et al (2002) *Blood* 99, 1986-1994). To this regard, the pegylation method employed here apparently satisfies such a requirement.

EXAMPLE 12: RBC ENCAPSULATION

[0177] RBC encapsulation of CocE can be accomplished via a linked PTD peptide. LMWP is selected as the PTD peptide to ferry mutant CocE into RBC, because of its potency in translocating proteins across cell membrane (Park et al. (2005) *FASEB Journal*, in press) and its lack of toxicity (Chang et al. (2001) *AAPS Journal* 3, Article #17, #18 and #19). To ensure that the encapsulated mutant CocE is permanently embedded in RBC, the linkage between CocE and LMWP can degrade automatically and rapidly once the mutant CocE-LMWP conjugates enter RBC. A linker such as a disulfide (S-S) bond that will be degraded quickly inside the RBC due to the presence of elevated cytosolic glutathione and reductase activity (Trouet et al. (1982)

Proceeding of the National Academy of Science 79, 626-629), ensures that CocE will stay in the RBC.

[0178] To produce mutant CocE- LMWP conjugates linked with S-S bonds, the amine group at the N-end of LMWP (this is the only -NH₂ group on LMWP) is first activated with SPDP, and the activated LMWP then mixed with mutant CocE in the presence of dithiothreitol (DTT) to allow for the formation of the S-S bond with one of the (four in wild-type) free cysteine residues on mutant CocE (Turner et al. (2002) Biochemistry 41, 12297-12307); according to a modified procedure previously developed (Liang et al. (2000) AAPS Pharmaceutical Science 2, Article 7). CocE is stable when being stored in DTT (Turner et al. (2002) Biochemistry 41, 12297-12307), suggesting that use of these free cysteine groups for conjugation is not likely to impair the catalytic activity of this enzyme (already performed mutation of each cysteine in wild-type CocE to a serine resulted in no diminution of activity). The final LWMP-CocE products is then purified via a heparin column, and is stored by lyophilization.

[0179] Encapsulation is achieved by incubating RBC with mutant CocE-LMWP for 30-60 minutes. Because PTD-mediated cell entry is temperature-independent (Schwarze et al. (1999) Science 285, 1569-1572), encapsulation is conducted at 4°C to maximally preserve the functionality of RBC. The process and extent of mutant CocE entrapment in RBC is monitored by confocal microscopy and flow cytometry analysis using FITC-labeled mutant CocE. The morphology of mutant CocE-entrapped RBC is also be examined by SEM.

[0180] Basic characterization—including assessment of the functionality of both RBC (e.g. oxygen-transfer activity) and mutant CocE (e.g. cocaine-hydrolyzing activity, kinetic properties such as Km, Vm, etc.), leakage of mutant CocE from RBC (i.e. by incubating mutant CocE-loaded RBC in buffer and then measuring enzyme activity in the supernatant), and stability of the entrapped mutant CocE against proteolytic degradation—is conducted

either in buffer or in plasma. Results obtained for the RBC-encapsulated mutant CocE are compared with those obtained for the free enzyme. In vivo functional tests of the mutant CocE-encapsulated RBC are conducted as described above.

[0181] Human RBC (from American Red Cross, Detroit, MI) are used for in vitro studies. For in vivo animal studies including the functional tests and pharmacokinetic studies, however, autologous RBC from the same animal species are used to avoid cell incompatibility and possible toxic effects.

[0182] Circulation half-life ($t_{1/2}$) of mutant CocE-entrapped RBC is determined by injecting ^{125}I -labeled mutant CocE (i.e. prior to its loading into RBC) into mice, according to the same procedures described above. Each set of experiments consists of 24 mice. Mice (3) are sacrificed at 3-, 6-, 12-, 24-hour and 3-, 6-, 10-, and 15-day time intervals. Blood and tissue samples are collected, weighed, and measured for radioactivity. PK parameters including the elimination $t_{1/2}$, as well as tissue distribution are calculated by using the KINFIT program as described above. Pharmacokinetic results obtained for the RBC-encapsulated mutant CocE are compared with those obtained for free mutant CocE.

[0183] Statistical analysis is performed on the results obtained from the RBC encapsulation experiments. Random block two-way ANOVA with Dennett's post-test is performed on data sets with two variables using GraphPad (San Diego, CA) Prism for Windows and GraphPad Software. Paired t-test is performed for experiments with two conditions.

[0184] To examine if the previously suggested could fulfill the latter two requirements, preliminary studies of PTD-mediated RBC encapsulation were conducted using L-asparaginase as a model enzyme. LMWVP, a PTD peptide previously developed in Dr. Yang's laboratory (Chang et al. (2001) AAPS Journal 3, Article #17, #1, and #19) with proven, potent membrane-penetrating activity (Park et al. (2005) FASEB Journal, in press), was linked to

asparaginase using a procedure similar to that described above. The LMWP-ASNase conjugates were then incubated with RBC (collected from DBA/2 mice) for 2 hrs at 4°C. For comparison, RBC-Ghosts containing encapsulated ASNase were also prepared according to the previously established procedures (Uptake et al. (1976) *Science* 193, 681-683). Preliminary results demonstrated that loading efficiency of the LMWP-mediated method was at least comparable to, if not better than, the conventional, osmosis-based cell rupturing technique. A major advantage of the PTD-mediated method, however, is that it only requires a single step for processing; unlike the other cell entry methods that all require multiple steps of the loading and washing procedures.

[0185] Figure 20 presents scanning electron microscopy (SEM) images taken from samples of normal RBC, ASNase-loaded RBC-Ghost, and LMWP-ASNase-loaded RBC. As seen, while the ASNase-loaded RBC from osmotic rupture/resealing method (i.e. RBC-Ghost) exhibited significant change in shape and morphology, the LMWP-ASNase-loaded RBC showed virtually indistinguishable shape and morphology (i.e. biconcave disk) from those of normal RBC. These findings are in agreement with those reported by many other investigators that PTD-mediated cell encapsulation does not cause any significant perturbation or alteration of the cell membrane (Dietz, GPH and Bahr, M (2004) *Molecular Cell Neurosciences* 27, 85-131; Schwarze et al. (1999) *Science* 285, 1569-1572; Suzuki et al. (2002) *Journal of Biological Chemistry* 25, 2437-2443).

[0186] To further evaluate these two RBC-encapsulation systems, preliminary clearance studies were carried out. The half-life of ASNase activity in plasma was evaluated after intravenous injection of: (1) ASNase-loaded RBC-Ghost, and (2) LMWP-ASNase-loaded RBC. Each animal group consisted of 4 DBA-2 mice, and each mouse was given 8 units of loaded ASNase activity. Blood samples were withdrawn at different time intervals from the tail vein, and the amount of ASNase activity in the whole blood was measured by direct Nesslerization of produced ammonia (Ho et al. (1970) *Journal of Biological*

Chemistry 245, 3708-15). Results demonstrated that there was almost a two-fold increase in the circulation half-life for the LMWP-ASNase-encapsulated RBC ($t_{1/2}$: 9.2 days) compared to that ($t_{1/2}$: 5.9 days) for the RBC-Ghost (see e.g., Figure 21). It is currently unknown what the difference between the half-lives of the encapsulated RBC and the normal, untreated RBC. Nonetheless, these results of 2-fold increase of $t_{1/2}$ over RBC-Ghost demonstrate the merit of this approach, as it was reported in the literature that even by utilizing the RBC-Ghost encapsulation method, the *in vivo* ASNase activity had already been prolonged from 26 hours for the free ASNase to 29 days for the RBC-Ghost-encapsulated ASNase (Kravtsoff et al. (1996) European Journal of Clinical Pharmacology 49, 465-470): already a 10-fold increase. Hence, another 2-fold increase of this $t_{1/2}$ by the encapsulation method described herein is particularly effective.

[0187] To confirm that the RBC-encapsulated ASNase could still retain its original biological functions, anti-tumor effects by both the RBC-Ghost-ASNase and the RBC-ASNase were examined on tumor-bearing mice. L5178Y mouse lymphoma cells were cultured, and to each DBA/2 mouse 7×10^5 cancer cells were injected intraperitoneally. Five days after tumor implantation, mice with similar bodyweights were selected and divided into three groups: (1) Control group given saline only; (2) ASNase-loaded RBC-Ghost; and (3) LMWP-ASNase-loaded RBC. Each group consisted of 5 mice, and each experimental mouse was given 0.1 mL of the drug-encapsulated RBC (or Ghost). Results showed that the average survival times for the untreated control, ASNase-RBC-Ghost-treated, and LMWP-ASNase-RBC-treated groups were 10.0, 12.6, and 14.4 days, respectively (see e.g., Figure 22). It should be noted that although the survival time among the three groups differed by only about 2 days, the effects of the treatment by the RBC-encapsulated ASNase was still quite dramatic; considering the fact that only 0.1 mL of the RBC suspension, which was equivalent to merely 5% of the total blood volume of the mouse, was given to each mouse for the anti-tumor treatment. Overall, these findings with the

ASNase model enzyme demonstrated that the RBC-encapsulated enzyme was still therapeutically active. It is noted that cocaine is more permeable across the RBC membrane than the asparaginase substrate.

[0188] Thus, utilization of RBC-encapsulated CocE in treating cocaine-related conditions is an effective approach because cocaine readily crosses the RBC membrane (and actually somewhat concentrates in RBC) (Javaid et al. (1978) Journal of Chromatography 15, 105-113), PTD-mediated encapsulation does not alter the physical and/or chemical properties of the RBC, and the RBC-encapsulated enzyme functions as if free.

EXAMPLE 13: ENDOTOXIN REMOVAL

[0189] Endotoxin contamination of CocE mutants can be performed in several ways. Ideally, endotoxin decontamination decreases the concentration to levels less than 10 EU/mg protein. Methods of decontamination include alternative ion-exchange column chromatography conditions, size exclusion, polyethyleneimine (PEI) and hydrophobic column chromatography, ultrafiltration and detergent extraction. An endotoxin detection system (PYROGENT 5000, Cambrex) is used to determine the endotoxin content of preparations. The assay is based on the anti-LPS factor from Limulus amoebocyte lysate (LAL). The sensitivity of the assay is between 0.01 and 100 EU/ml, well within the levels required. The spectrophotometric assay is designed in a 96-well microtiter-plate format. Fractionation of endotoxin and CocE and/or mutant CocE by the following procedures can be assayed for both endotoxin level and cocaine esterase activity. Cocaine esterase activity is measured utilizing a spectrophotometric assay that takes advantage of the intrinsic absorption of cocaine at 240 nm. Upon hydrolysis, the absorption spectra reveals a dramatic reduction in the 240 nm peak (Turner et al. (2002) Biochemistry 41, 12297-12307).

[0190] Anion exchange chromatography: Current conditions involve the use of fast performance liquid chromatography (FPLC) on a Q-Sepharose

column at pH 8.0 for endotoxin decontamination. The buffer conditions (pH) can be optimized to maximize the both the adsorption of CocE and/or mutant CocE and separation of endotoxin. Fluted fractions are assessed by activity measure of CocE or mutant CocE activity (spectroscopic assay of the hydrolysis of cocaine, absorption at 240 nm). Endotoxin levels are assessed using PYROGENT 5000 (above).

[0191] Size Exclusion Chromatography and Ultrafiltration: Endotoxin can exist as monomeric forms ($MW \sim 1-2 \times 10^4$) or in micellar form ($MW \sim 4 \times 10^5$ to 1×10^6), depending on the buffer conditions. The presence of detergents such as cholate favors the monomeric form whereas divalent cations (e.g. Ca^{2+}) favor the micellar form (Hirayama, C and Sakata, M (2002) Journal of Chromatography B Analytical Technology Biomedical Life Science 781, 419-432). This property of endotoxin is used to separate the micellar (i.e. in the presence of divalent cations such as Mg^{2+} or Ca^{2+}) from CocE and/or mutant CocE by Gel filtration chromatography using either a Sephadex 75 or Sephadex 200 (Pharmacia) column resin. Micellar endotoxin should not be retained on the column and should pass through the void whereas CocE and/or mutant CocE should elute as a monodispersed protein corresponding to a 65 kDa protein. Similarly, the capacity of ultrafiltration units to separate the micellar forms of endotoxin from CocE and/or mutant CocE will be assessed. Ultrafiltration units are now available with molecular weight cutoffs of 3×10^5 to 1×10^6 , well within the range required to retain micellar endotoxin but not CocE itself.

[0192] Polyethyleneimine Chromatography: Mitzner et al. (1993) and Morimoto et al. (1985) have used PEI-immobilized on cellulose beads or on cellulose fiber, respectively to remove endotoxin from BSA preparations. PEI-chromatography column is a very weak anion exchanger that may in fact take advantage of some hydrophobic properties of endotoxin and thus preferentially adsorb it. Various PEI-silica bead preparations are available from Sigma, depending on the bead size. While silica-based beads are more classically

associated with HPLC applications we will pack columns for lower pressure work and will select the 200 um mesh sizes.

[0193] Triton X-114 Extraction: Triton X-114 phase separation has been successfully used to separate endotoxin from albumin and catalase (Aida, Y and Pabst, MJ (1990) J Immunological Methods 132, 191-195). Aida and Pabst report a 1000-fold decrease in endotoxin concentration following a single Triton X-114 extraction step. CocE and mutant CocE samples are incubated with equal volumes of Triton 114 and allowed to incubate first on ice and then at 37°C for 15 minutes. The Triton X-114 phase that contains the endotoxin is removed by centrifugation. As noted earlier it has been demonstrated that CocE is considerably thermal labile, but may be protected somewhat by the presence of the substrate, cocaine. If incubation of CocE with Triton X-114 at 37°C results in significant CocE inactivation, the enzyme will be stabilized by including excess substrate during the extraction.

EXAMPLE 14: MUTAGENESIS OF THE COCE HOST ORGANISM

[0194] There exists several bacterial strains that exhibit temperature sensitive growth on cocaine esterase byproducts, including several *Pseudomonas* strains, and these organisms can be adapted for temperature sensitive growth on cocaine by addition of the CocE gene encoded within suitable plasmid vectors. For example, the CocE gene was originally sequenced from *Rhodococcus* MB1 by subcloning gene fragments into *Rhodococcus erythropolis* CW25, a bacterium unable to metabolize cocaine but able to grow on the cocaine esterase byproducts ecgonine methyl ester and benzoate (Bresler et al. (2000) Applied & Environmental Microbiology 66, 904-908). The CocE gene has been subcloned into shuttle vectors pJAK-14 and pMMB67EH (see e.g., Example 1). These plasmids are capable of expression in any gram-negative bacterium including *Pseudomonas*, and additionally the pMMB67EH plasmid enables high levels of expression and ease of transformation by bacterial conjugation with the use of helper plasmid pRK2013. Transformation of

either plasmid into bacteria that exhibit temperature sensitive growth on cocaine esterase byproducts (e.g., *Pseudomonas* strains), enables temperature sensitive growth on plates containing cocaine as a sole source of carbon. Mutagenesis of both the original *Rhodococcus* MB1 and the CocE plasmid-containing *Pseudomonas* strains is performed using UV-light radiation at 260nm. Exposure is titrated such that 90-95% of cells are killed. The remaining cells are recovered in nutrient media for 1 hr at 26°C, collected and enriched in the presence of cocaine as previously described (Britt, et al. (1992) *Journal of Bacteriology* 174, 2087-2094). Enrichment is performed at 37°C to select for thermostable variants of the CocE. Finally, cells are plated onto minimal media agar plates containing 10mM cocaine and incubated at 37°C. Single colonies are grown and tested for CocE activity at 37°C. PCR amplification of the CocE gene is performed on mutants found to produce active and soluble CocE at 37°C, and the amplification products are subcloned into the pET-22B(+) plasmid for further characterization. As a prelude to CocE mutagenesis, it may be necessary to mutagenize the native *Pseudomonas* strains in order to preselect for no temperature sensitivity at 37°C when grown on cocaine hydrolysis products, and then demonstrate temperature sensitivity on cocaine at 37°C after cloning in CocE.

EXAMPLE 15: HIGH THROUGHPUT SCREENING METHOD FOR IDENTIFICATION OF THERMOSTABLE COCE MUTANTS

[0195] Several high throughput screening methods for identification of thermostable CocE variants were implemented. Because the wild-type enzyme is known to have thermal-instability at temperatures above 30°C, after transformation into *E. coli* BL21 cells, colonies are subcultured and protein expression induced at 16°C. Expressed proteins are then tested for esterase activity at temperatures 30°C and above. After several rounds of mutagenesis and testing at increasing temperatures thermostable mutants are achieved. Each

individual mutant CocE is then prepared, purified, and tested for activity and thermostability at 37°C, as described above (see Examples 1 and 4)

[0196] Bacterial colonies containing mutant polypeptides are screened from directly from agar plates by nitrocellulose filter imprint of replica plates followed by lysis of the bacteria and protein fixation. Determination of enzymatic activity at various temperatures is achieved by monitoring the accumulation of benzoic acid, the acidic by product of (-)cocaine. A moist nitrocellulose imprint is placed onto a dried filter paper previously saturated with a mixture of cocaine at pH 7.4 with no buffer and a pH indicator that transitions from colorless to color upon acidification e.g., methyl red. Active enzyme is identified by color change and colonies are appropriately harvested. The detection method based on acidification through the formation of benzoic acid is employed for the detection of cell expression of catalytic antibodies that hydrolyze cocaine at the benzoyl ester group, the same site cleaved by CocE. Alternatively, detection by nitrocellulose imprint is achieved by exposure to a cocaine thiol-derivative and subsequent detection of sulfhydryl groups via a precipitating heavy-metal (e.g., mercury-based) indicator system.

[0197] Bacterial colonies containing mutant polypeptides are also screened by subculturing into liquid medium and testing directly for cocaine esterase activity using a cocaine thiol-derivative and detection using the colorimetric thiol indicator, Ellman's reagent (see e.g., Figure 6). Ellman's reagent rapidly forms a disulfide bond with free thiol-groups and releases a colored thiolate ion which absorbs at 412 nm. Cultures incubated overnight at 16°C in the presence of IPTG to induce protein expression (20µl) are mixed with 1mM benzoylthioester cocaine derivative, and 500uM Ellman's reagent in 100mM sodium phosphate pH 7.4 to a final volume of 200µl. Results (see e.g., Figure 6) indicate that cells containing the wild-type CocE enzyme are able to cleave the benzoylthioester at much higher levels than cells alone.

[0198] Finally bacterial colonies containing mutant polypeptides are screened by subculturing into liquid medium followed by lysis and isolation of

mutant polypeptides using an affinity medium. For example, lysed cells are washed through nickel-agarose filter-plates allowing collection and subsequent elution of 6xHIS tagged proteins (for example, Ni-NTA Superflow 96-Bio-robot kit (Qiagen)). Alternatively, cells are lysed within nickel-coated microtiter plates allowing binding of 6xHIS fusion proteins and subsequent removal of contaminants (for example, immobilizer nickel chelate plates (Nunc) or NEN Nickel-chelate flash plates (Perkin Elmer)). Similarly, lysed cells are incubated with nickel coated microbeads (such as Ni-NTA magnetic agarose beads (Qiagen)), followed by subsequent removal of contaminating proteins. Isolated esterase protein activity are then tested using any of the assays previously mentioned (such as the spectrophotometric activity assay (Examples 1 and 4), the tritiated cocaine activity assay (Example 1), the benzoic acid pH indication activity assay, the thiol-derivative cocaine detection systems, the use of cocaine aptamers (Stojanovic, M. N., de Prada, P. & Landry, D. W. (2001) J Am Chem Soc 123, 4928-31; Stojanovic, M. N. & Landry, D. W. (2002) J Am Chem Soc 124, 9678-9) by monitoring changes in fluorescence upon degradation of cocaine, or by the use of a generic esterase substrate such as 4-nitrophenyl acetate and monitoring of colorimetric changes at 420nm as described previously (Halgasova, N. et al. (1994) Biochem J 298 Pt 3, 751-5; O'Conner, C.J. & Manuel, R.D. (1993) J Dairy Sci. 76:3674-3682).

EXAMPLE 16: PRELIMINARY ANALYSIS OF N197K MUTANT POLYPEPTIDE

[0199] Preliminary analysis of the N197K mutant polypeptide (SEQ ID NO: 42) showed good stability after 1 hour at 37°C on day 0. Vmax and Km values are shown in Table 5. A recheck on day 3 showed similar stability. Vmax and Km values are shown in Table 6. Higher Km values on day 3 were due to reuse of old cocaine. Gel filtration of samples incubated at 37°C showed formation of aggregates.

TABLE 5: N197K initial test (Day 0)

	N197K (0)	N197K (60)
Equation 1		
Best-fit values		
VMAX	2928	2187
KM	34.77	24.24

TABLE 6: N197K repeat test (Day 3)

	N197K (0)	N197K (60)
Equation 1		
Best-fit values		
VMAX	3357	3085
KM	102.2	113.5

EXAMPLE 17: IDENTIFICATION OF THERMALLY STABLE MUTANT CocE POLYPEPTIDES

[0200] Thermally stable mutant CocE polypeptides were identified by determining $\tau_{1/2}$ of the mutant CocE polypeptides. Briefly, enzymes were preincubated at 37°C for varying times. Activity measurements were determined at RT (25°C). Mutant enzymes with $\tau_{1/2}$ of greater than 12 minutes (the $\tau_{1/2}$ of wild type CocE) were considered thermally stable (See, e.g., Figure 27 and Table 7). As demonstrated by the combination mutant CocE polypeptide T172R/G173Q, in various embodiments, the combination of two single mutations with lower or no stability can result in a thermally stable combination.

TABLE 7: Thermally stable mutant CocE polypeptides

Mutant	Stability @ 37°C (t _{1/2})
T122A	No
Q123E	No
S159A	No
S140A	No
S167A/W52L	No
T172R	~46 min
V121D	No
L163V	No
F189A	No
F189A/T172R	~40 min (Similar to T172R)
C107S	No
W220A	No
F189L	No
A193D	No
T172R/A193D	~40 min (Similar to T172R)
G173Q	~25 min
T254R	No
N42V	No
T172R/G173Q	~ 326 min
G171Q/T172R/G173Q	No
G171A	No
G173A	No
wt-I175-G-D185	No
wt-T176-G-G-D185	No
T172R/G173Q-I175-G-D185	
T172R/G173Q-I175-G-G-A186	~75 min
T172R/G173Q-T176-G-G-D185	~75 min
S177Q	No
D45R	No
F47R	No

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L169K	~ 274 min
L174R	No
A181K	No
S179R	No
F189K	25 min
V190K	No
A194K	No
R182K	No

EXAMPLE 18: PROTECTION OF AGGREGATION OF THERMAL STABLE COCAINE ESTERASE MUTANTS

[0201] Protection from aggregation of thermal stable CocE mutants was assessed using size exclusion chromatography. Briefly, enzymes were preincubated at 37°C for 0 minutes or 60 minutes and resolved by size exclusion chromatography. Results for wild-type CocE, T172R and T172R/G173Q are shown in Figure 28.

EXAMPLE 19: LOW-UV SPECTRA

[0202] Low-UV CD spectra data were obtained using an Aviv Spectropolarimeter Mode 400, with the help of Norma Greenfield, UMDNJ, using a 5-cell holder and 0.2mg/ml protein concentrations. Raw data values obtained were blanked on control PBS, smoothed, and deconvoluted using the CCA algorithm as described by A. Perczel, K. Park, and G.D. Fasman, [Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm: a practical guide. Analytical Biochemistry 203, 83-93 (1992).] This algorithm finds the minimum number of curves needed to reconstruct a data set, and expresses the percentage each curve contributes to the data set as a function of temperature.

[0203] Below is presented a low UV CCA deconvolution analysis of CocE WT and 4 mutants, obtained in a single CD spectra melt using a 5-cell holder. The experiment was performed over the period of 8 hours, from 0-80°C.

[0204] Temperature dependent melting was observed between the wavelengths tested (200-250nm), and the smoothed spectra for each mutant are shown in Figure 29. Deconvolution via the CCA algorithm indicated that each spectra was best described by a set of three curves, as shown in Figure 30. This suggests that the melting of CocE is at least a two step process, moving from an original curve (curve 1) to an intermediate unfolding step (curve 2), and finally fully denatured protein (curve 3). The % contribution that each temperature played in describing these three curves is shown in Figure 31. Dose response analysis was used to approximate the temperature at which the initial spectra melts (1), the formation and melt of the intermediate state (2), and the accumulation of the fully melted protein (3). These numbers were collected and plotted in Figure 32 and shown in Table 8.

TABLE 8: Melting points of each step

	#1 melt	#2 formation	#2 melt	#3 formation
—●— WT	37.04	34.57	50.98	59.86
—■— T172R	39.22	38.11	54.56	56.85
—▲— T172R-A193D	38.11	46.47	47.68	58.72
—×— T172R-F189K	38.8	46.81	48.42	57.23
—*— T172R-G173Q	40.62	45.61	46.17	56.61

[0205] The most thermostable mutant T172R-G173Q (as determined in other assays) showed the highest temperature melting of the original curve 1 (40°C vs 37°C for wild-type), and the lowest temperature for both disappearance

of the curve 2 intermediate (46°C vs 50°C for wild-type) and appearance of the fully melted curve 3 (56°C vs 59°C for wild-type).

[0206] In summary, it appears that all mutants undergo a 2-step melting process.

EXAMPLE 20: STABILIZATION USING PRODUCTS AND INHIBITORS

[0207] Cocaine esterase (CocE) cleaves cocaine to produce Benzoic acid and Ecgonine methyl ester. Briefly, alternative substrates and inhibitors of cocaine, as well as compounds able to thermostabilize the enzyme, were investigated generally by substituting amide and thiol analogues at the reactive ester bond, or removing the bond (for inhibitors), substituting benzoyl analogues in place of the benzoic acid leaving group, and/or removing or altering the methyl-ester group on the ecgonine portion of the molecule. As discussed below, it was determined that some substrates, products, and inhibitors stabilized thermal denaturation of wild type CocE, as well as prevented thermally-induced aggregation in gel electrophoresis.

[0208] Cocaine is the natural substrate of Cocaine esterase (CocE). Cocaine cleavage was monitored by a drop in absorbance at 240nm. Cocaine (mM range) prevented 37°C-induced formation of high molecular weight CocE aggregates (0.1 mg/ml enzyme concentrations) (see, e.g., Figure 33). Cocaine (uM quantities) stabilized 37°C-induced activity loss (see, e.g., Figure 34), although the mechanism of this stabilization is complicated due to substrate inhibition at higher concentrations.

[0209] Benzoic acid is the natural product of CocE and a weak inhibitor of CocE cleavage of 4-nitrophenyl acetate (K_i 310uM). Benzoic acid prevented 37°C-induced formation of high molecular weight CocE aggregates (0.1 mg/ml enzyme concentrations) (see, e.g., Figure 35). Benzoic acid (uM quantities) stabilized 37°C-induced activity loss (see, e.g., Figure 36), although the

mechanism of this stabilization is complicated due to substrate inhibition at higher concentrations.

[0210] CocE catalyzes the cleavage of 4-nitrophenyl acetate (4NPA) to 4-nitrophenol (4NP) and acetate. The cleavage reaction was monitored by observation of product formation at 400 nm. Both 4NP and 4NPA (mM range) prevented 37°C-induced formation of high molecular weight CocE aggregates (0.1 mg/ml enzyme concentrations).

[0211] Phenylboronic acid is a potent inhibitor of CocE (K_i 250nM). Phenylboronic acid stabilized 37°C-induced aggregation of CocE with an EC_{50} of 0.2UM by densitometry analysis.

[0212] Using the above, a screening assay is developed to screen for small molecules that might similarly stabilize the enzyme but not necessarily occupy the active site. Molecules identified as stabilizing molecules are used to stabilize the proteins disclosed herein until ready for use.

CLAIMS

What is claimed is:

1. An isolated mutant cocaine esterase (CocE) polypeptide comprising an amino acid sequence of SEQ ID NO: 1, wherein at least one amino acid residue is substituted such that the mutant CocE polypeptide has esterase activity with increased thermostability at 37°C as compared to wild-type CocE.

2. The isolated CocE polypeptide of claim 1, wherein at least two amino acid residues are substituted.

3. The isolated CocE polypeptide of claim 2, wherein at least three amino acid residues are substituted.

4. The isolated CocE polypeptide of claim 3, wherein at least four amino acid residues are substituted.

5. The isolated CocE polypeptide of claim 4, wherein at least five amino acid residues are substituted.

6. An isolated mutant cocaine esterase polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3 (L163V); SEQ ID NO: 7 (V225I); SEQ ID NO: 8 (I218L); SEQ ID NO: 9 (A310D); SEQ ID NO: 10 (A149S); SEQ ID NO: 11 (S159A); SEQ ID NO: 12 (S265A); SEQ ID NO: 13 (S56G); SEQ ID NO: 14 (W220A); SEQ ID NO: 16 (S140A); SEQ ID NO: 17 (F189L); SEQ ID NO: 18 (A193D); SEQ ID NO: 19 (T254R); SEQ ID NO: 20 (N42V); SEQ ID NO: 21 (V262L); SEQ ID NO: 22 (L508G); SEQ ID NO: 23 (Y152H); SEQ ID NO: 24 (V160A); SEQ ID NO: 25 (T172R); SEQ ID NO: 26 (Y532F); SEQ ID NO: 27 (T74S); SEQ ID NO: 28 (W285T); SEQ ID NO: 29 (L146P); SEQ ID NO: 30 (D533S); SEQ ID NO: 31 (A194R); SEQ

ID NO: 32 (G173Q); SEQ ID NO: 33 (C477T); SEQ ID NO: 34 (K531A); SEQ ID NO: 35 (R41I); SEQ ID NO: 36 (L119A); SEQ ID NO: 37 (K46A); SEQ ID NO: 38 (F84Y), SEQ ID NO: 39 (T172R-G173Q); SEQ ID NO: 40 (L169K); SEQ ID NO: 41 (F189A), SEQ ID NO: 42 (N197K), SEQ ID NO: 43 (R182K), SEQ ID NO: 44 (F189K), SEQ ID NO: 45 (V190K), SEQ ID NO: 46 (Q191K), and SEQ ID NO: 47 (A194K), or a functional fragment thereof.

7. An isolated nucleic acid encoding the mutant CocE polypeptide of any one of claims 1-6.

8. An isolated nucleic acid comprising a nucleotide sequence that hybridizes to SEQ ID NO: 2 or the complement of SEQ ID NO: 2 under high stringency conditions, wherein the isolated nucleic acid encodes a mutant CocE polypeptide having esterase activity with increased thermostability at 37°C as compared to wild-type CocE.

9. An isolated nucleic acid encoding a mutant CocE polypeptide comprising an amino acid sequence that has at least 85% sequence identity with the nucleic acid sequence of SEQ ID NO: 2, wherein the encoded mutant CocE polypeptide has esterase activity with increased thermostability at 37°C as compared to wild-type CocE.

10. The isolated nucleic acid of claim 7 wherein the sequence identity is selected from the group consisting of at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%.

11. The polypeptide or nucleic acid of any one of claims 1-10 wherein the increase in mutant CocE polypeptide thermostability over wild-type CocE is at least about 2.1 kcal/mol, at least about 2.2 kcal/mol, at least about 2.3 kcal/mol, at least about 2.4 kcal/mol, at least about 2.5 kcal/mol, at least about 2.6 kcal/mol, at least about 2.7 kcal/mol, at least about 2.8 kcal/mol, at least about

2.9 kcal/mol, at least about 3.0 kcal/mol, at least about 3.1 kcal/mol, at least about 3.2 kcal/mol, at least about 3.3 kcal/mol, at least about 3.4 kcal/mol, at least about 3.5 kcal/mol, at least about 3.6 kcal/mol, at least about 3.7 kcal/mol, at least about 3.8 kcal/mol, at least about 3.9 kcal/mol, at least about 4.0 kcal/mol, at least about 4.1 kcal/mol, at least about 4.2 kcal/mol, at least about 4.3 kcal/mol, at least about 4.4 kcal/mol, or at least about 4.5 kcal/mol.

12. The polypeptide or the nucleic acid of any one of claims 1-11 wherein the mutant CocE polypeptide has reduced immunogenicity as compared to wild-type CocE.

13. The polypeptide or the nucleic acid of any one of claims 1-12 wherein the mutant CocE polypeptide has at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 100%, at least about 105%, at least about 110%, at least about 115%, at least about 120%, at least about 125%, at least about 130%, at least about 135%, or at least about 140% of the esterase activity of wild-type CocE polypeptide.

14. The polypeptide or the nucleic acid of any one of claims 1-12 wherein the mutant CocE polypeptide retains at least substantially the same or greater catalytic efficiency of wild-type CocE polypeptide.

15. A pharmaceutical composition comprising the mutant CocE polypeptide or the mutant CocE polypeptide encoded by the nucleic acid of any one of claims 1-14 and a pharmaceutically acceptable carrier or excipient.

16. The polypeptide, polypeptide encoded by the nucleic acid, or the pharmaceutical composition of any one of claims 1-15, wherein the mutant CocE polypeptide is pegylated.

17. The polypeptide, polypeptide encoded by the nucleic acid, or the pharmaceutical composition of any one of claims 1-16, wherein the mutant CocE polypeptide is encapsulated in a red blood cell.

18. The polypeptide, polypeptide encoded by the nucleic acid, or the pharmaceutical composition of any one of claims 1-17, wherein the mutant CocE polypeptide is stabilized by a substrate or inhibitor.

19. The polypeptide, polypeptide encoded by the nucleic acid, or the pharmaceutical composition of any one of claims 1-5 and 7-18, wherein the polypeptide comprises a plurality of amino acid residue substitutions selected from the group consisting of: T122A; 123E; S159A; S140A; S167A/W52L; T172R; V121D; L163V; F189A; F189A/T172R; C107S; W220A; F189L; A193D; T172R/A193D; G173Q; T254R; N42V; T172R/G173Q; G171Q/T172R/G173Q; G171A; G173A; wt-I175-G-D185; wt-T176-G-G-D185; T172R/G173Q-I175-G-D185; T172R/G173Q-I175-G-G-A186; T172R/G173Q-T176-G-G-D185; S177Q; D45R; F47R; L169K; L174R; A181K; S179R; F189K; V190K; A194K; and R182K.

20. A method of treating a cocaine-induced condition comprising administering to a subject in need thereof an effective amount of the mutant CocE polypeptide, the mutant CocE polypeptide encoded by the nucleic acid, or the pharmaceutical composition of any one of claims 1-19.

21. The method of claim 20, wherein the cocaine-induced condition is selected from the group consisting of cocaine overdose, cocaine toxicity, cocaine addiction, and cocaine dependence.

22. A high throughput screening method for identification of thermostable mutant CocE polypeptides comprising the steps of:

stably transforming a cell with a nucleic acid encoding a mutant CocE polypeptide;
expressing the mutant CocE polypeptide in the cell;
isolating the expressed CocE polypeptide;
measuring esterase activity of the isolated mutant CocE polypeptide at one or more predetermined temperatures of at least about 30°C up to about 50°C to determine thermostability of the isolated mutant CocE polypeptides;
selecting isolated mutant CocE polypeptide with esterase activity at the predetermined temperature(s).

23. The high throughput screening method of claim 22 wherein measuring esterase activity of the isolated mutant polypeptides comprises:
contacting the isolated mutant CocE polypeptide with (i) cocaine and a pH indicator or (ii) a thio-derivative of cocaine and a thiol indicator;
detecting a change in the pH indicator or the thiol indicator; wherein a change of the pH indicator or the thiol indicator is correlated with formation of benzoic acid from hydrolysis of cocaine or cocaine derivative by the mutant CocE polypeptide.

24. The high throughput screening method of any one of claims 22-23 further comprised of repeating one or more steps of the screening method at least once at an increased temperature for measuring esterase activity.

25. The high throughput screening method of claim 24 wherein a first screening cycle has a first temperature for measuring esterase activity of about 30°C and a subsequent screening cycle has a temperature for measuring esterase activity of about 45°C.

26. The high throughput screening method of any one of claims 22-25 wherein expression of mutant CocE polypeptide occurs at a temperature at which wild type CocE substantially retains catalytic activity.

27. The high throughput screening method of any one of claims 22-25 wherein expression of mutant CocE polypeptide occurs at a temperature at which wild-type CocE polypeptide substantially partitions into inclusion bodies.

FIG. 1

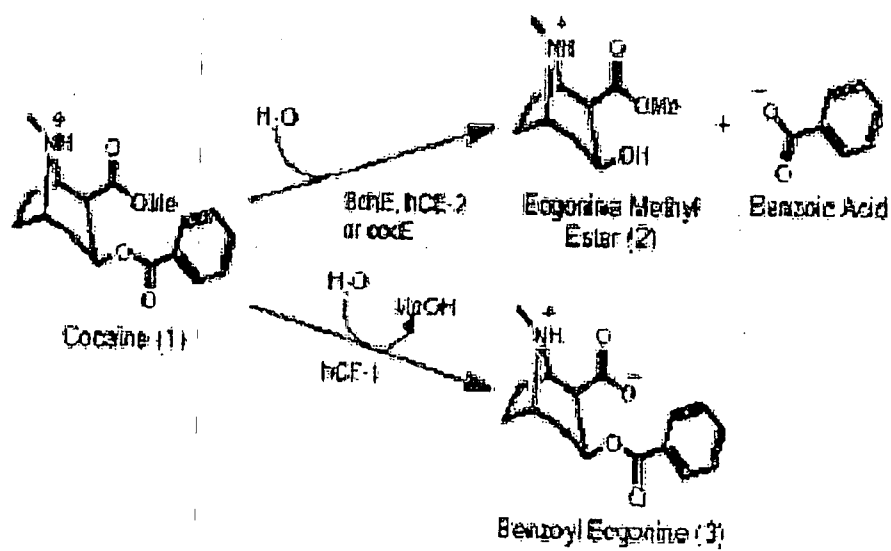


FIG. 2

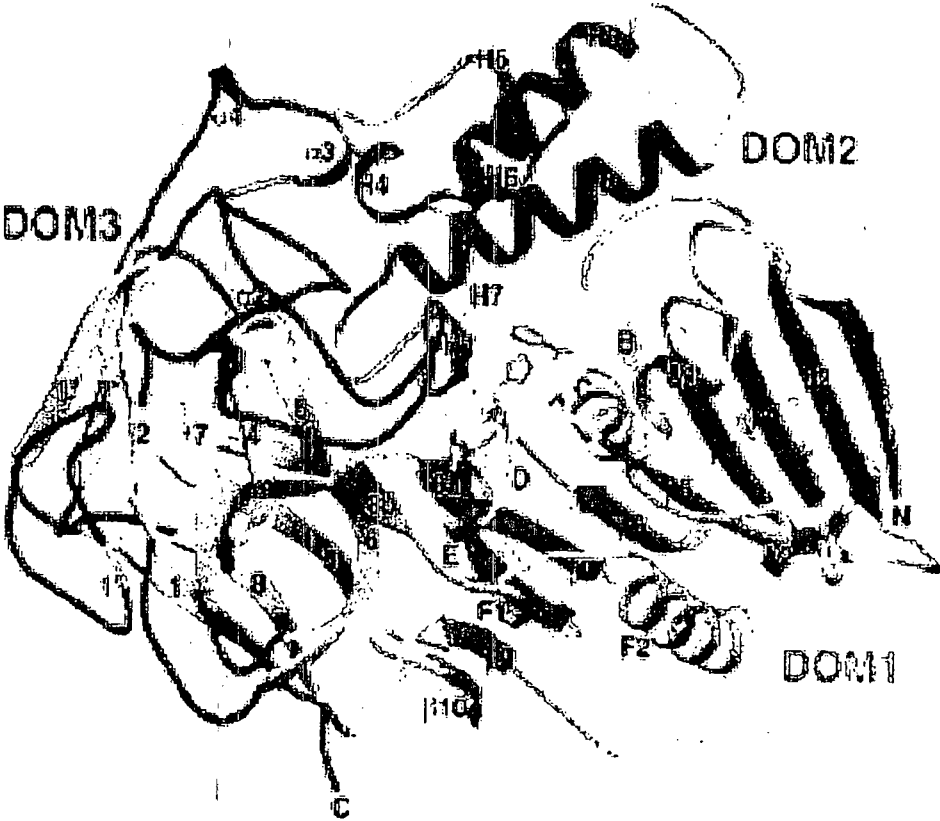


FIG. 3

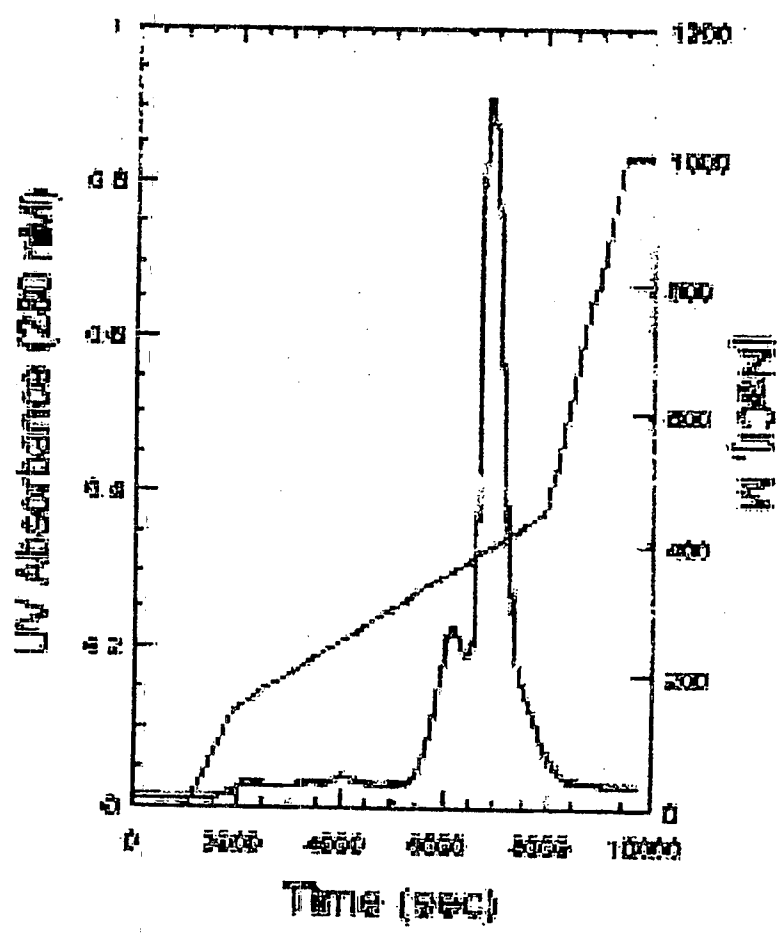


FIG. 4

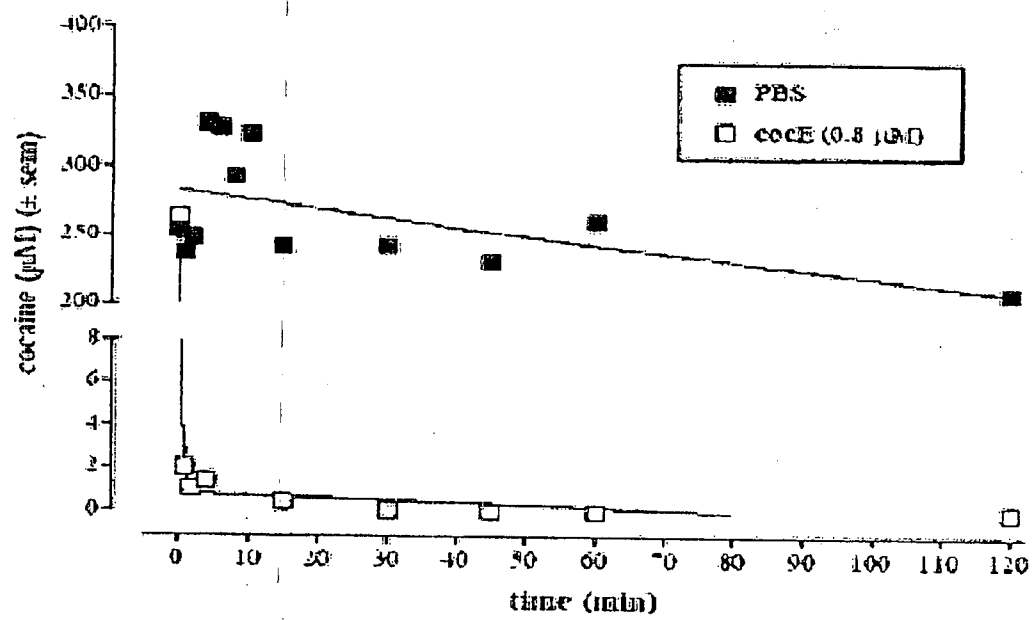
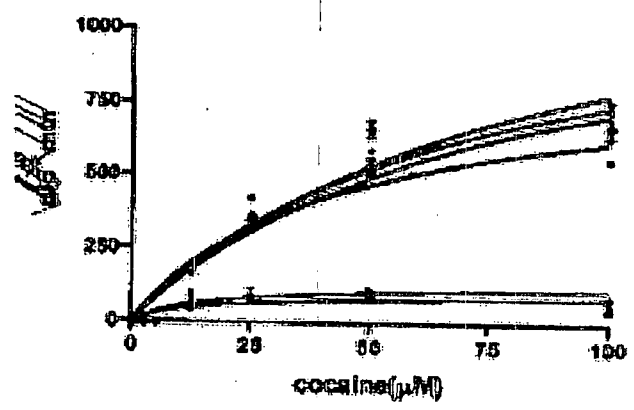


FIG. 5

Repeat of mutants S159A & T172R (3-6-06)



- T172R
- S159A
- T172R(40min @ 37°C)
- wt CocE
- S159A(40min @ 37°C)
- wt CocE(40min @ 37°C)

FIG. 6

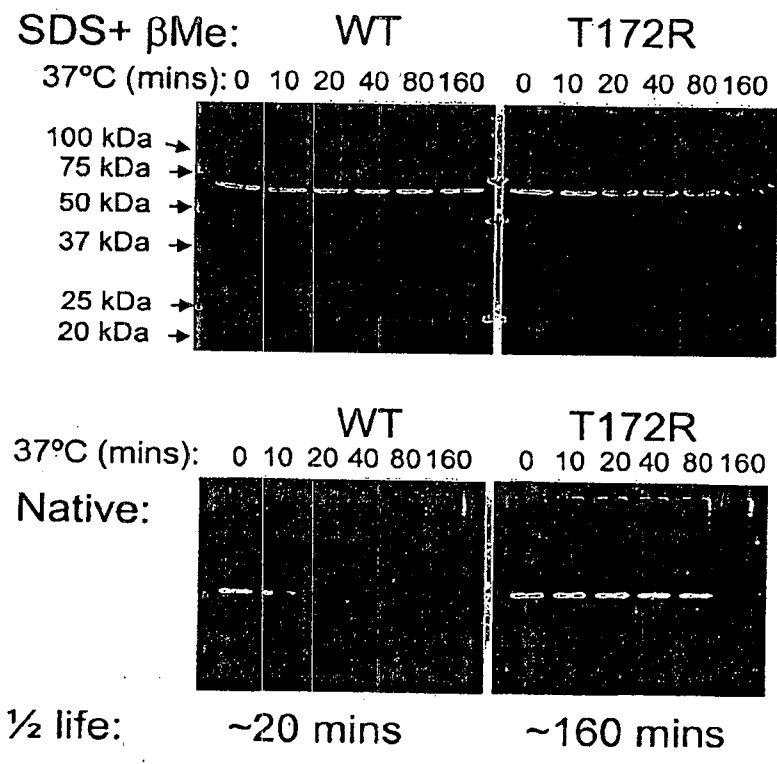


FIG. 7

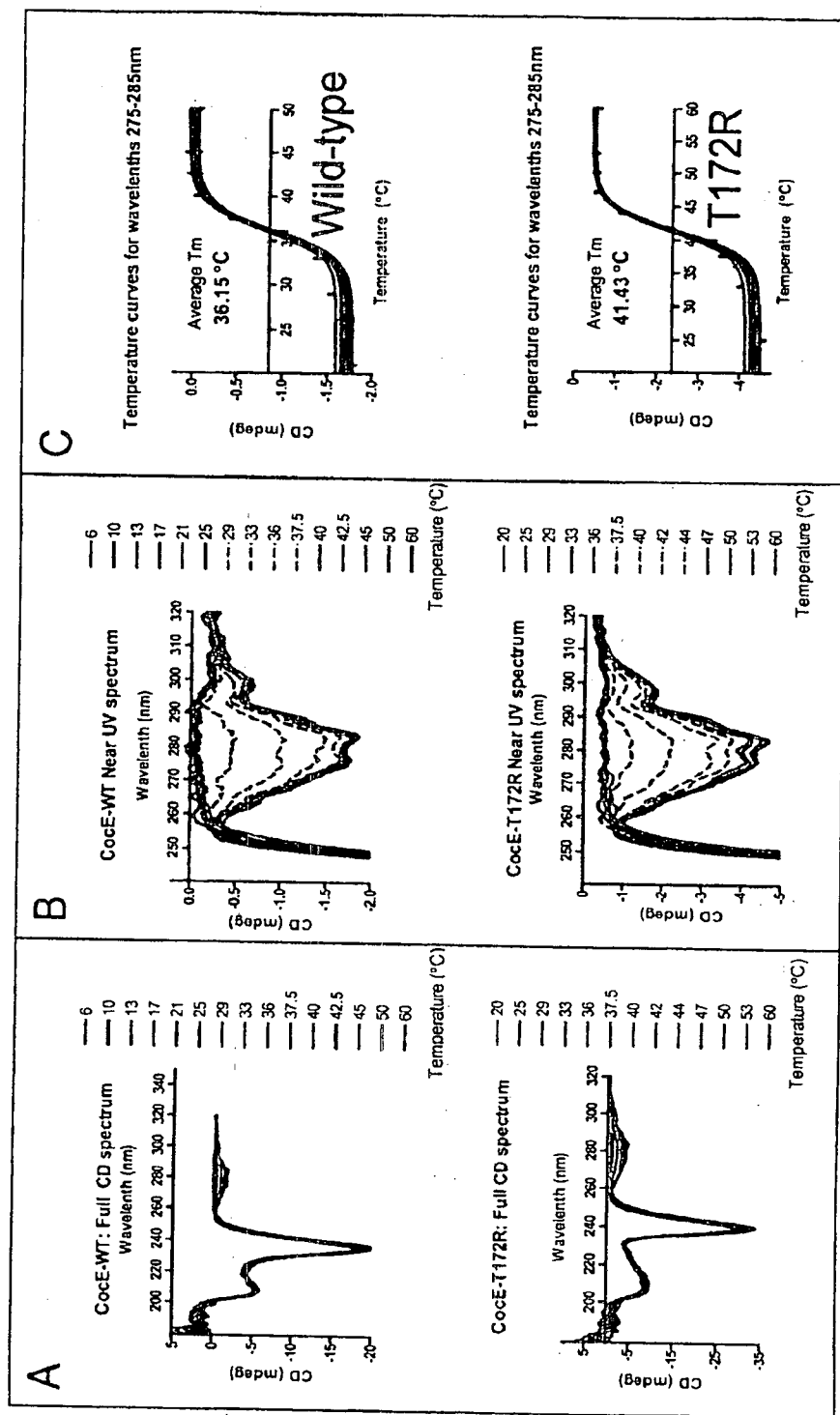


FIG. 8

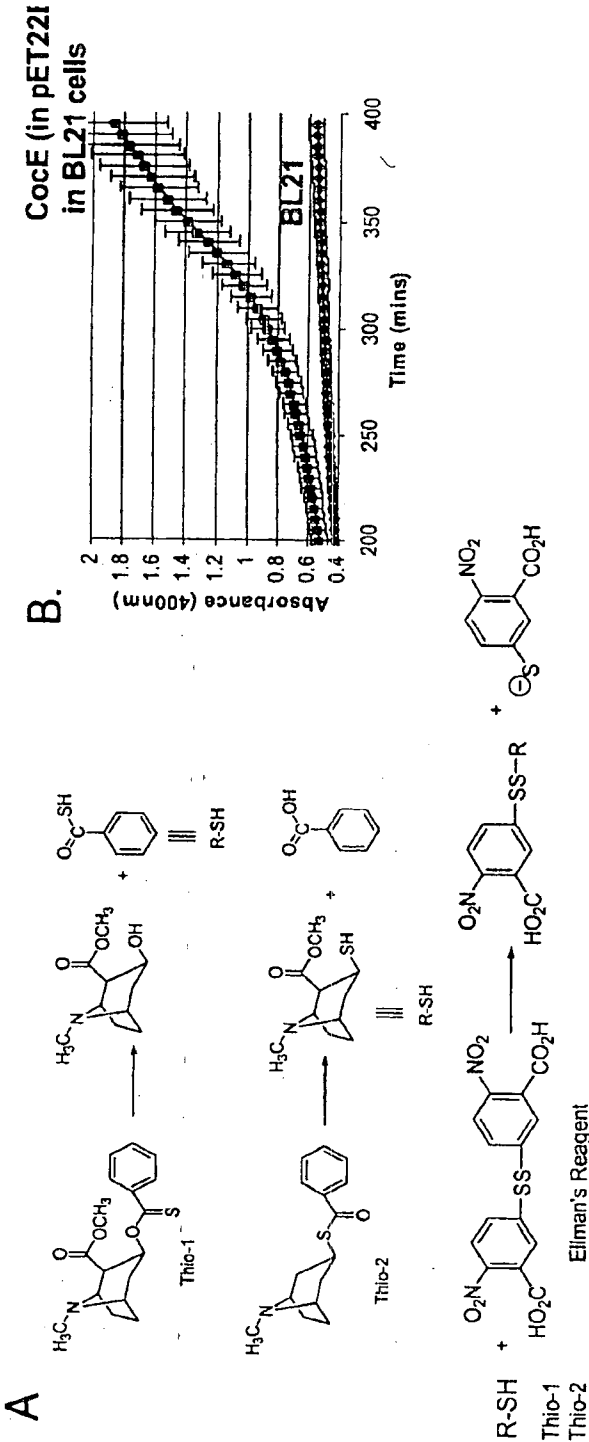


FIG. 9

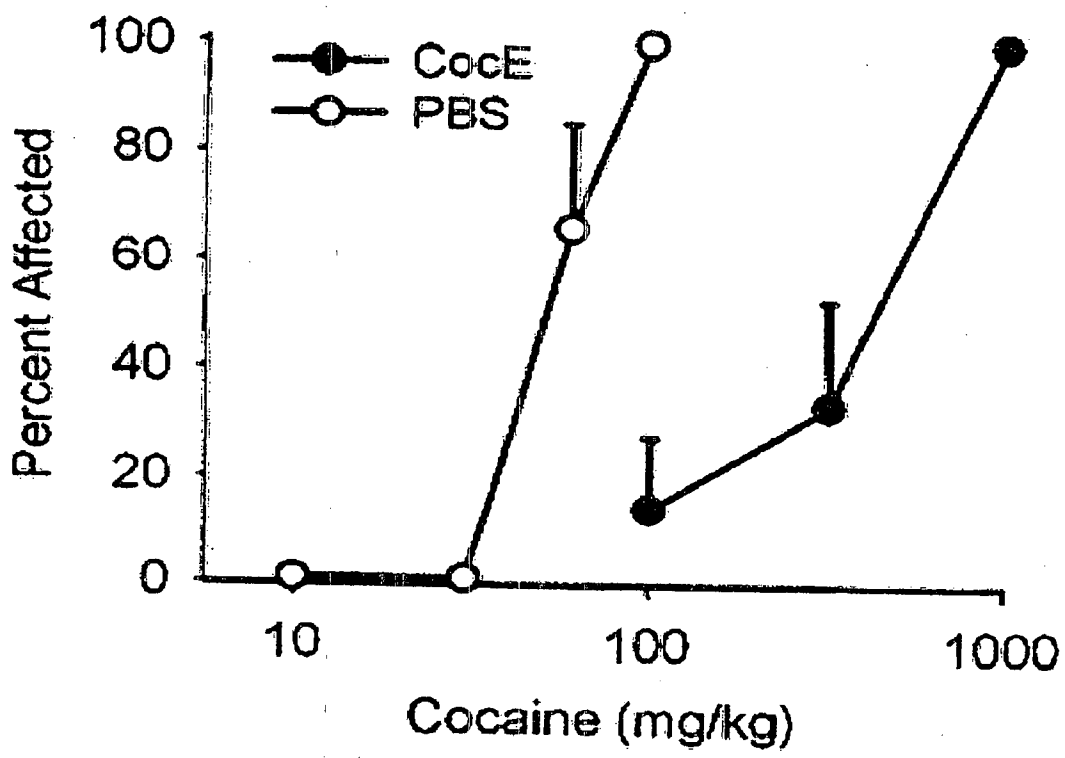


FIG. 10

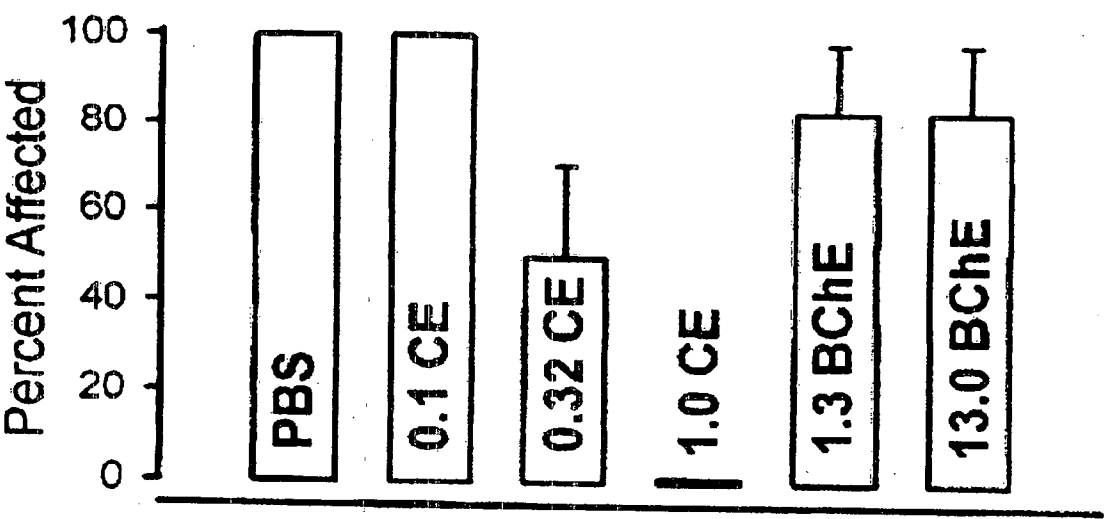


FIG. 11

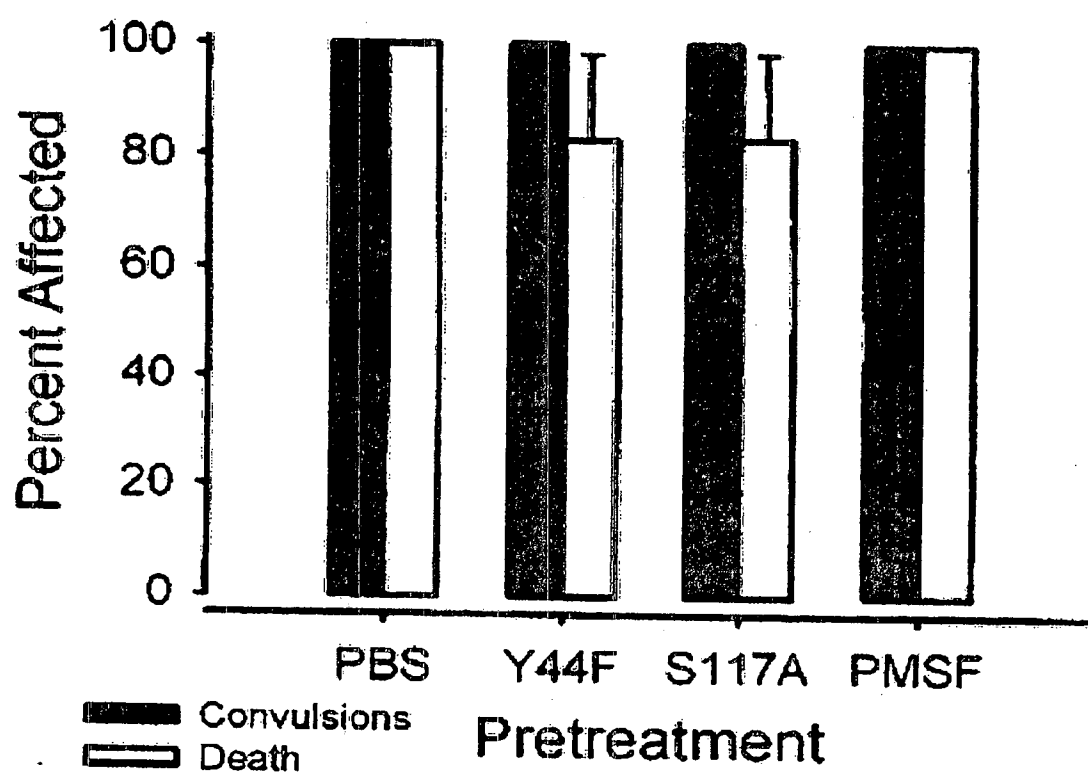


FIG. 12

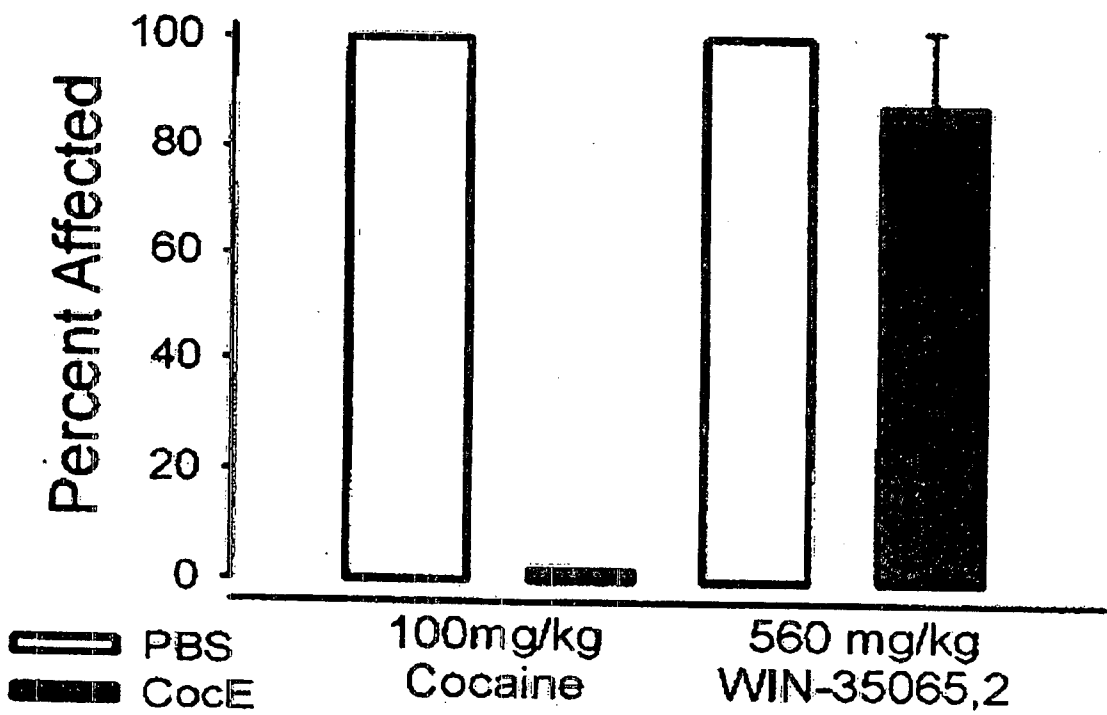


FIG. 13

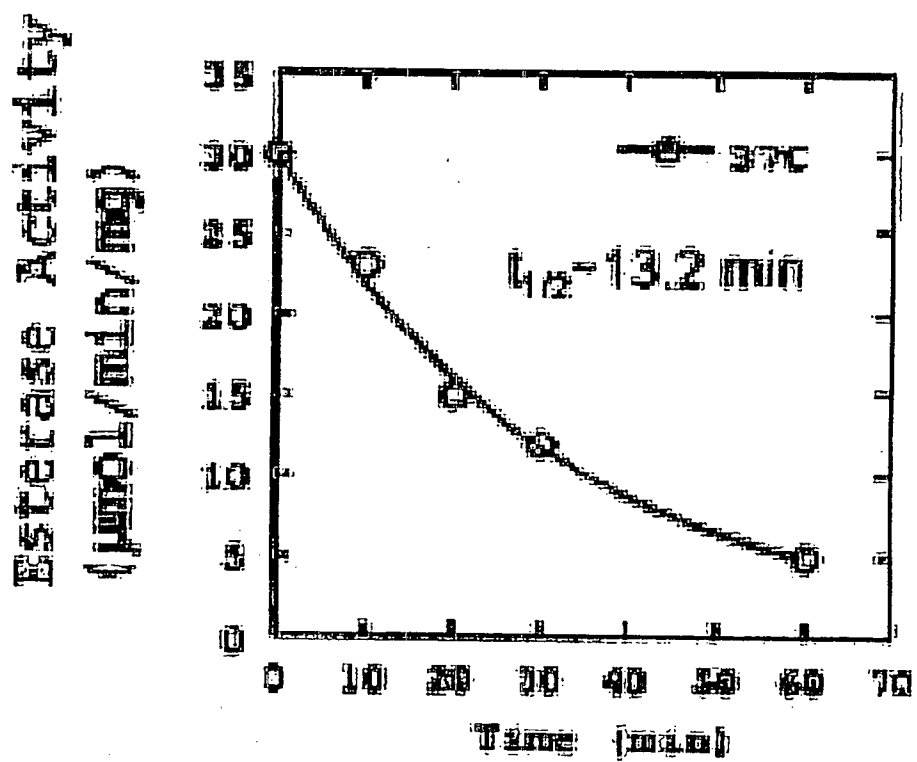


FIG. 14

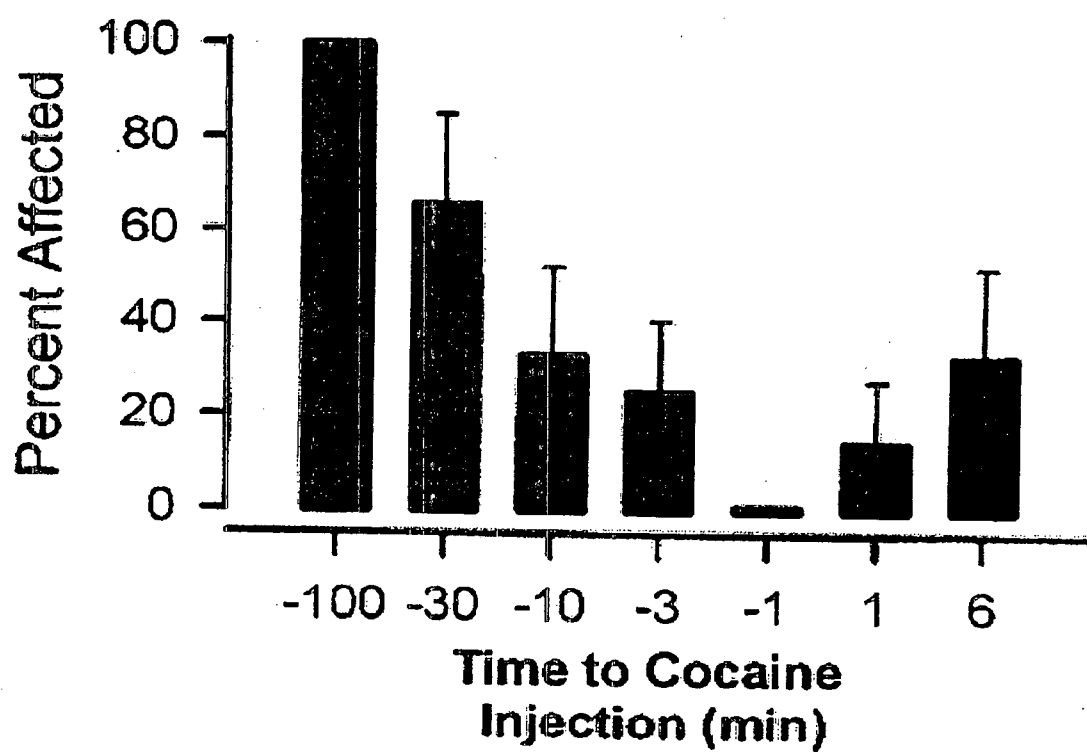


FIG. 15

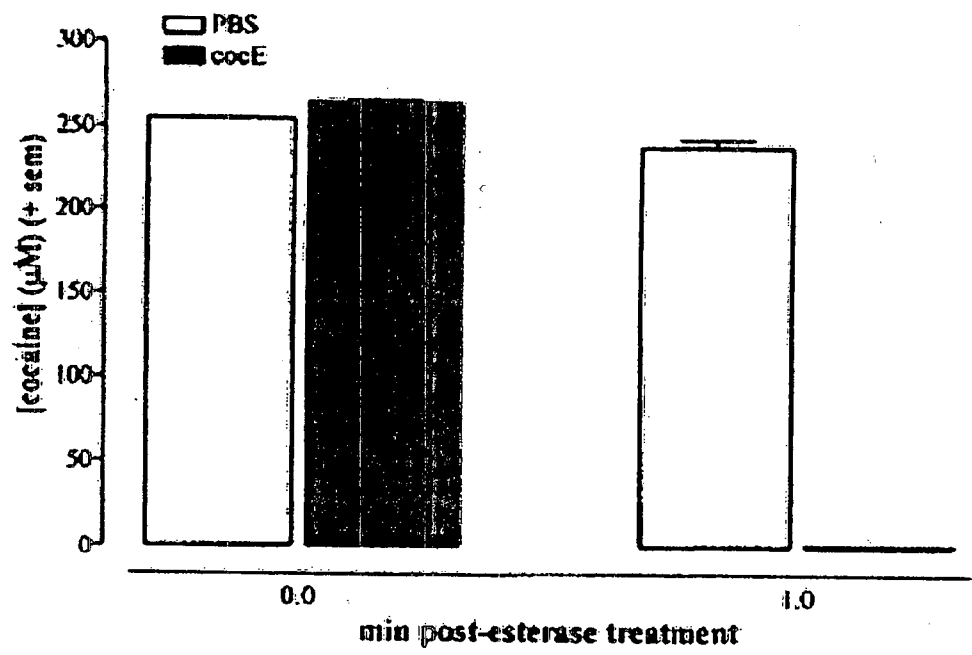


FIG. 16

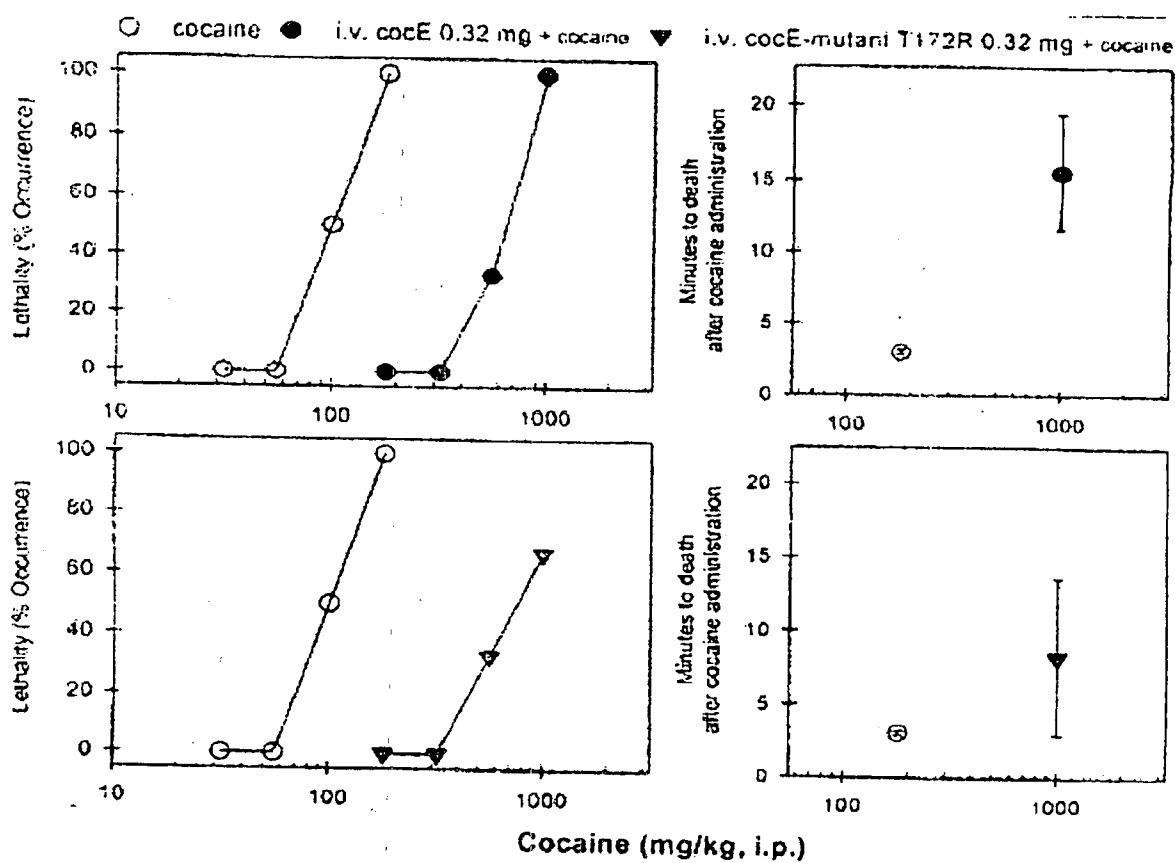


FIG. 17

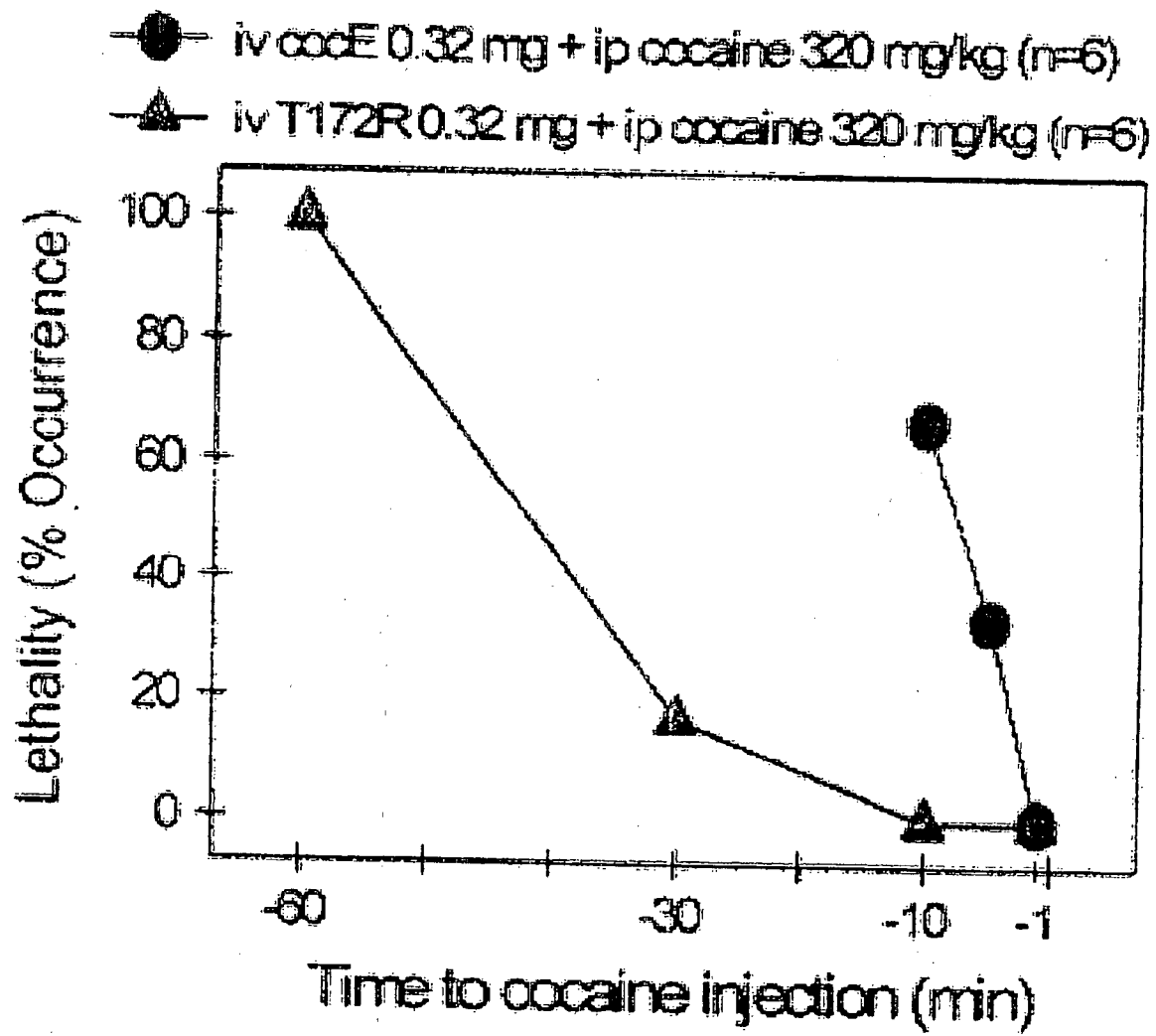


FIG. 18

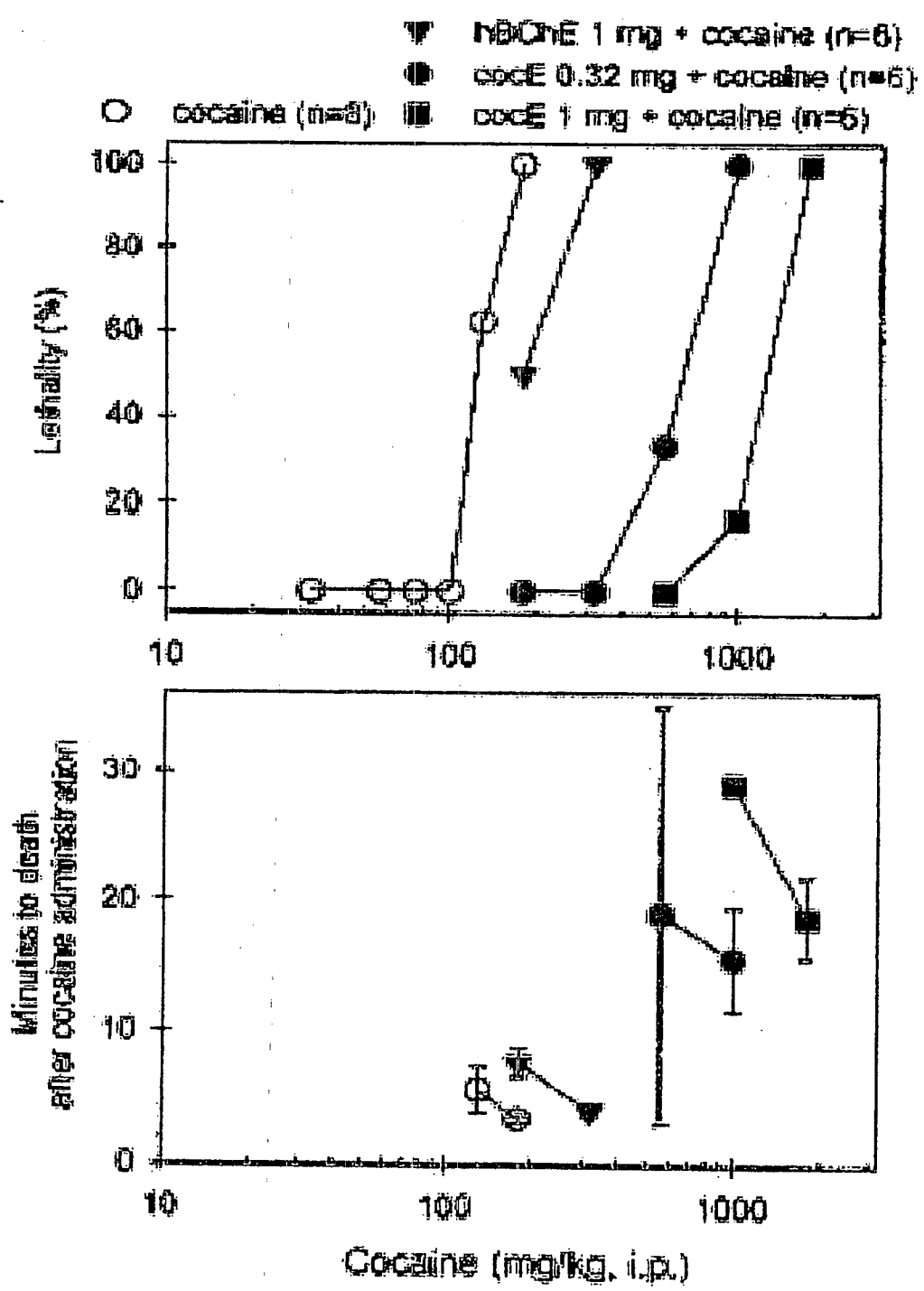


FIG. 19

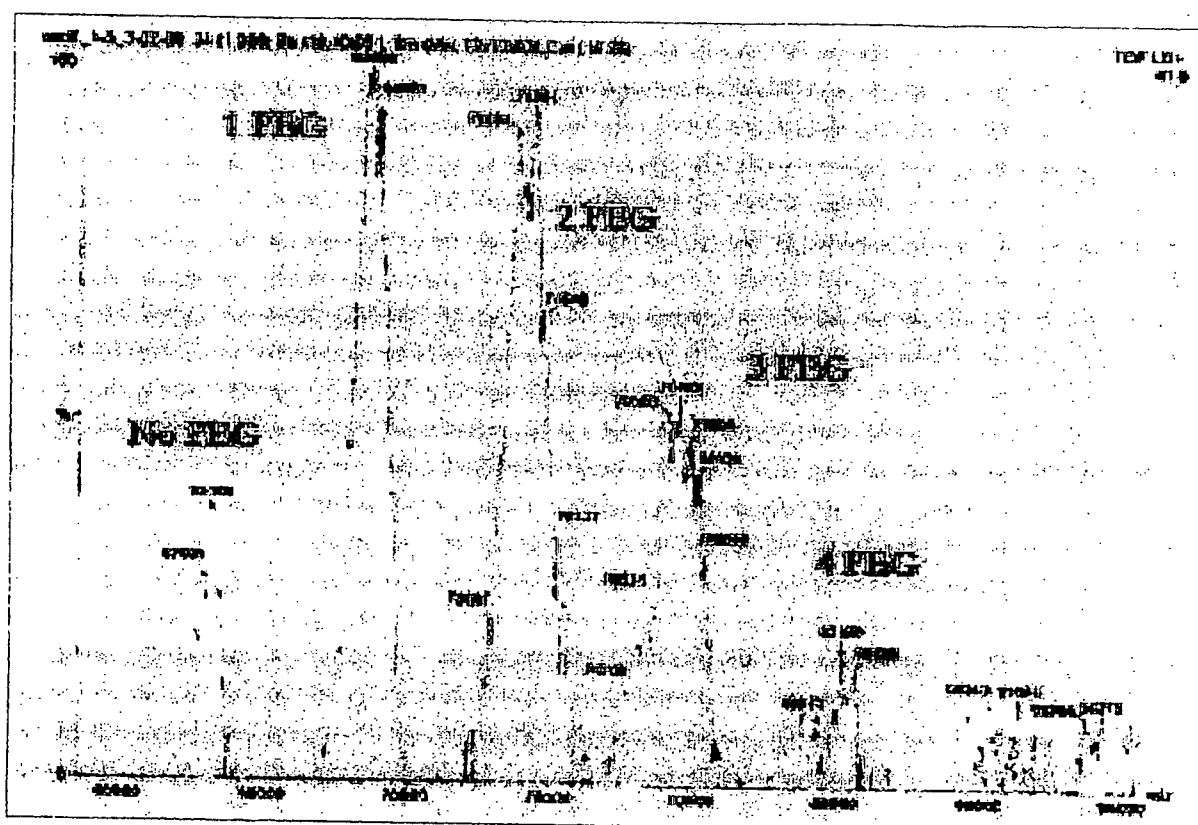


FIG. 20

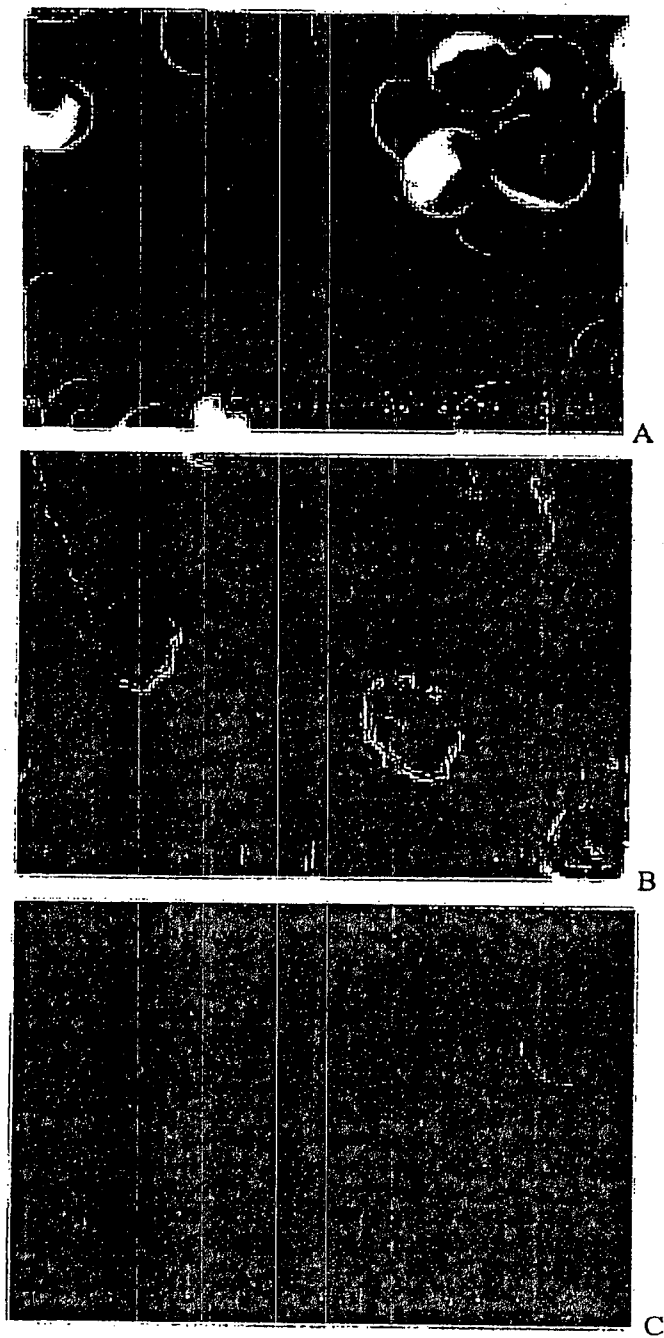


FIG. 21

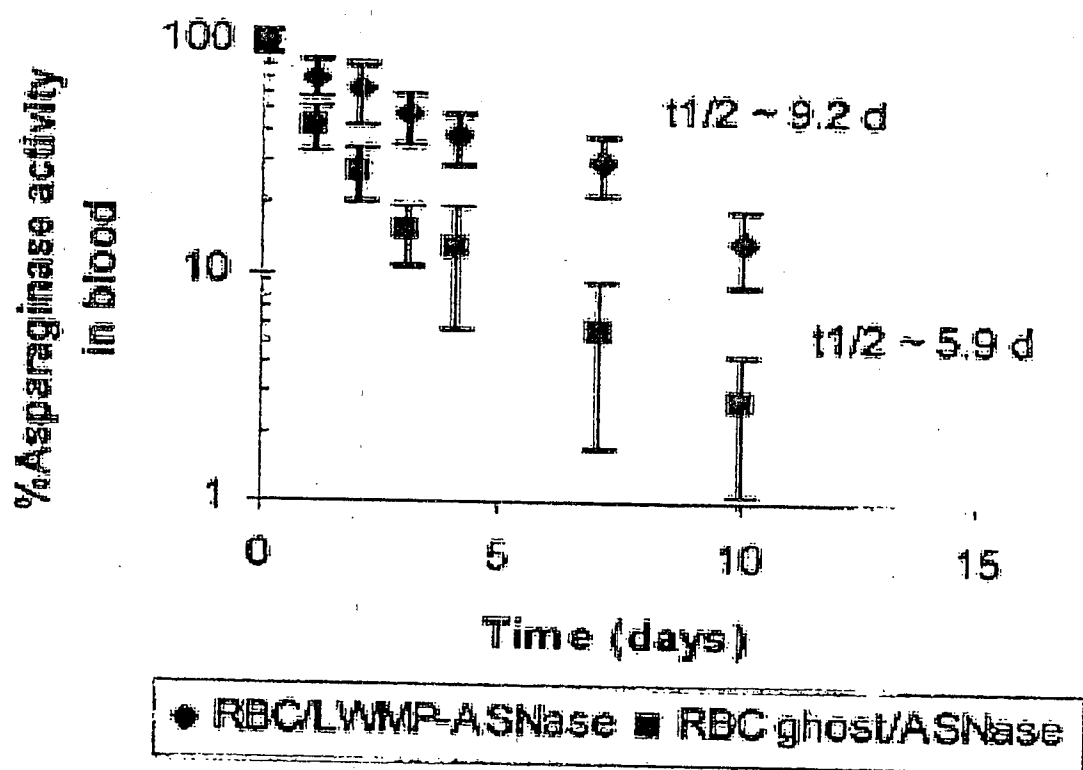


FIG. 22

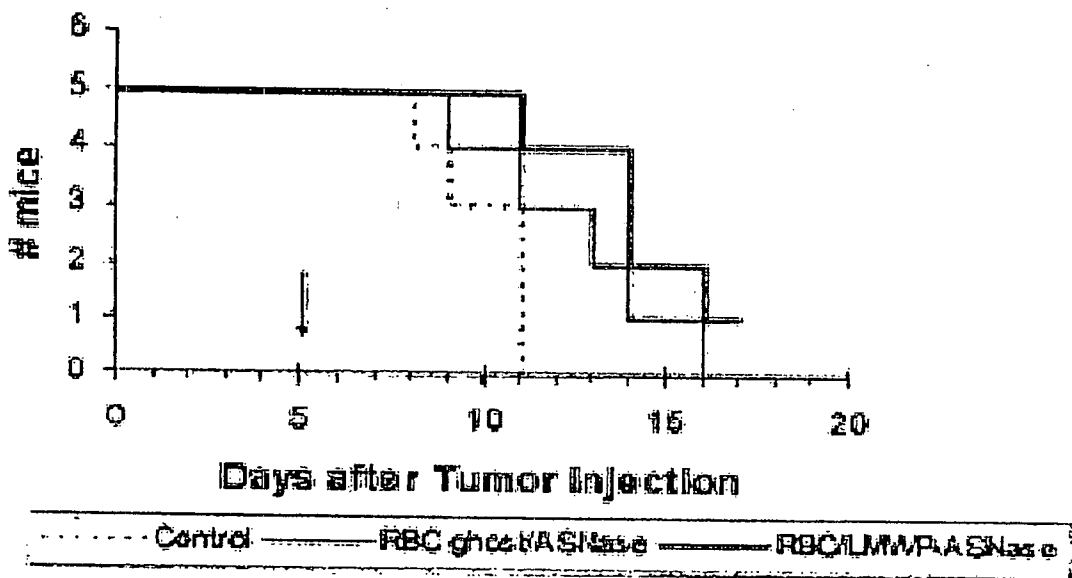


FIG. 23

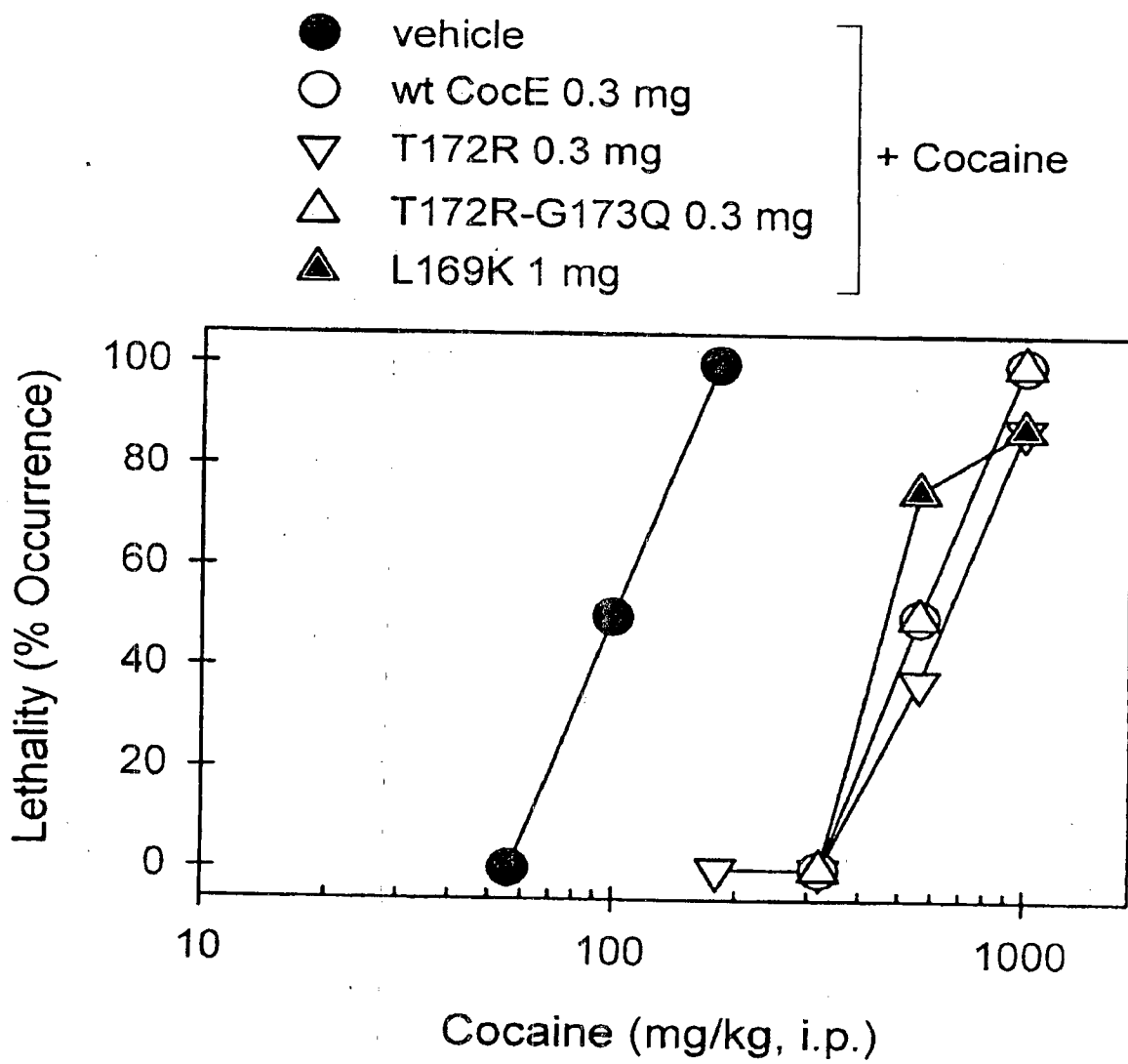


FIG. 24

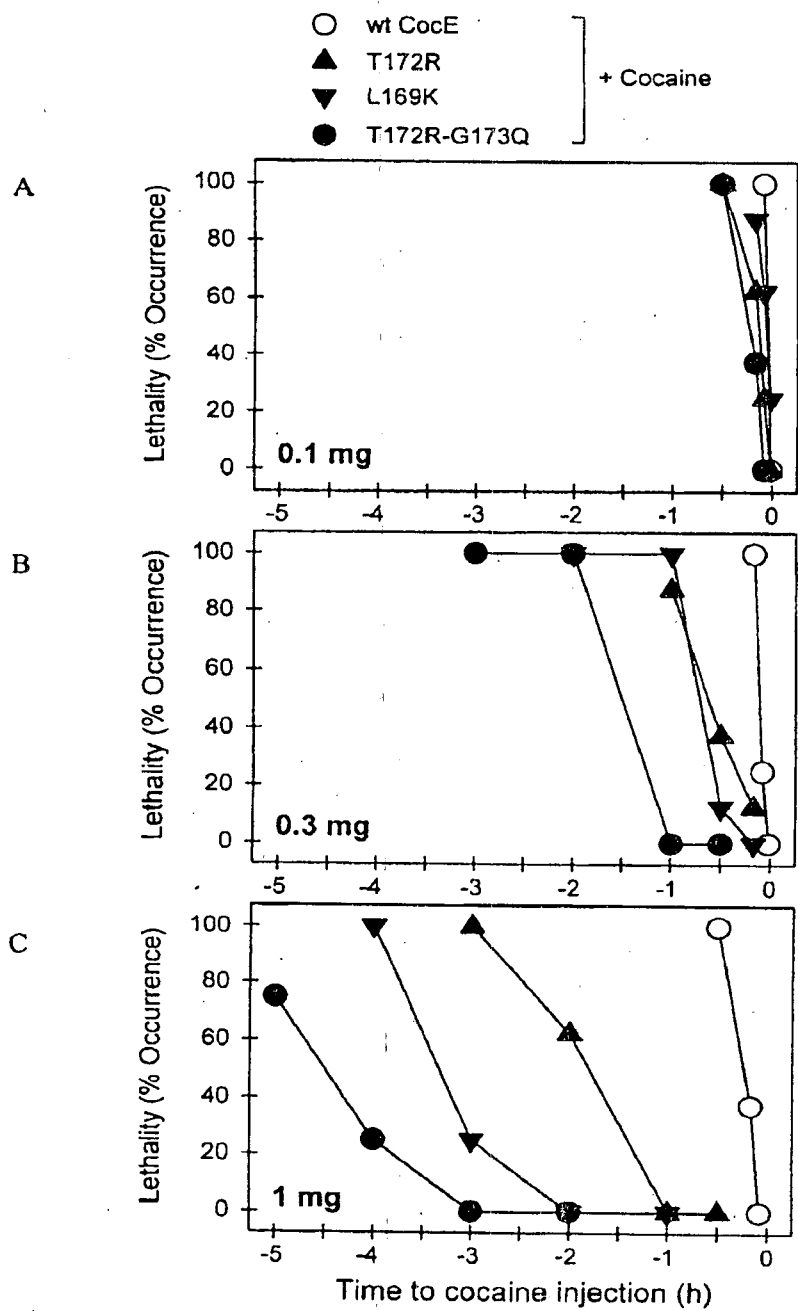


FIG. 25

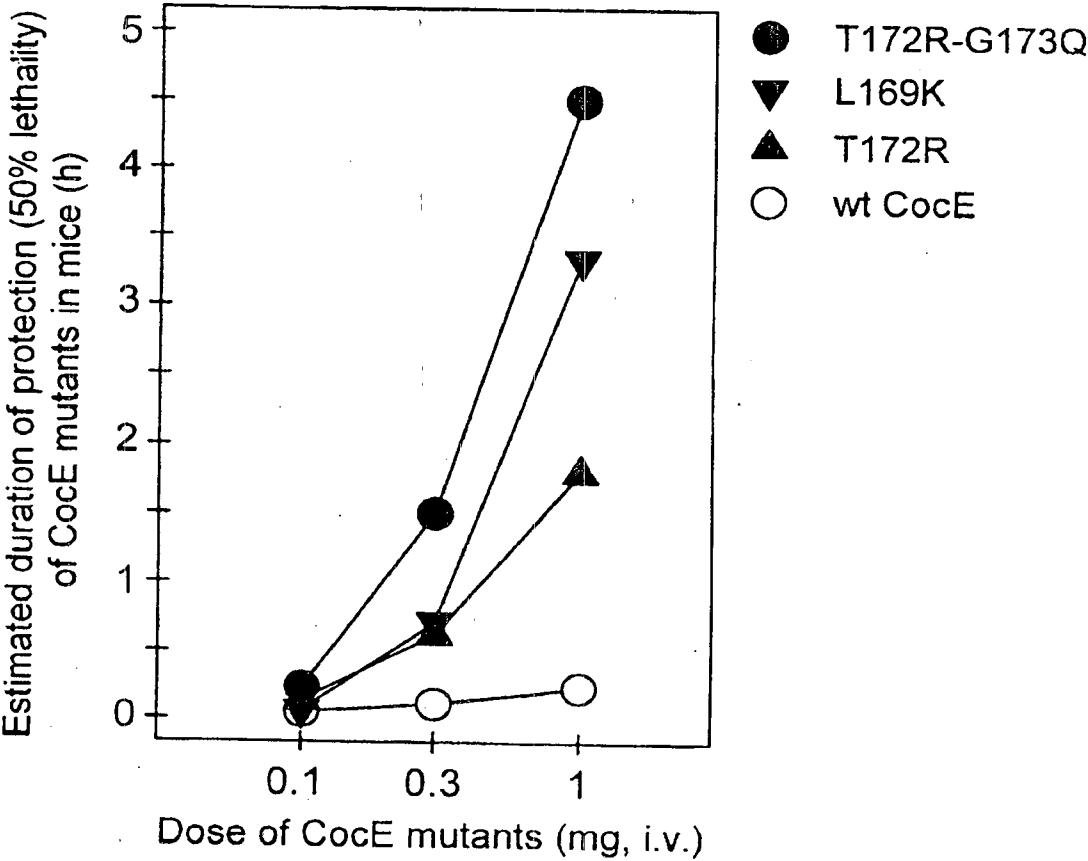


FIG. 26

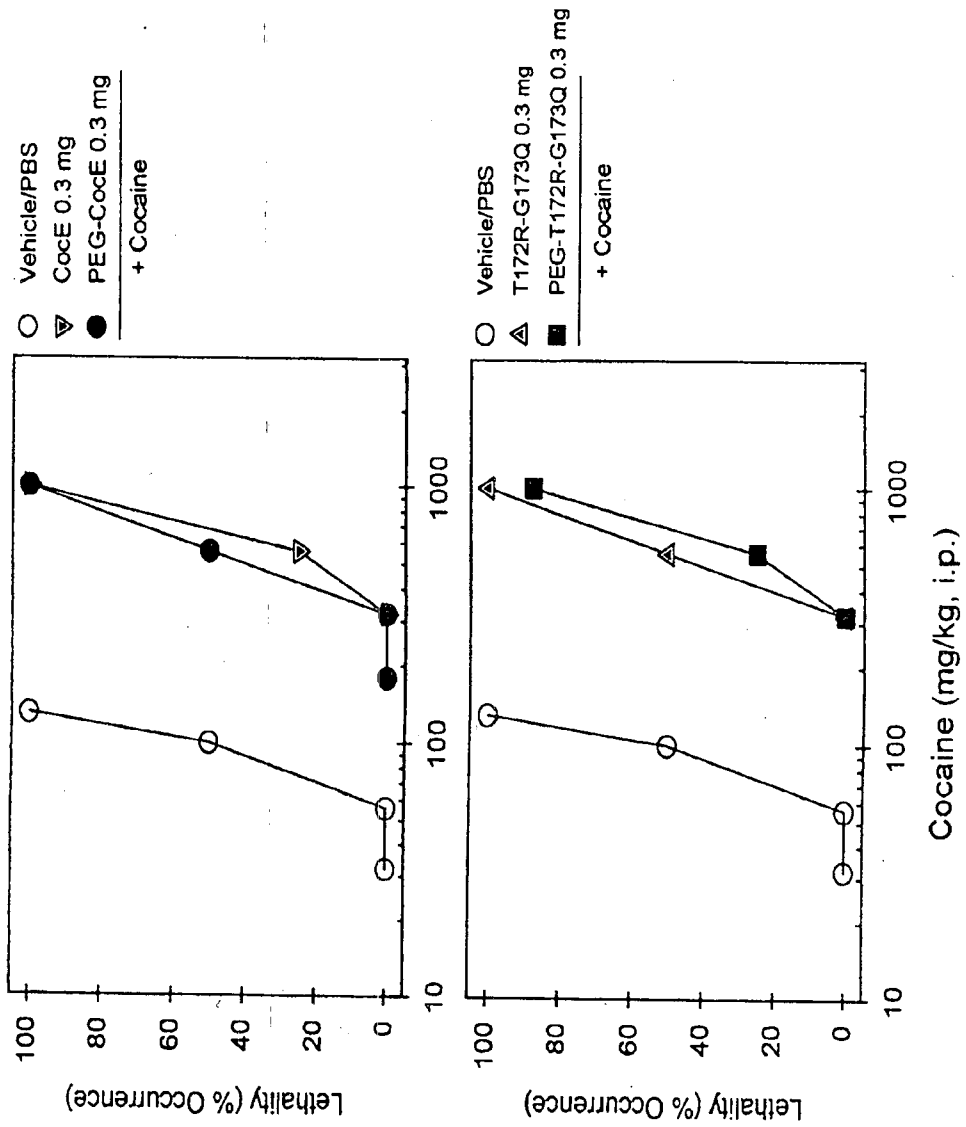


FIG. 27

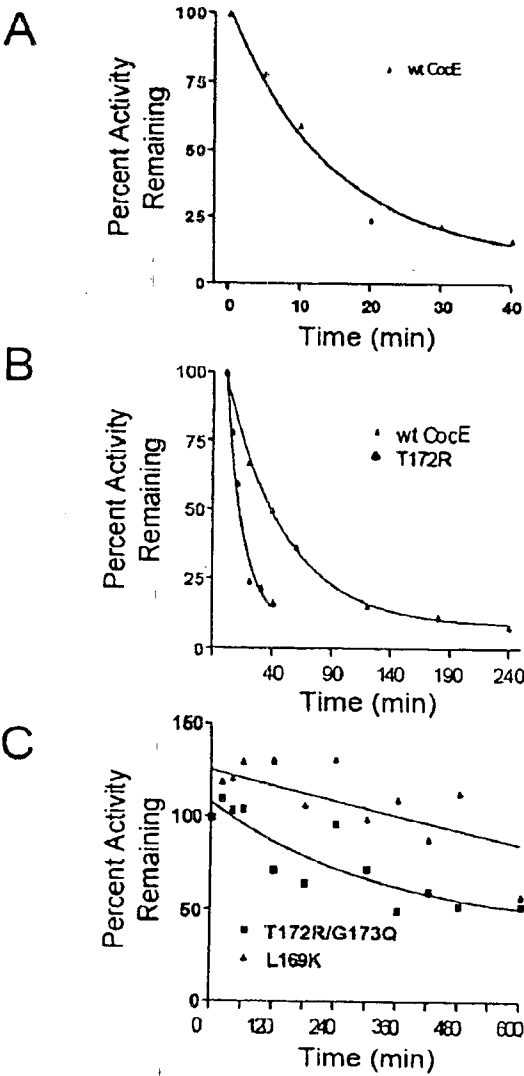


FIG. 28

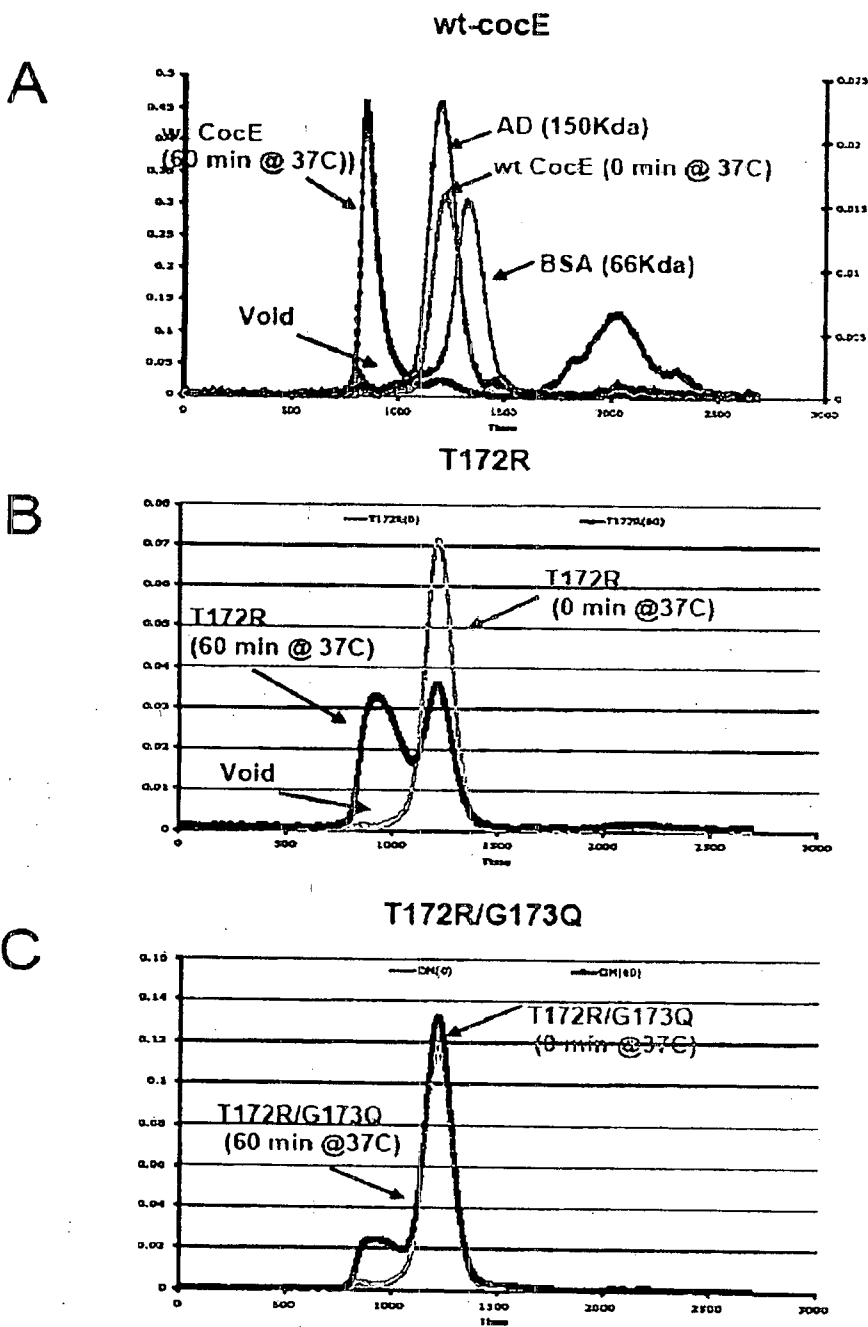


FIG. 29

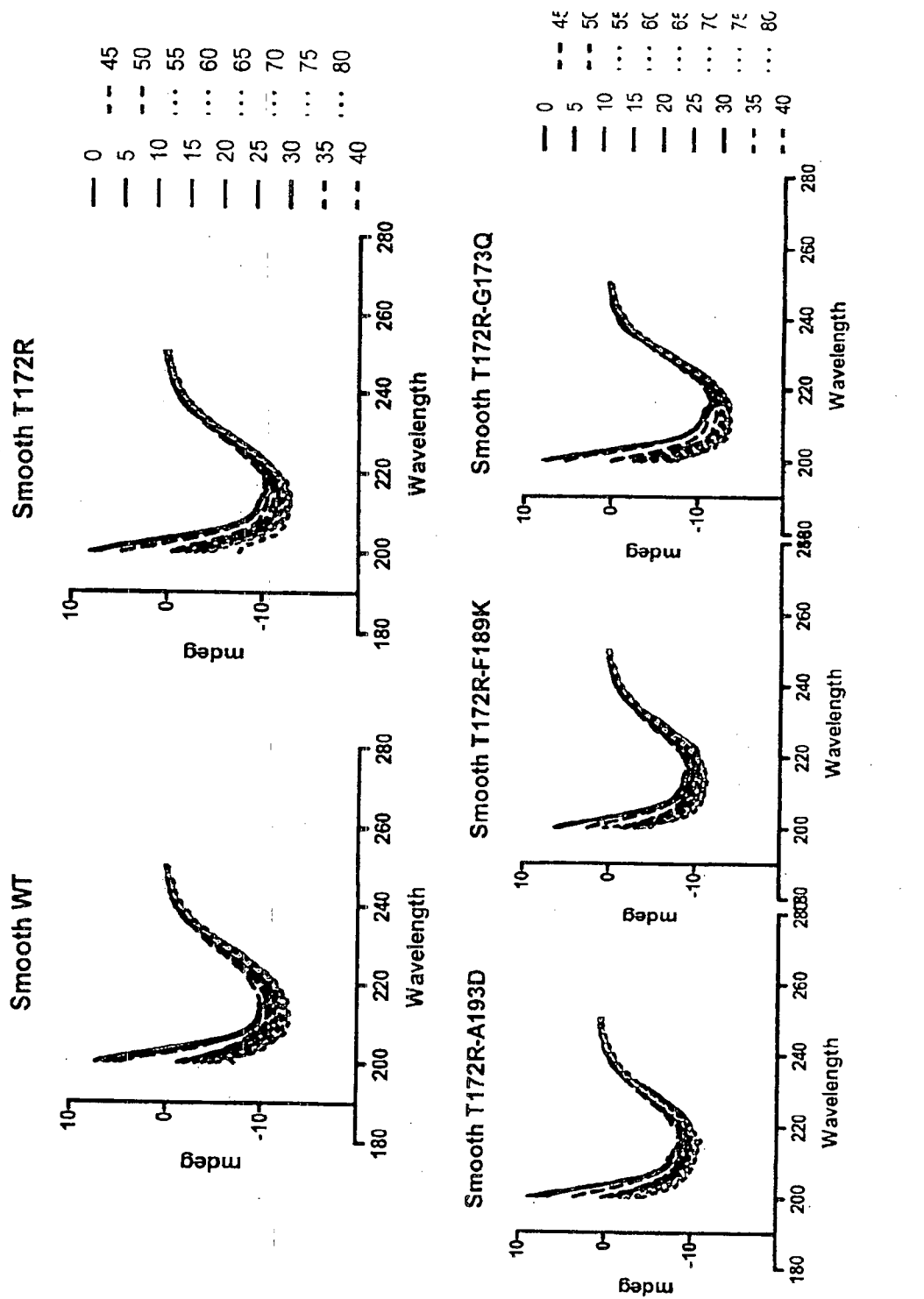


FIG. 30

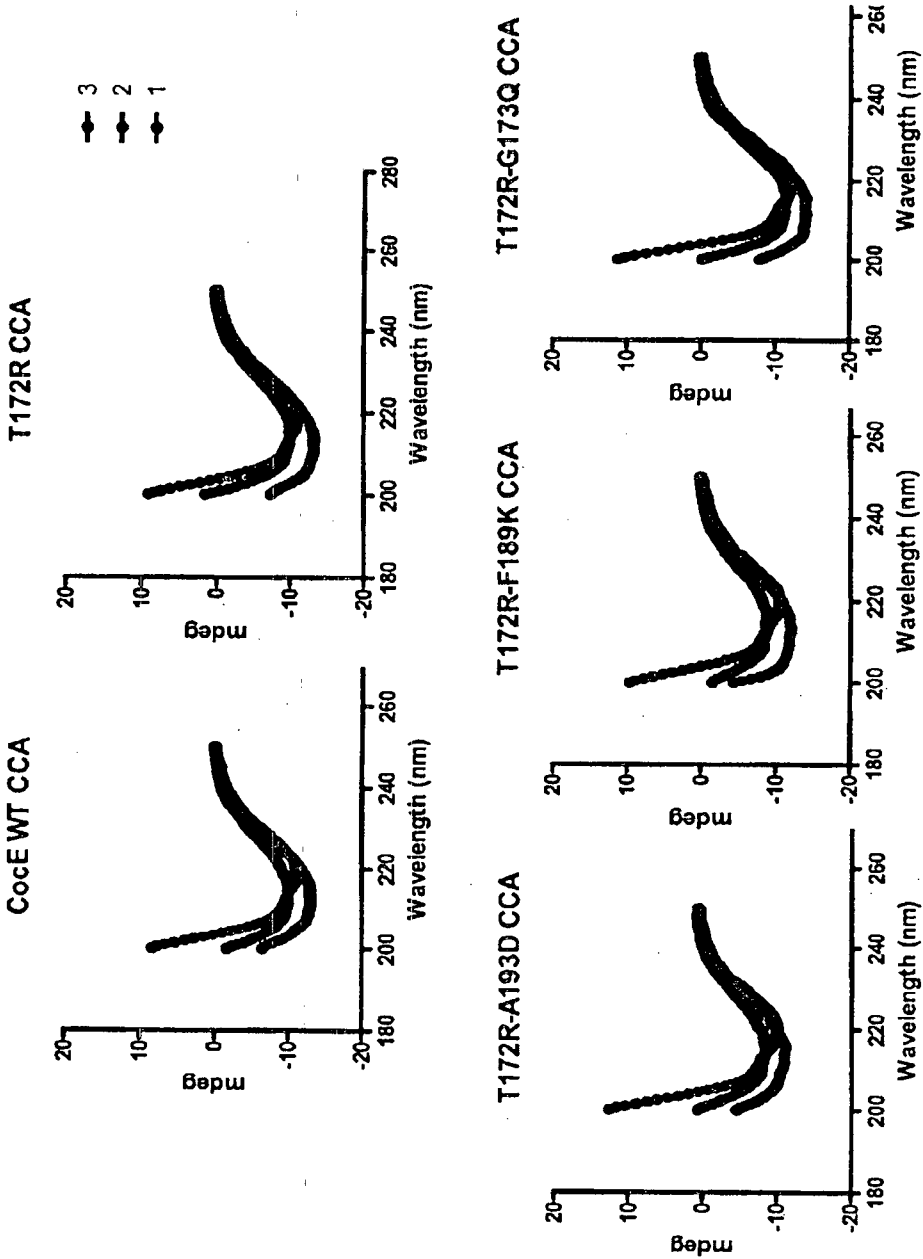


FIG. 31

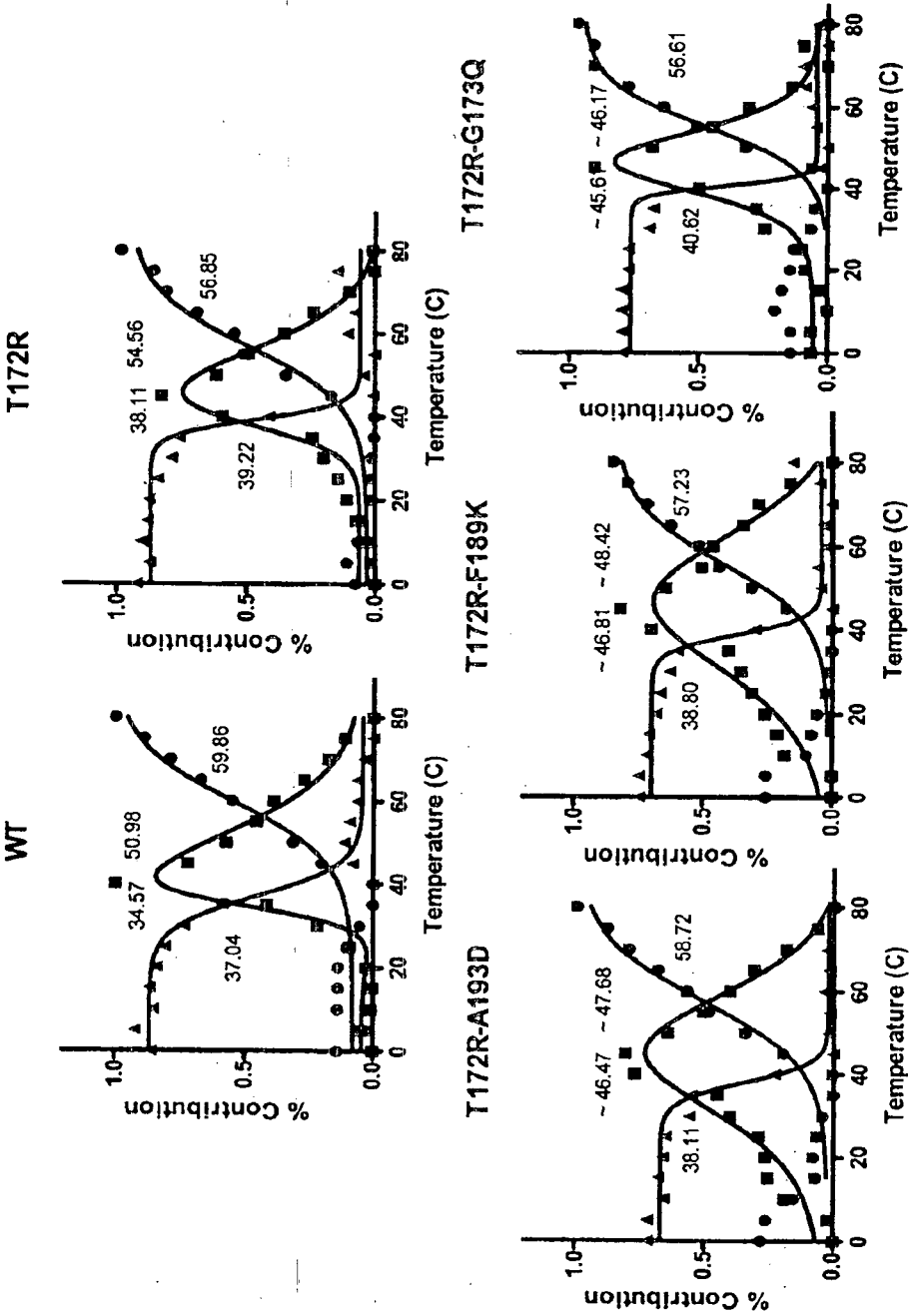
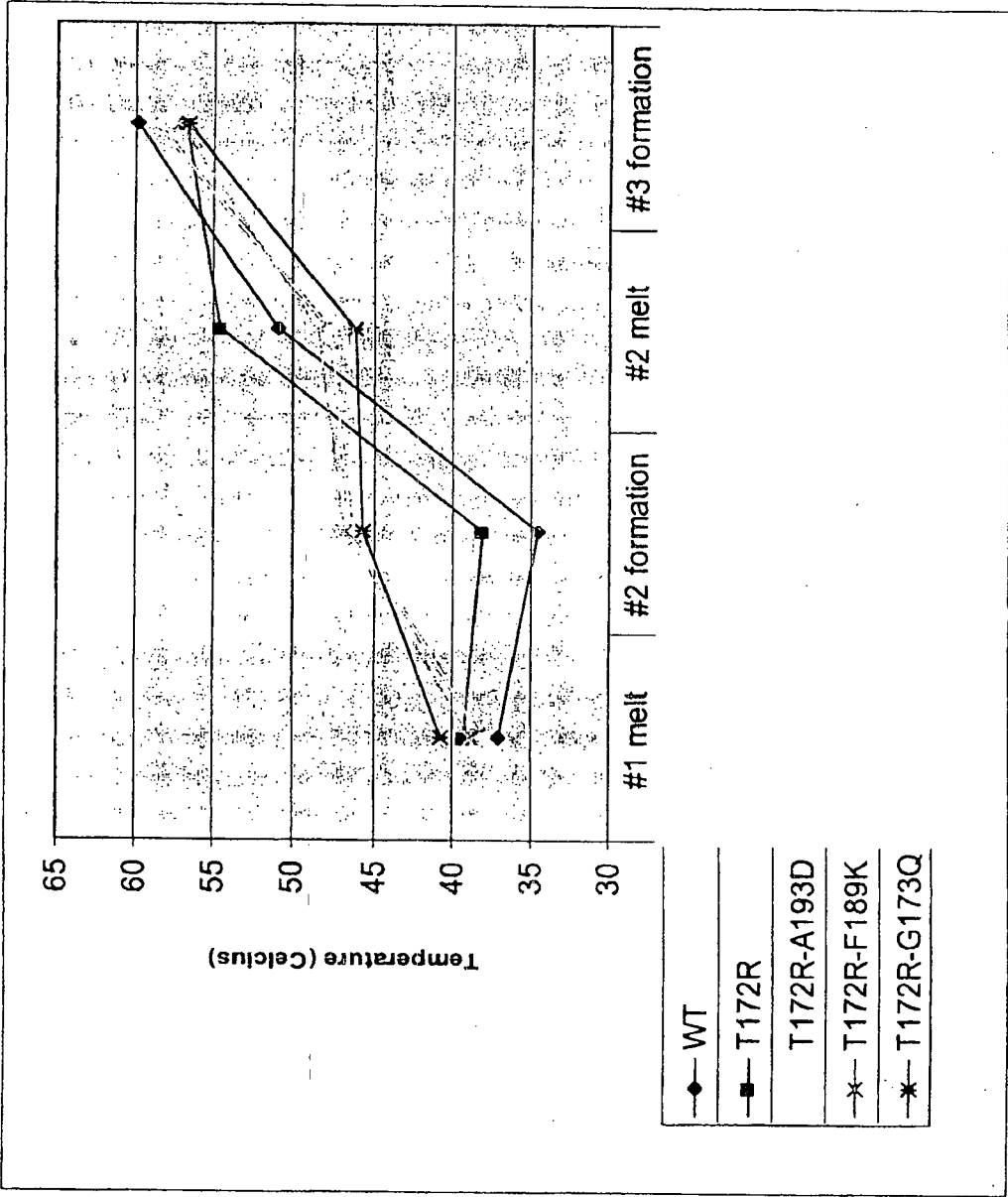


FIG. 32



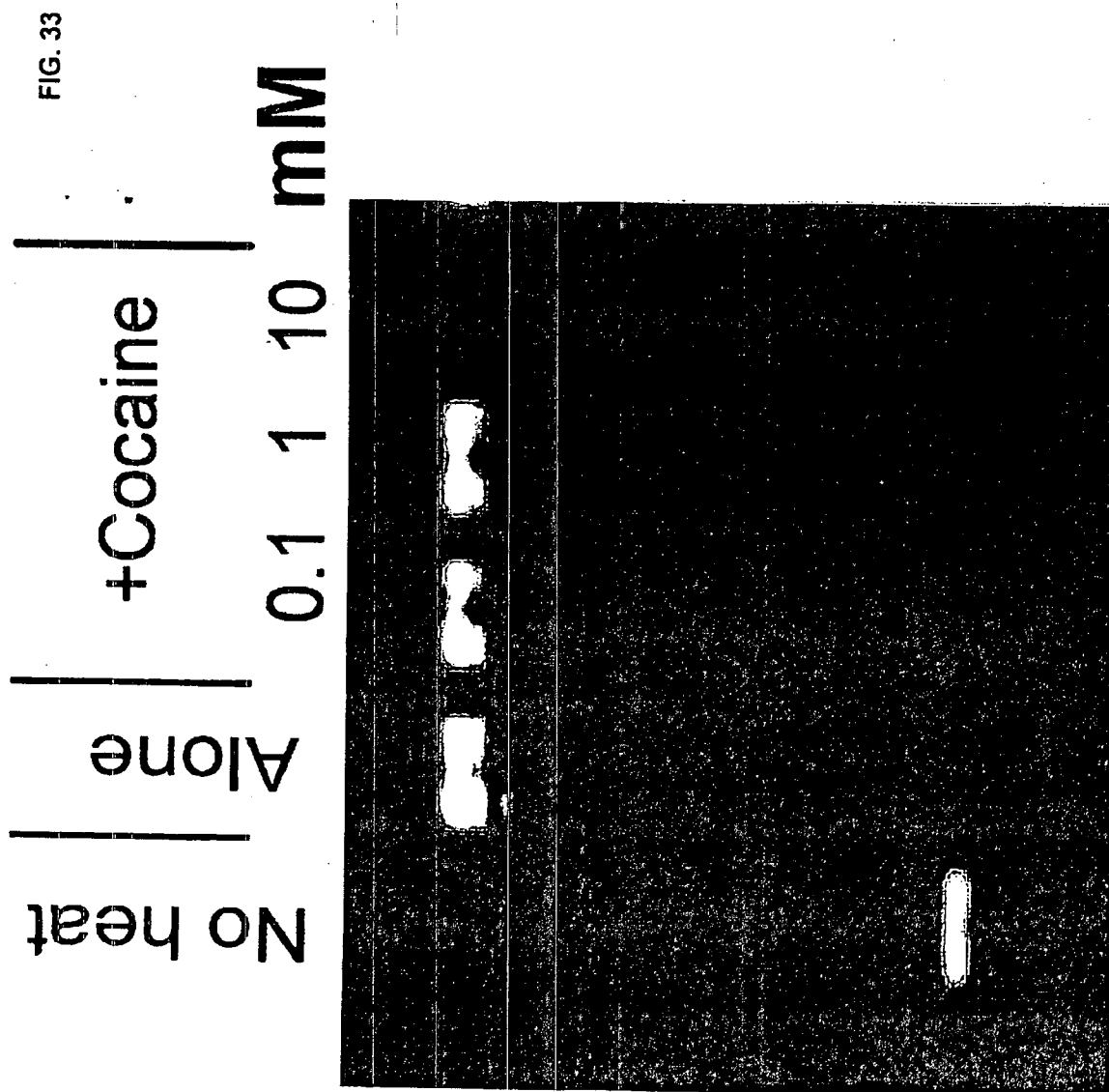


FIG. 34

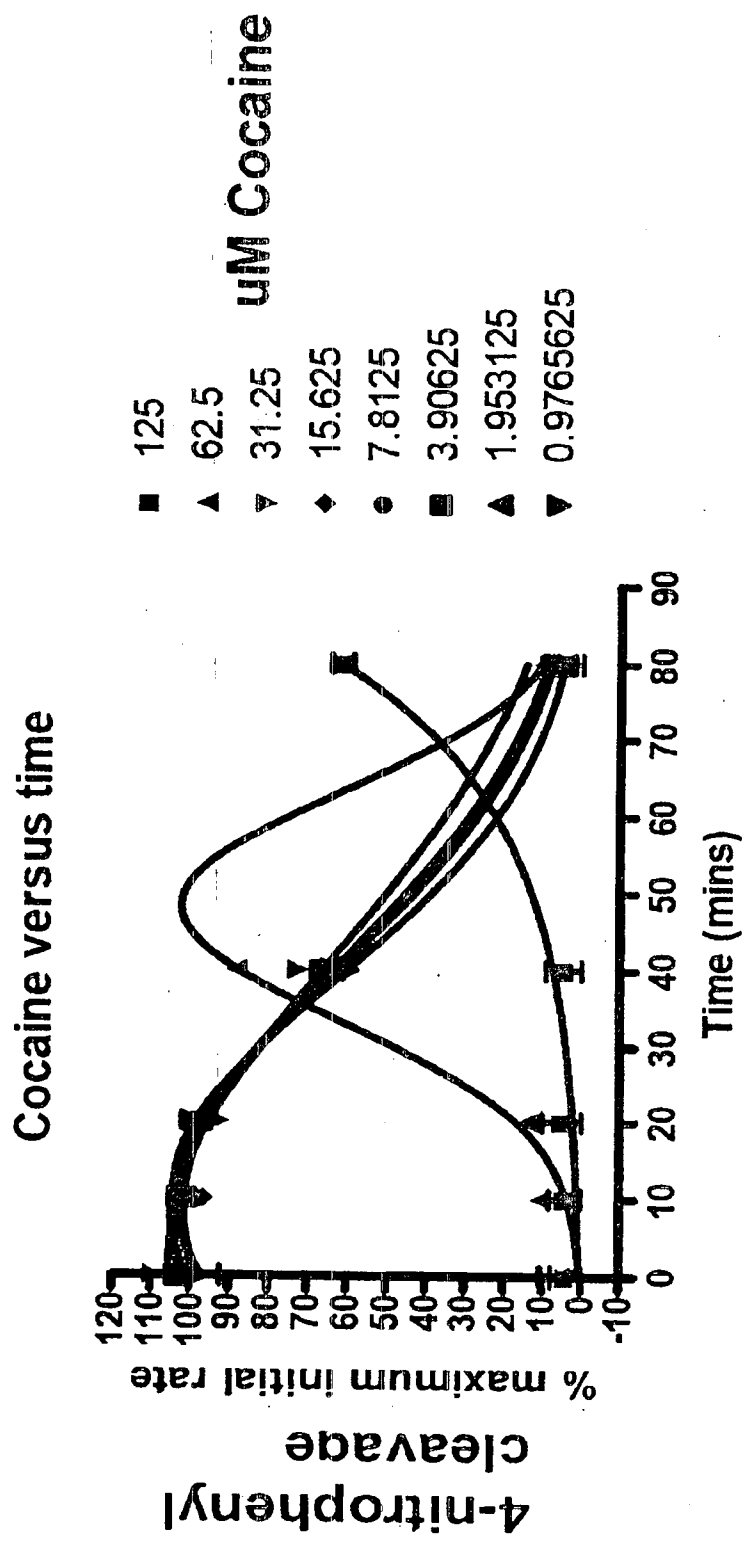
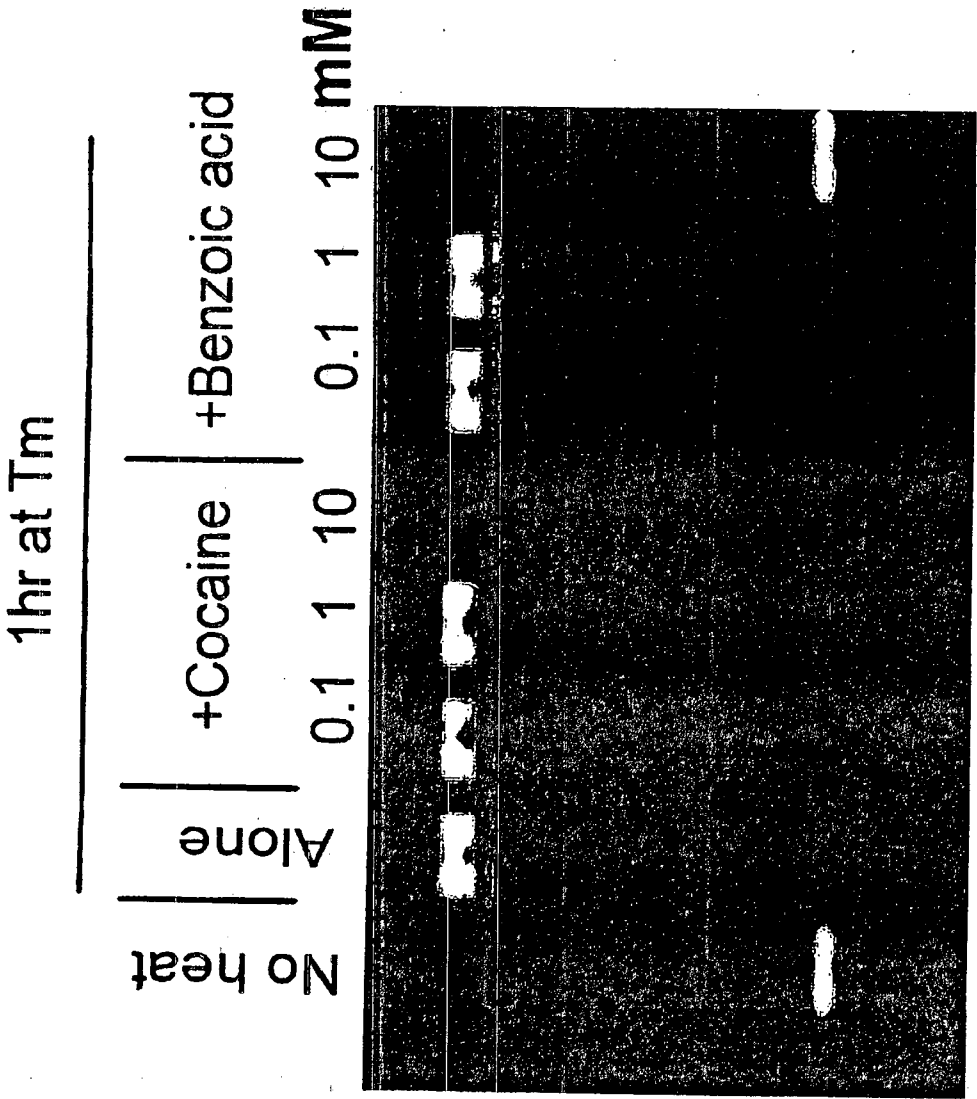


FIG. 35



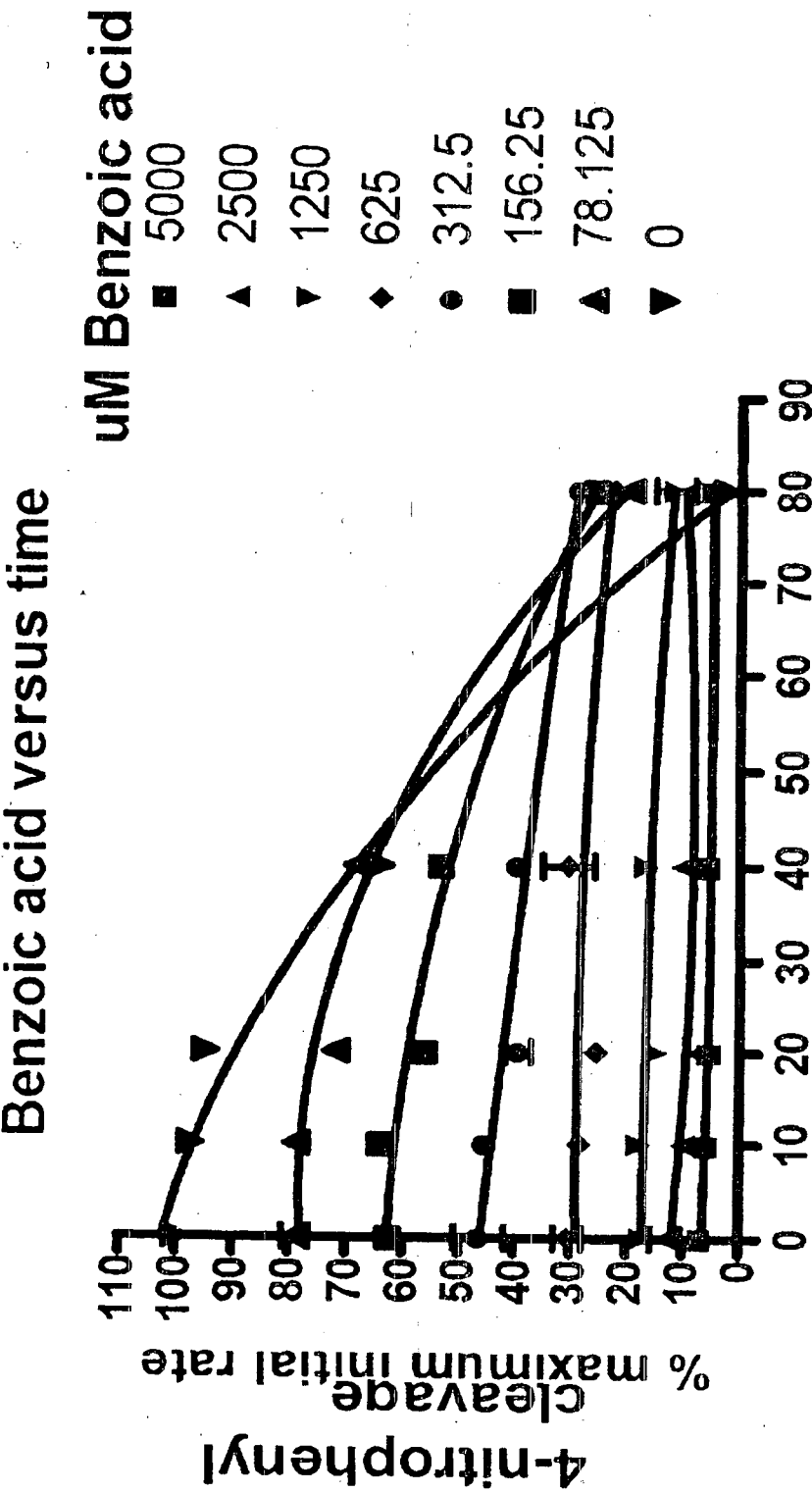


FIG. 36

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(81) Países designados (a menos que se indique lo contrario para cada clase de protección disponible): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Países designados (a menos que se indique lo contrario para cada clase de protección disponible): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publicado:

- con informe internacional de búsqueda
- con parte del listado de secuencias de la descripción publicada separadamente en formato electrónico y disponible, previa solicitud, de la Oficina Internacional

(88) Fecha de publicación del informe de búsqueda internacional:

24 de diciembre de 2008

(54) Título: COMPOSICIONES ANTI-COCAÍNA Y TRATAMIENTO

(57) Extracto: Incorporaciones de la invención divulgada en esta especificación se refieren generalmente a terapéutica anti-cocaína. Específicamente, algunas incorporaciones de la invención se refieren a mutantes de cocaína esterasa (CocE) altamente eficientes, termoestables y duraderos, que pueden proteger contra los efectos tóxicos y reforzadores de la cocaína en sujetos. Se proveen en esta especificación polipéptidos CocE mutantes, que exhiben actividad esterasa termoestable. Se proveen igualmente métodos de tratar afecciones inducidas por la cocaína, en un sujeto necesitado de ello, mediante administración de CocE mutante, así como métodos para la detección selectiva de gran productividad de polipéptidos de esterasa candidatos.

WO 2008/008358 A3

COMPOSICIONES ANTI-COCAÍNA Y TRATAMIENTO

REFERENCIA CRUZADA A SOLICITUDES RELACIONADAS

[0001] Esta solicitud reivindica prioridad a la solicitud provisional de los Estados Unidos No. 60/819,569, presentada el 10 de julio de 2006, la cual se incorpora aquí por referencia en su totalidad.

DECLARACIÓN CON RESPECTO A INVESTIGACIÓN O DESARROLLO PATROCINADOS CON RECURSOS FEDERALES

[0002] Esta invención se realizó en parte con apoyo del Gobierno de los Estados Unidos bajo la Donación NIDA No. DA021416. El Gobierno de los Estados Unidos tiene ciertos derechos en la invención.

INCORPORACIÓN POR REFERENCIA DE MATERIAL PRESENTADO EN UN DISCO COMPACTO

[0003] El Listado de Secuencias, que constituye una parte de la presente divulgación, incluye un formato de lectura por computador y un listado de secuencias escrito, que comprende secuencias de nucleótidos y/o aminoácidos de la presente invención. La información del listado de secuencias registrado en formato de lectura por computador es idéntica al listado de secuencias escrito. El asunto del Listado de Secuencias se incorpora en esta especificación por referencia en su totalidad.

CAMPO DE LA INVENCION

[0004] La invención divulgada en esta especificación se refiere generalmente a terapéutica de la cocaína.

ARTE PREVIO

[0005] El abuso de cocaína es un problema médico y social persistente, que se resiste a la curación mediante farmacoterapia. La cocaína actúa bloqueando la reabsorción de monoaminas, dopamina, norepinefrina y serotonina, prolongando y magnificando los efectos de estos neurotransmisores en el sistema nervioso central (Benowitz NL (1993) *Pharmacol Toxicol* 72, 3-12). La toxicidad de la cocaína se caracteriza tanto por convulsiones como por disfunción cardíaca (por ejemplo, infarto del miocardio, arritmias cardíacas, aumento de la presión sanguínea, derrame cerebral, o aneurisma disecante y elevada demanda de oxígeno del miocardio), debido a efectos sobre los sistemas neurotransmisores y bloqueo de canal de sodio miocárdico (Bauman JL and DiDomenico RJ (2002) *J Cardiovasc Pharmacol Ther* 7, 195-202; Wilson LD and Shelat C (2003) *J Toxicol Clin Toxicol* 41, 777-788; Knuepfer MM (2003) *Pharmacol Ther* 97, 181-222). Debido a la capacidad de la cocaína de cruzar fácilmente la barrera hematoencefálica y sus efectos generalizados sobre los sistemas nerviosos central y periférico, la sobredosis puede resultar en muerte súbita (véase Bauman JL and DiDomenico RJ (2002) *J Cardiovasc Pharmacol Ther* 7, 195-202, para una revisión).

[0006] Aunque el mecanismo de acción de la cocaína es bien entendido, esta información no se ha traducido aún en el desarrollo de un antagonista efectivo de la cocaína que pudiera emplearse en situaciones de abuso y sobredosis. Los efectos rápidos y pleiotrópicos de la cocaína plantean un problema complejo para el tratamiento de la toxicidad aguda de la cocaína (Carroll FI, Howell LL and Kuhar M.J (1999) *J Med Chem* 42, 2721-2736). Los dos tipos de terapias disponibles para el tratamiento del abuso de opioideos, antagonismo (por ejemplo, naltrexona) y reemplazo (por ejemplo, metadona), no registran paralelos en el caso de la cocaína, si bien se están considerando intentos hacia éstos últimos (por ejemplo, J. Grabowski et al. (2004) *Addictive Behaviors* 29, 1439-1464). Un enfoque

es impedir que la cocaína alcance sitios de acción, o reducir su impacto, administrando esterasas endógenas, anticuerpos específicos de la cocaína, o un anticuerpo catalítico.

[0007] La cocaína de origen natural se hidroliza con el éster de benzoilo por la butirilcolinesterasa sérica (BChE) en ecgonina metil éster no tóxico y ácido benzoico. En el hígado, la carboxilesterasa hCE-2 hidroliza el metil éster para producir benzoilecgonina y metanol (véase a este respecto, Figura 1). La semi-vida de eliminación de la cocaína en la sangre varía de 0.5 1.5 horas (T. Inaba (1989) Canadian Journal of Physiology & Pharmacology 67, 1154-1157). Ha habido unos cuantos intentos de emplear BChE de origen natural o BChE genéticamente modificada para aumentar la degradación de la cocaína (véase a este respecto, Carmona et al. (2000) Drug Metabolism & Disposition 28, 367-371; Xie et al. (1999) Molecular Pharmacology 55, 83-91; Sun et al. (2002a) Molecular Pharmacology; Sun et al. (2002b) Pharmacology & Experimental Therapeutics 302, 710-716; Duysen et al. (2002) Journal of Pharmacology & Experimental Therapeutics 302, 751-758; Gao Y and Brimijoin S (2004) Journal of Pharmacology & Experimental Therapeutics 310, 1046-1052; Gao et al. (2005) Molecular Pharmacology 67, 204-211). Otros investigadores han utilizado un anticuerpo monoclonal, Mab 15A10, como un anticuerpo catalítico contra la cocaína (véase por ejemplo, Landry et al, 1993; Mets et al., 1998; Baird et al., 2000; Larsen et al., 2004), mientras que otros están explorando la posibilidad del uso de vacunas (véase por ejemplo, Kosten et al. (2002) Vaccine 20, 1196-1204).

Tabla 1: Cinética de varias enzimas que hidrolizan cocaína contra (-) cocaína.

Enzima	Kcat(min-1)	Km (µM)	Eficiencia (kcat/Km)	Referencia
BChE	4.1	4.5	9.1 x 10 ⁶	Sun et al., 2002a
Ala328W/Y332A	154	18	8.5 x 10 ⁶	Sun et al., 2002a

Mab15A10	2.2	220	1×10^4	Larsen et al., 2004
AME 359	620	20	3.1×10^7	Gao et al., 2005
CocE	468	0.64	7.2×10^8	Turner et al., 2002

[0008] Una bacteria, *Rhodococcus* sp. MB 1, nativa del suelo que rodea la planta de coca, ha desarrollado la capacidad de utilizar cocaína como su sola fuente de carbono y nitrógeno. La bacteria expresa una cocaína esterasa (CocE) que actúa en la misma forma que la BChE para hidrolizar el éster de benzoilo de la cocaína, produciendo ecgonina metil éster y ácido benzoico (véase a este respecto, Figura 1) (Bresler et al. (2000) *Appl Environ Microbiol* 66, 904-908; Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Struct Biol* 9, 17-21). El gen para CocE se ha aislado y clonado (Bresler et al. (2000) *Appl Environ Microbiol* 66, 904-908), y la estructura cristalina de CocE se ha determinado (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Struct Biol* 9, 17-21). La estructura de CocE (véase a este respecto, Figura 2) revela una serina esterasa clásica plegada además de los otros dos dominios que se combinan para formar un receptáculo de unión de cocaína ("*a cocaine binding pocket*"). La alteración de cualquiera de los tres aminoácidos (Asp, His, o Ser) en la tríada catalítica en el sitio activo (para una revisión, véase por ejemplo Dodson G and Wlodawer A (1998) *Trends Biochem Sci* 23, 347-352) inactiva la actividad esterasa contra la cocaína. Más aún, la mutación de residuos que hacen contacto con la fracción benzoato de la cocaína (por ejemplo, Tyr44) desestabiliza igualmente la hidrólisis de la cocaína, presumiblemente deteriorando la estabilización del oxianión en el sitio de transición (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Structural Biology* 9, 17-21). La enzima purificada (MW ~65kDa) cataliza la cocaína muy eficientemente con una cinética de Michaelis-Menten $k_{cat} = 7.2 \text{ s}^{-1}$ y $K_m = 640 \text{ nM}$ (Turner et al. (2002) *Biochemistry* 41, 12297-12307; Larsen et al. (2002) *Nature Structural Biology* 9, 17-21), casi tres órdenes de magnitud mayor que las esterases endógenas y, muy probablemente,

actuaría suficientemente rápido para desintoxicar humanos que se hayan tomado una dosis excesiva de cocaína (Landry et al. (1993) Science 259, 1899-1901; Mets et al. (1998) National Academy of Sciences of the United States of America 95, 10176-10181). Adicionalmente, la esterasa metaboliza asimismo cocaetilo, un potente metabolito de la cocaína y alcohol, casi tan eficientemente como metaboliza la cocaína ($k_{cat} = 94 \text{ s}^{-1}$ y $K_m = 1600 \text{ nM}$) (Turner et al. (2002) Biochemistry 41, 12297-12307; Larsen et al. (2002) Nature Structural Biology 9, 17-21).

[0009] En consecuencia, sería deseable proveer una CocE estable para terapéutica anti-cocaína.

SUMARIO DE LA INVENCION

[0010] Por consiguiente, los presentes inventores han tenido éxito en descubrir mutantes de cocaína esterasa altamente eficientes, termoestables y de larga duración, que pueden proteger contra los efectos tóxicos y reforzantes de la cocaína en sujetos.

[0011] Un aspecto de la invención provee polipéptidos de cocaína esterasa (CocE) mutantes aislados de la secuencia de aminoácidos de CocE tipo silvestre (por ejemplo, SEQ ID NO: 1), pero con por lo menos un residuo de aminoácido sustituido. Los polipéptidos CocE mutantes tienen actividad esterasa con termoestabilidad creciente a 37°C, comparados con la CocE tipo silvestre.

[0012] Varias incorporaciones incluyen polipéptidos CocE mutantes con por lo menos dos, tres, cuatro, cinco, o más sustituciones de la secuencia de aminoácidos de la CocE tipo silvestre. Ejemplos de polipéptidos CocE mutantes en el ámbito de la invención incluyen aquellos con una secuencia de aminoácidos de SEQ ID NO: 3 (L163V); SEQ ID NO: 7 (V225I); SEQ ID NO: 8 (I218L); SEQ ID NO: 9 (A310D); SEQ ID

NO: 10 (A149S); SEQ ID NO: 11 (S159A); SEQ ID NO: 12 (S265A); SEQ ID NO: 13 (S56G); SEQ ID NO: 14 (W220A); SEQ ID NO: 16 (S140A); SEQ ID NO: 17 (F189L); SEQ ID NO: 18 (A193D); SEQ ID NO: 19 (T254R); SEQ ID NO: 20 (N42V); SEQ ID NO: 21 (V262L); SEQ ID NO: 22 (L508G); SEQ ID NO: 23 (Y152H); SEQ ID NO: 24 (V160A); SEQ ID NO: 25 (T172R); SEQ ID NO: 26 (Y532F); SEQ ID NO: 27 (T74S); SEQ ID NO: 28 (W285T); SEQ ID NO: 29 (L146P); SEQ ID NO: 30 (D533S); SEQ ID NO: 31 (A194R); SEQ ID NO: 32 (G173Q); SEQ ID NO: 33 (C477T); SEQ ID NO: 34 (K531A); SEQ ID NO: 35 (R41I); SEQ ID NO: 36 (L119A); SEQ ID NO: 37 (K46A); SEQ ID NO: 38 (F84Y), T172R-G173Q (SEQ ID NO: 39); L169K (SEQ ID NO: 40); F189A (SEQ ID NO: 41), N197K (SEQ ID NO: 42), R182K (SEQ ID NO: 43), F189K (SEQ ID NO: 44), V190K (SEQ ID NO: 45), Q191K (SEQ ID NO: 46), y A194K (SEQ ID NO: 47), o un fragmento funcional) de las mismas. Polipéptidos ejemplares adicionales de CocE mutante incluyen F189A/T172R, T172R/A193D, T172R/G173Q-I175-G-G-A186, T172R/G173Q-T176-G-G-D185, y similares. Dadas las convenciones de denominación y las secuencias polipeptídicas divulgadas en esta especificación, una persona experta en el arte podría determinar las secuencias polipeptídicas para los polipéptidos CocE mutantes ya mencionados.

[0013] Otro aspecto de la invención provee composiciones farmacéuticas que incluyen, entre sus componentes un polipéptido CocE mutante en el ámbito de la invención y una sustancia portadora o excipiente farmacéuticamente aceptable.

[0014] Otro aspecto de la invención provee ácidos nucleicos aislados que codifican el polipéptido CocE mutante descrito en esta especificación. En varias incorporaciones, los ácidos nucleicos incluyen aquellos con secuencias que se hibridan al ácido nucleico que codifica CocE tipo silvestre (por ejemplo, SEQ ID NO: 2), o al complemento del mismo, bajo condiciones de alta restricción. Tal ácido nucleico aislado

codifica un polipéptido CocE mutante que tiene actividad esterasa con termoestabilidad incrementada a 37°C, comparada con la CocE tipo silvestre. Varias incorporaciones de la secuencia de ácido nucleico aislado tiene por lo menos alrededor del 85% de identidad de secuencia con la secuencia de ácido nucleico de la CocE tipo silvestre (por ejemplo, SEQ ID NO: 2). Por ejemplo, la secuencia de ácido nucleico aislado tiene por lo menos alrededor de 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, y 99% de identidad de secuencia de la CocE tipo silvestre (por ejemplo, SEQ ID NO: 2).

[0015] En varias incorporaciones, polipéptidos CocE mutantes (o polipéptidos codificados por ácidos nucleicos en el ámbito de la invención) tienen temperaturas de fusión mayores, comparados con la CocE tipo silvestre. En varias incorporaciones, polipéptidos CocE mutantes (o polipéptidos codificados por ácidos nucleicos en el ámbito de la invención) aumentan la termoestabilidad de los polipéptidos CocE mutantes sobre la CocE tipo silvestre en por lo menos alrededor de 2.0 kcal/mol. Por ejemplo, la termoestabilidad incrementada puede ser por lo menos alrededor de 2.1 kcal/mol, por lo menos alrededor de 2.2 kcal/mol, por lo menos alrededor de 2.3 kcal/mol, por lo menos alrededor de 2.4 kcal/mol, por lo menos alrededor de 2.5 kcal/mol, por lo menos alrededor de 2.6 kcal/mol, por lo menos alrededor de 2.7 kcal/mol, por lo menos alrededor de 2.8 kcal/mol, por lo menos alrededor de 2.9 kcal/mol, por lo menos alrededor de 3.0 kcal/mol, por lo menos alrededor de 3.1 kcal/mol, por lo menos alrededor de 3.2 kcal/mol, por lo menos alrededor de 3.3 kcal/mol, por lo menos alrededor de 3.4 kcal/mol, por lo menos alrededor de 3.5 kcal/mol, por lo menos alrededor de 3.6 kcal/mol, por lo menos alrededor de 3.7 kcal/mol, por lo menos alrededor de 3.8 kcal/mol, por lo menos alrededor de 3.9 kcal/mol, por lo menos alrededor de 4.0 kcal/mol, por lo menos alrededor de 4.1 kcal/mol, por lo menos alrededor de 4.2 kcal/mol, por lo menos alrededor de 4.3 kcal/mol, por lo menos alrededor de 4.4 kcal/mol, o por lo menos alrededor de 4.5 kcal/mol.

[0016] En varias incorporaciones, polipéptidos CocE mutantes (o polipéptidos codificados por ácidos nucleicos en el ámbito de la invención) tienen una inmunogenicidad reducida, comparados con la CocE tipo silvestre.

[0017] En algunas incorporaciones, los polipéptidos CocE mutantes termoestables tienen menos actividad esterasa que la CocE tipo silvestre. Por ejemplo, mutantes de CocE termoestable pueden tener alrededor de 10%, alrededor de 20%, alrededor de 30%, alrededor de 40%, alrededor de 50%, alrededor de 60%, alrededor de 65%, alrededor de 70%, alrededor de 75%, alrededor de 80%, alrededor de 85%, alrededor de 90%, alrededor de 95%, o alrededor de 99% de la actividad esterasa de la CocE tipo silvestre. En otras incorporaciones, los polipéptidos CocE mutantes tienen la misma eficiencia catalítica, o mayor, de los polipéptidos CocE tipo silvestre. Por ejemplo, mutantes de CocE termoestables pueden tener alrededor de 100%, alrededor de 110%, alrededor de 120%, alrededor de 130%, alrededor de 140%, alrededor de 150%, o más, de la actividad esterasa de CocE tipo silvestre.

[0018] En varias incorporaciones, los polipéptidos CocE mutantes se pegilan. En varias incorporaciones, el polipéptido CocE mutante se encapsula en un glóbulo rojo. Por ejemplo, un polipéptido(s) CocE mutante pegilado (o una composición farmacéutica que contenga un polipéptido(s) CocE mutante pegilado) puede encapsularse en un glóbulo rojo (RBC - *red blood cell*).

[0019] En algunas incorporaciones, los polipéptidos CocE mutantes se estabilizan por un sustrato, producto, y/o inhibidor.

[0020] Otro aspecto de la invención provee los métodos de tratar una afección inducida por la cocaína. En tales métodos, una cantidad

terapéuticamente efectiva de un polipéptido(s) CocE mutante (o una composición farmacéutica que contenga un polipéptido(s) CocE mutante) en el ámbito de la invención se administra a un sujeto necesitado de ello. En varias incorporaciones, la afección inducida por la cocaína incluye sobredosis de cocaína, toxicidad de la cocaína, adicción a la cocaína, dependencia de la cocaína, y/o alguna combinación de las mismas.

[0021] Aún otro aspecto de la invención posibilita un método de detección selectiva de gran productividad ("*a high throughput screening*") para la identificación de polipéptidos CocE mutantes termoestables. En tal método de detección selectiva, una célula se transforma en forma estable con un ácido nucleico que codifica un polipéptido CocE mutante candidato. El polipéptido CocE mutante se expresa en la célula. El polipéptido CocE mutante expresado se aísla o despliega. La actividad esterasa del polipéptido CocE mutante aislado se mide a una o más temperaturas para determinar la termoestabilidad de los polipéptidos CocE mutantes aislados. Tal temperatura(s) puede ser desde alrededor de 30°C a alrededor de 50°C. Se selecciona(n) el polipéptido(s) CocE mutantes con actividad esterasa a la temperatura(s) predeterminada.

[0022] En algunas incorporaciones del método de detección selectiva, la medición de la actividad esterasa de los polipéptidos mutantes aislados puede llevarse a cabo contactando el polipéptido CocE mutante aislado con (i) cocaína y un indicador de pH o (ii) un tio-derivado de cocaína y un indicador de tiol. A continuación se detecta cualquier cambio en el indicador de pH o el indicador de tiol. Tal cambio se correlaciona con la formación de ácido benzoico de la hidrólisis de cocaína o un derivado de cocaína por el polipéptido CocE mutante.

[0023] Tales incorporaciones del método de detección selectiva incluyen además conducir varios ciclos del procedimiento de detección selectiva a temperaturas incrementadas para medir la actividad esterasa. Por

ejemplo, el primer ciclo puede emplear una temperatura para medir la actividad esterasa de alrededor de 30°C, mientras que un ciclo subsiguiente puede emplear una temperatura para medir la actividad esterasa de alrededor de 45°C.

[0024] En algunas incorporaciones del método de detección selectiva, la expresión de un polipéptido CocE mutante ocurre a una temperatura a la cual la CocE tipo silvestre conserva sustancialmente la actividad catalítica. En otras incorporaciones del método de detección selectiva, la expresión del polipéptido CocE mutante ocurre a una temperatura a la cual el polipéptido CocE tipo silvestre se divide sustancialmente en cuerpos de inclusión. Por ejemplo, la temperatura de expresión puede ser por lo menos alrededor de 35°C, por lo menos alrededor de 36°C, por lo menos alrededor de 37°C, por lo menos alrededor de 38°C, por lo menos alrededor de 39°C, o por lo menos alrededor de 40°C.

[0025] Otros objetos y características serán en parte evidente y en parte se destacarán en más adelante.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

[0026] Los expertos en el arte entenderán que los dibujos, descritos a continuación, son para fines ilustrativos únicamente. Los dibujos no tienen como finalidad limitar el ámbito de las presentes enseñanzas en modo alguno.

[0027] Figura 1 es un diagrama que ilustra el metabolismo de la cocaína por varias reacciones enzimáticas.

[0028] Figura 2 es un diagrama de cintas que ilustra la estructura de CocE. Se indican el dominio 1 (DOM1), el dominio 2

(DOM2) y el dominio 3 (DOM 3), junto con el sitio activo localizado en la intersección de los tres dominios. En el sitio activo se muestra una molécula de ácido benzoico.

[0029] Figura 3 es un gráfico de curvas que describe el perfil de elución para CocE por FPLC (Q-Sepharose), en el que se muestra la absorbencia de UV y la concentración de cloruro de sodio con el transcurso del tiempo. Para una mayor información sobre la metodología, véase Ejemplo 1.

[0030] Figura 4 es un gráfico de curvas y diagrama de dispersión que muestra la degradación *in vitro* de cocaína en la presencia de CocE en plasma humano. Para una mayor información sobre la metodología, véase Ejemplo 2.

[0031] Figura 5 es un gráfico de la cinética enzimática Michaelis-Menten para la CocE tipo silvestre, la CocE mutante T172R (SEQ ID NO: 26), y la CocE mutante S159A (SEQ ID NO: 11) para reacciones llevadas a cabo a 30°C y 37°C. Para una mayor información sobre la metodología, véase Ejemplo 4.

[0032] Figura 6 es un conjunto de fotografías de la coloración total de proteína luego de la separación por PAGE (*polyacrylamide-gel electrophoresis*) de la CocE tipo silvestre y mutante T172R empleando condiciones desnaturalizantes (SDS+βMe) o no desnaturalizantes (Native) luego de la incubación a 37°C durante varios puntos de tiempo. Para mayor información, véase Ejemplo 4.

[0033] Figura 7 es un gráfico de curvas que describe el dicroísmo circular determinado con temperaturas de fusión de CocE tipo silvestre y T172R. El espectro total se muestra en A, el espectro cercano a UV se muestra en B, y la temperatura de fusión estimada para cada mutante

se muestra en C. Para mayor información, véase Ejemplo 4.

[0034] Figura 8 contiene (A) un dibujo de la hidrólisis del derivado benzoiltioéster de cocaína (tio-1) y la des-carbometoxi cocaína (tio-2) seguida por reacción de los tiols liberados (R-SH) con reactivo de Ellman; y (B) un gráfico de curvas que muestra la reacción colorimétrica de células BL21 de control y células BL21 que contienen CocE incubada con el derivado benzoiltioéster (tio-1) y reactivo de Ellman. Para mayor información, véase Ejemplo 15.

[0035] Figura 9 es un gráfico de curvas y diagrama de dispersión que muestra el efecto de 1.0 mg CocE (círculos cerrados) o PBS (vehículo, círculos abiertos) sobre la mortalidad inducida por cocaína cuando se administra un minuto después de dosis crecientes de cocaína (n = 6-7). Los datos presentados se expresan como porcentaje y su error estándar. Para una mayor información sobre la metodología, véase Ejemplo 5.

[0036] Figura 10 es un gráfico de barras que muestra el efecto de dosis crecientes de CocE (CE) o BChE humana (BChE), o PBS, sobre la mortalidad inducida por la cocaína cuando se administran un minuto antes 180 mg/kg cocaína (n = 6-7). Los datos presentados se expresan como porcentaje y su error estándar. Para una mayor información sobre la metodología, véase Ejemplo 5.

[0037] Figura 11 es un gráfico de barras que muestra el efecto de los mutantes de CocE S117A y Y44F, o CocE tratada con PMSF sobre las convulsiones inducidas por cocaína y la mortalidad cuando se administran 1 minuto antes 180 mg/kg de cocaína (n = 5-6). Para una mayor información sobre la metodología, véase Ejemplo 5.

[0038] Figura 12 es un gráfico de barras que muestra el efecto

de 1.0 mg CocE o PBS administrado un minuto después de los 560 mg/kg WIN-35065-2, la LD100 determinada del compuesto ($n = 6-8$). Para una mayor información sobre la metodología, véase Ejemplo 5.

[0039] Figura 13 es un gráfico de curvas y un diagrama de dispersión que muestran la inactivación en función del tiempo de CocE (125 ng/ml) *in vitro*. El decrecimiento exponencial $t_{1/2}$ se calculó como 13.2. Para una mayor información sobre la metodología, véase Ejemplo 6.

[0040] Figura 14 es un gráfico de barras que muestra los efectos protectores en función del tiempo de 1 mg CocE cuando se administra varias veces antes y después de 100 mg/kg de cocaína ($n = 6-8$). Para una mayor información sobre la metodología, véase Ejemplo 6.

[0041] Figura 15 es un gráfico de barras que muestra las concentraciones de cocaína en plasma humano tratado con 0.8 μ M de cocaína esteras o vehículo de esterasa en el minuto 0 y en el minuto 1 luego de la administración de esterasa. Para una mayor información sobre la metodología, véase Ejemplo 6.

[0042] Figura 16 es una serie de gráficos de curvas y diagramas de dispersión que muestran el efecto de 0.32 mg CocE (círculos cerrados), 0.32 mg de CocE mutante T172R (triángulos cerrados), o PBS (vehículo, círculos abiertos) sobre la mortalidad inducida por cocaína cuando se administra en el minuto 1 luego de dosis crecientes de cocaína (panel izquierdo) y minutos antes de la muerte luego de la administración de de cocaína (panel derecho). Para una mayor información sobre la metodología, véase Ejemplo 7.

[0043] Figura 17 es un gráfico de curvas y un diagrama de dispersión que muestran el efecto de 0.32 mg/kg de CocE (círculos cerrados) o CocE mutante T172R (triángulos cerrados) sobre la mortalidad

inducida por cocaína cuando se administra en los minutos 1, 10, 30 y 60 antes de la administración de 320 mg/kg de cocaína (n=6). Para una mayor información sobre la metodología, véase Ejemplo 7.

[0044] Figura 18 es una serie de gráficos de curvas y diagramas de dispersión que muestran el efecto de 1 mg de hBChE (triángulo cerrado), 0.32 mg de CocE (círculo cerrado), o 1 mg de CocE (rectángulo/cuadrado cerrado) sobre la mortalidad inducida por cocaína. Figura 18A muestra la mortalidad como una función de la concentración de la cocaína. Figura 18B muestra los minutos hasta la muerte luego de la administración de cocaína como una función de la concentración de la cocaína. Para una mayor información sobre la metodología, véase Ejemplo 8.

[0045] Figura 19 es una trazado del espectro de masas MALDI-TOF de CocE pegilada. La diferencia de masa entre picos corresponde a - 5500 Da; equivalentes al peso molecular de una sola cadena PEG. Para una mayor información sobre la metodología, véase Ejemplo 11.

[0046] Figura 20 es una imagen SEM de glóbulos rojos fijados con gluteraldehído. Figura 20A es RBC normales sin tratamiento. Figura 20B es un RBC de ruptura osmótica-resellado cargado con L-ASNasa. Figura 20C es RBC cargado con LMWP-ASNasa. Para una mayor información sobre la metodología, véase Ejemplo 12.

[0047] Figura 21 es un diagrama de dispersión que muestra la actividad porcentual de la asperaginasa en la sangre como una función del tiempo (días) para RBC/LMP-ASNasa o RBC Ghost/ASNasa. Para una mayor información sobre la metodología, véase Ejemplo 12.

[0048] Figura 22 es un gráfico de curvas que muestra la supervivencia de ratones DBA/2 que portan células de linfoma L5178Y. El día 5 se administró enzima o solución salina, momento en el cual los

síntomas estaban presentes. Para una mayor información sobre la metodología, véase Ejemplo 12.

[0049] Figura 23 es un gráfico de curvas y diagramas de dispersión que demuestran los efectos protectores de CocE y sus mutantes contra la toxicidad inducida por la cocaína. La figura presenta el porcentaje de ratones que exhiben mortalidad inducida por la cocaína como una función de la concentración de cocaína inyectada en ratones a los que se administró CocE tipo silvestre (0.3 mg), T172R (0.3 mg), T172R-G173Q (0.3 mg), o L169K (1 mg). CocE o mutantes (mg) se administraron intravenosamente 1 minuto antes de la administración de cocaína (mg/kg, i.p.). Diferentes símbolos representan curvas de respuesta a la dosis de mortalidad inducida por la cocaína en la ausencia o presencia de CocE o mutantes. Cada punto de datos representa el porcentaje de ratones (n=8 para cada estado de dosificación) que exhiben mortalidad inducida por la cocaína.

[0050] Figura 24 es una serie de gráficos de líneas y diagramas de dispersión que presentan el transcurso del tiempo de los efectos protectores de CocE contra la toxicidad de la cocaína. CocE o mutantes (0.1, 0.3, y 1 mg, i.v.) se aplicaron en diferentes puntos de tiempo antes de la administración de 180 mg/kg de cocaína i.p. Figura 24A indica el porcentaje de ratones que presentaron mortalidad inducida por la cocaína como una función del tiempo de administración de CocE tipo silvestre (0.1 mg), T172R (0.1 mg), L169K (0.1 mg), o T172R-G173Q (0.1 mg) antes de la aplicación de 180 mg/kg de cocaína i.p. Figura 24B presenta el porcentaje de ratones que exhibieron mortalidad inducida por la cocaína como una función del tiempo de aplicación de CocE tipo silvestre (0.3 mg), T172R (0.3 mg), L169K (0.3 mg), o T172R-G173Q (0.1 mg) antes de la aplicación de 180 mg/kg de cocaína i.p.. Figura 24C indica el porcentaje de ratones que presentaron mortalidad inducida por la cocaína como una función del tiempo de aplicación de CocE tipo silvestre (1 mg), T172R (1

mg), L169K (1 mg), o T172R-G173Q (1 mg) antes de la administración de 180 mg/kg de cocaína i.p. Cada punto de datos representa el porcentaje de ratones (n=8 para cada condición de dosificación) que presentaron mortalidad inducida por la cocaína.

[0051] Figura 25 es un gráfico de curvas y diagrama de dispersión que demuestran la duración estimada de protección para el 50% de mortalidad. La figura presenta la duración estimada (horas) de protección (50% de mortalidad) de mutantes de CocE en ratones como una función de la dosificación (mg, i.v.) de T172R-G173Q, L169K, T172R y CocE tipo silvestre. El tiempo requerido en alcanzar el 50% de mortalidad se determinó a partir de la Figura 24.

[0052] Figura 26 es una serie de gráficos de curvas y diagramas de dispersión que demuestran los efectos protectores de CocE, T172R-G173Q y sus formas pegiladas contra la toxicidad inducida por la cocaína. Cada enzima (0.3 mg) se aplicó por vía intravenosa 1 minuto antes de la administración de cocaína (mg/kg, i.p.). Diferentes símbolos representan curvas de respuesta a la dosis de la mortalidad inducida por la cocaína en la ausencia o presencia de la enzima. Cada punto de datos representa el porcentaje de ratones (n=8 para cada condición de dosificación) que exhibieron mortalidad inducida por la cocaína. Figura 26A presenta la ocurrencia porcentual de mortalidad como una función de la dosificación de cocaína (mg/kg, i.p.) para vehículo/PBS, CocE tipo silvestre (0.3. mg), y PEG-CocE tipo silvestre (0.3 mg). Figura 26B presenta la ocurrencia porcentual de la mortalidad como una función de la dosificación de cocaína (mg/kg, i.p.) para vehículo/PBS, T172R-G173Q (0.3. mg) y PEG-T172R-G173Q (0.3. mg).

[0053] Figura 27 es una serie de gráficos de curvas y diagramas de dispersión que muestran la termoestabilidad de la cocaína esterasa y sus mutantes: (A) una CocE tipo silvestre, (B) T172R, (C) T272R/G173Q y

L169K. Para una mayor información sobre la metodología, véase Ejemplo 17.

[0054] Figura 28 es una serie de cromatogramas que muestran enzimas preincubadas a 37°C durante 0 minutos o 60 minutos y resueltas por cromatografía de exclusión molecular ("*size exclusion chromatography*") (A) CocE tipo silvestre, (B) T172R, (C) T172R/G173Q. Estándares de peso molecular, BSA (66Kda) y AD (150Kda) se incluyen en A. Para una mayor información sobre la metodología, véase Ejemplo 18.

[0055] Figura 29 es una serie de espectros CD emparejados (alisados estadísticamente) de CocE tipo silvestre y cuatro mutantes, que muestran la fusión dependiente de la temperatura observada entre 200 y 250nm. Para una mayor información sobre la metodología, véase Ejemplo 19.

[0056] Figura 30 es una serie de espectros para CocE tipo silvestre y cuatro mutantes restaurados a 3 curvas ("*deconvoluted to 3 curves*") mediante el algoritmo CCA, que sugieren que la fusión de CocE es por lo menos un proceso de dos pasos que transcurre desde la curva original (curva 1) a un paso de desdoblamiento intermedio (curva 2) a una proteína completamente desnaturalizada (curva 3). Para una mayor información sobre la metodología, véase Ejemplo 19.

[0057] Figura 31 es una serie de gráficos de curvas y diagramas de dispersión que muestran la contribución porcentual de cada temperatura en describir las 3 curvas CCA restauradas, mostradas en Figura 30. Para una mayor información sobre la metodología, véase Ejemplo 19.

[0058] Figura 32 es un gráficos de curvas y un diagrama de dispersión que muestra las temperaturas de fusión y formación para (1) las fusiones de espectros iniciales, (2) la formación y fusión del estado

intermedio, y (3) la acumulación de la proteína completamente fundida. Para una mayor información sobre la metodología, véase Ejemplo 19.

[0059] Figura 33 es una fotografía de un gel que muestra que la cocaína (rango mM impidió la formación inducida por 37°C de agregados de CocE de alto peso molecular (concentraciones de enzima de 0.1mg/ml. Para una mayor información sobre la metodología, véase Ejemplo 20.

[0060] Figura 34 es un gráfico de curvas y un diagrama de dispersión que muestran que la cocaína (cantidades uM) estabilizó la pérdida de actividad inducida por 37°C. Para una mayor información sobre la metodología, véase Ejemplo 20.

[0061] Figura 35 es una fotografía de un gel que muestra que el ácido benzoico (rango mM impidió la formación inducida por 37°C de agregados de CocE de alto peso molecular (concentraciones de enzima 0.1mg/ml. Para una mayor información sobre la metodología, véase Ejemplo 20.

[0062] Figura 36 es un gráfico de curvas y un diagrama de dispersión que muestra que la cocaína (cantidades uM) estabilizó la pérdida de actividad inducida por 37°C. Para una mayor información sobre la metodología, véase Ejemplo 20.

DESCRIPCIÓN DETALLADA DE LA INVENCION

[0063] Las incorporaciones de la invención divulgadas en esta especificación proveen composiciones y métodos para una metodología de degradación catalítica a la terapéutica anti-cocaína. La tecnología descrita en esta especificación se basa en parte en el descubrimiento de mutantes de cocaína esterasa altamente eficientes, termoestables y de larga duración,

que pueden proteger contra los efectos tóxicos y reforzantes de la cocaína en sujetos. Tales mutantes ofrecen opciones de tratamiento para afecciones inducidas por cocaína tales como sobredosis de cocaína y adicción a la cocaína.

POLIPÉPTIDOS COCE MUTANTES

[0064] A pesar de la potencia de la CocE tipo silvestre (véase a este respecto, SEQ ID NO: 1, No. de Acceso AF173165) en metabolizar cocaína (véase a este respecto, Ejemplo 2; Ejemplo 4; Ejemplo 5), la aplicación de CocE tipo silvestre como agente terapéutico en el tratamiento de sobredosis de cocaína puede limitarse debido a su baja estabilidad térmica a la temperatura fisiológica (véase a este respecto, Ejemplo 4; Ejemplo 6). La inestabilidad térmica contribuye a la corta vida media plasmática de la CocE tipo silvestre. La caída significativa (>50%) de la actividad de la CocE se observó luego de la incubación de la enzima en plasma a 37°C, o luego de su administración intravenosa a un ratón. El $t_{1/2}$ de la CocE a 37°C es de aproximadamente 15 minutos mientras que a 4°C el $t_{1/2}$ es superior a 6 meses. Estudios preliminares en ratas demostraron una duración relativamente corta del efecto anti-cocaína de poco más de 30 minutos para CocE.

[0065] En consecuencia, un aspecto de la invención provee polipéptidos CocE mutantes purificados, que exhiben estabilidad térmica y vida media plasmática incrementadas, comparados con la CocE tipo silvestre. Los polipéptidos CocE mutantes de la invención poseen un valor clínico significativo, debido a su capacidad de hidrolizar eficientemente la cocaína, exhibiendo al mismo tiempo una termoestabilidad y/o vida media plasmática incrementadas.

[0066] La invención provee polipéptidos CocE mutantes en los cuales se sustituye por lo menos un residuo de aminoácido de la CocE tipo

silvestre, donde la CocE mutante tiene una termoestabilidad incrementada conservando al mismo tiempo una eficiencia catalítica relativamente alta. En algunas incorporaciones, los polipéptidos CocE mutantes mantienen sustancialmente la actividad esterasa funcional del polipéptido CocE tipo silvestre (es decir, hidrólisis de la cocaína). Los polipéptidos CocE mutantes tienen una secuencia peptídica que difiere de un polipéptido CocE nativo en uno o más aminoácidos. La secuencia peptídica de tales mutantes puede exhibir una sustitución, eliminación, o adición de uno o más aminoácidos de un polipéptido CocE nativo. Las inserciones de aminoácidos son preferiblemente de alrededor de 1, 2, 3, y 4 a 5 aminoácidos contiguos, y las eliminaciones son preferiblemente de alrededor de 1, 2, 3, 4, 5, 6, 7, 8, y 9 a 10 aminoácidos contiguos. En varias incorporaciones, el polipéptido CocE mutante puede contener por lo menos una, dos, tres, cuatro, o más sustituciones, eliminaciones, o adiciones de aminoácidos, donde el polipéptido CocE mutante resultante posee una termoestabilidad incrementada.

[0067] El término aminoácido, tal como se emplea en esta especificación, tiene como finalidad incluir aminoácidos de origen natural así como aminoácidos de origen no natural, incluidos análogos y derivados de aminoácidos. Los últimos incluyen moléculas que contienen una fracción de aminoácido. Una persona experta en el arte reconocerá que la referencia en esta especificación a un aminoácido incluye, por ejemplo, L-aminoácido proteógenos de origen natural; D-aminoácidos; aminoácidos químicamente modificados tales como análogos y derivados de aminoácidos; aminoácidos no proteógenos de origen natural, y compuestos químicamente sintetizados que tienen propiedades conocidas en el arte por ser características de los aminoácidos. Para todas las secuencias de aminoácidos divulgadas en esta especificación, se entiende que nucleótidos y aminoácidos equivalentes pueden sustituirse en las secuencias sin afectar la función de las secuencias. Tal sustitución está dentro de la capacidad de una persona de habilidad ordinaria en el arte.

[0068] La invención provee asimismo polipéptidos CocE mutantes purificados con las siguientes sustituciones: L163V (SEQ ID NO: 3); V121D (SEQ ID NO: 4); S167A (SEQ ID NO: 5); Q123E (SEQ ID NO: 6); V225I (SEQ ID NO: 7); I218L (SEQ ID NO: 8); A310D (SEQ ID NO: 9); A149S (SEQ ID NO: 10); S159A (SEQ ID NO: 11); S265A (SEQ ID NO: 12); S56G (SEQ ID NO: 13); W220A (SEQ ID NO: 14); T122A (SEQ ID NO: 15); S140A (SEQ ID NO: 16); F189L (SEQ ID NO: 17); A193D (SEQ ID NO: 18); T254R (SEQ ID NO: 19); N42V (SEQ ID NO: 20); V262L (SEQ ID NO: 21); L508G (SEQ ID NO: 22); Y152H (SEQ ID NO: 23); V160A (SEQ ID NO: 24); T172R (SEQ ID NO: 25); Y532F (SEQ ID NO: 26); T74S (SEQ ID NO: 27); W285T (SEQ ID NO: 28); L146P (SEQ ID NO: 29); D533S (SEQ ID NO: 30); A194R (SEQ ID NO: 31); G173Q (SEQ ID NO: 32); C477T (SEQ ID NO: 33); K531A (SEQ ID NO: 34); R41I (SEQ ID NO: 35); L119A (SEQ ID NO: 36); K46A (SEQ ID NO: 37); F84Y (SEQ ID NO: 38); T172R-G173Q (SEQ ID NO: 39); L169K (SEQ ID NO: 40); F189A (SEQ ID NO: 41); N197K (SEQ ID NO: 42); R182K (SEQ ID NO: 43); F189K (SEQ ID NO: 44); V190K (SEQ ID NO: 45); Q191K (SEQ ID NO: 46); y A194K (SEQ ID NO: 47). Por ejemplo, el polipéptido CocE mutante T172R (SEQ ID NO: 25) tiene una termoestabilidad incrementada, V_{max} y K_m incrementados a 37°C, temperatura de fusión incrementada (T_m), vida media incrementada en el plasma, mayores reducciones en la mortalidad debida a la toxicidad de la cocaína, y efectos anti-cocaína más prolongados, comparados con la CocE tipo silvestre (véase a este respecto, Ejemplo 4, Ejemplo 7).

[0069] El incremento resultante en la termoestabilidad del polipéptido CocE mutante es por lo menos alrededor de 2 kcal/mol. La termoestabilidad de un polipéptido dado puede evaluarse por una variedad de métodos conocidos en el arte, incluidos por ejemplo espectroscopía de dicroísmo circular (CD) o calorimetría de barrido diferencial. Por ejemplo, el aumento resultante en la termoestabilidad puede ser por lo menos

alrededor de 2.1, por lo menos alrededor de 2.2, por lo menos alrededor de 2.3, por lo menos alrededor de 2.4, por lo menos alrededor de 2.5, por lo menos alrededor de 2.6, por lo menos alrededor de 2.7, por lo menos alrededor de 2.8, por lo menos alrededor de 2.9, por lo menos alrededor de 3.0, por lo menos alrededor de 3.1, por lo menos alrededor de 3.2, por lo menos alrededor de 3.3, por lo menos alrededor de 3.4, por lo menos alrededor de 3.5, por lo menos alrededor de 3.6, por lo menos alrededor de 3.7, por lo menos alrededor de 3.8, por lo menos alrededor de 3.9, por lo menos alrededor de 4.0, por lo menos alrededor de 4.1, por lo menos alrededor de 4.2, por lo menos alrededor de 4.3, por lo menos alrededor de 4.4, o por lo menos alrededor de 4.5 kcal/mol. Se contemplan aumentos de la termoestabilidad aún mayores. Se cree que reducir la energía en alrededor de 2.1 a alrededor de 4.5 kcal/mol puede ampliar el tiempo de vida media de la proteína alrededor de 30 a alrededor de 1000 veces más a temperatura de la sala.

[0070] Generalmente, los polipéptidos CocE mutantes tienen una actividad esterasa con termoestabilidad incrementada, comparada con la CocE tipo silvestre. En algunas incorporaciones, los polipéptidos CocE mutantes termoestables pueden tener menos actividad esterasa que la CocE tipo silvestre. Por ejemplo, mutantes CocE termoestables pueden tener alrededor de 10%, alrededor de 20%, alrededor de 30%, alrededor de 40%, alrededor de 50%, alrededor de 60%, alrededor de 65%, alrededor de 70%, alrededor de 75%, alrededor de 80%, alrededor de 85%, alrededor de 90%, alrededor de 95%, o alrededor de 99% de la actividad esterasa de la CocE tipo silvestre. En otras incorporaciones, los polipéptidos CocE mutantes tienen aproximadamente la misma eficiencia catalítica, o mayor, de los polipéptidos CocE tipo silvestre. Por ejemplo, los mutantes de CocE termoestables pueden tener alrededor de 100%, alrededor de 110%, alrededor de 120%, alrededor de 130%, alrededor de 140%, alrededor de 150%, o más de la actividad esterasa de la CocE tipo silvestre.

[0071] Variantes de los polipéptidos CocE mutantes tales como fragmentos, análogos y derivados se encuentran igualmente dentro de esta invención. Fragmentos del polipéptido CocE correspondientes a uno o más motivos y/o dominios particulares o a tamaños arbitrarios, por ejemplo, por lo menos 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 50, 75, 100, 125, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1150 y 1200 aminoácidos de longitud pretenden quedar dentro del ámbito de la invención divulgada en esta especificación. Porciones peptídico aisladas de polipéptidos CocE pueden obtenerse detectando selectivamente péptidos producidos recombinantemente a partir del fragmento correspondiente del ácido nucleico que codifica tales péptidos. Adicionalmente, fragmentos pueden sintetizarse químicamente empleando técnicas conocidas en el arte, tales como química f-Moc o t-Boc de fase sólida Merrifield convencional. Por ejemplo, un polipéptido CocE, como se describe en esta especificación, puede dividirse arbitrariamente en fragmentos de longitud deseada sin traslapamiento de los fragmentos, o dividirse preferiblemente en fragmentos traslapantes de una longitud deseada.

[0072] Otro aspecto de la invención divulgada en esta especificación concierne a formas recombinantes del polipéptido CocE. En algunas incorporaciones, moléculas de ácido nucleico aisladas de la invención incluyen aquellos polinucleótidos que codifican los polipéptidos CocE descritos anteriormente. En otras incorporaciones, los polipéptidos recombinantes de la invención divulgados en esta especificación se codifican por un ácido nucleico que tiene por lo menos 85% de identidad de secuencia (por ejemplo, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, y 99%) con la secuencia de ácido nucleico de SEQ ID NO: 2, donde el polipéptido CocE recombinante expresado conserva sustancialmente la misma eficiencia catalítica, o mayor, del polipéptido CocE tipo silvestre y tiene una termoestabilidad incrementada, comparada con la CocE tipo silvestre.

[0073] Pueden utilizarse asimismo en la invención ácidos nucleicos que se hibridan bajo condiciones de alta restricción a los ácidos nucleicos de SEQ ID NO: 2 o los complementos de SEQ ID NO: 2. Por ejemplo, aquellos ácidos nucleicos que se hibridan a SEQ ID NO: 2 o al complemento de SEQ ID NO: 2 bajo condiciones de baja restricción, condiciones de restricción moderada, o condiciones de alta restricción y codifican además un polipéptido CocE mutante, que tiene actividad esterasa con termoestabilidad incrementada, comparada con CocE tipo silvestre, se encuentran dentro de la invención. Ácidos nucleicos preferidos son aquellos que tienen una secuencia de nucleótidos que es el complemento de la totalidad o una porción de SEQ ID NO: 2. Otras variantes del gen CocE nativo dentro de la invención son polinucleótidos que comparten por lo menos 65% (por ejemplo, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, y 99%) de identidad de secuencia con SEQ ID NO: 2 o el complemento de SEQ ID NO: 2. Ácidos nucleicos que se hibridizan bajo condiciones de restricción a, o comparten por lo menos 65% de identidad de secuencia con SEQ ID NO: 2 o el complemento de SEQ ID NO: 2, pueden obtenerse por técnicas conocidas en el arte tales como introduciendo mutaciones en el gen CocE nativo, o mediante aislamiento de un organismo que expresa tal ácido nucleico (por ejemplo, una variante alélica).

[0074] Moléculas de ácido nucleico que codifican proteínas de fusión CocE mutantes se encuentran igualmente dentro de la invención. Tales ácidos nucleicos pueden elaborarse preparando un constructo (por ejemplo, un vector de expresión) que exprese la proteína de fusión CocE mutante cuando se introduzca en un hospedero apropiado. Por ejemplo, tal a constructo puede hacerse ligando un primer polinucleótido que codifica la proteína CocE mutante fusionada sin alteración en el marco de lectura ("*in frame*") con un segundo polinucleótido que codifica otra proteína de tal

modo que la expresión del constructo en un sistema de expresión adecuado produzca una proteína de fusión.

[0075] Las moléculas de ácido nucleico de la invención pueden modificarse en una fracción base, fracción azúcar, o el esqueleto fosfato, por ejemplo, para mejorar la estabilidad de la molécula, la hibridación, y similares. Las moléculas de ácido nucleico utilizadas en incorporaciones de la invención divulgada en esta especificación pueden estar en la forma de ARN o en la forma de ADN (por ejemplo, ADNc, ADN genómico y ADN sintético). El ADN puede ser bicatenario o monocatenario y, si monocatenario, puede ser la hebra codificante (transcrita) o la hebra no codificante (complementaria). La secuencia codificante que codifica un polipéptido CocE mutante puede ser idéntica a la secuencia nucleotídica reivindicada, o puede ser también una secuencia codificante diferente que, como resultado de la redundancia o información repetida del código genético, codifica el mismo polipéptido que los polinucleótidos de SEQ ID NOS: 3-37.

DISEÑO DE CocE MUTANTE

[0076] Mutaciones termoestabilizadoras de CocE pueden diseñarse o bien para aumentar la estabilidad termodinámica de un polipéptido disminuyendo ΔG (desdoblado $\rightarrow$ plegado) o disminuir la tasa de desdoblamiento incrementando la energía libre de activación del proceso de desdoblamiento. La ΔG (desdoblado $\rightarrow$ plegado) es la diferencia de energía libre entre el estado desdoblado y el estado plegado. Para un polipéptido termodinámicamente estable, ΔG (desdoblado $\rightarrow$ plegado) debe ser un valor negativo. Generalmente, entre menor sea el valor ΔG (desdoblado $\rightarrow$ plegado), más estable es el estado plegado. Tal energía libre de activación del proceso de desdoblamiento es la diferencia de energía libre entre el estado plegado y el estado de transición del desdoblamiento (Steipe, 1999).

[0077] Polipéptidos CocE mutantes con termoestabilidad incrementada pueden diseñarse y generarse por una variedad de métodos conocidos en el arte incluidos, por ejemplo, diseño racional, evolución dirigida (por ejemplo, mutagénesis aleatoria, mutagénesis del organismo hospedero de CocE), o una combinación de las mismas. La evolución dirigida puede lograrse mediante mutación y recombinación, seguidas por detección sistemática del rasgo deseado o aplicando una presión selectiva para obtener el rasgo de interés (véase a este respecto, Lehmann and Wyss, 2001). La mutagénesis puede llevarse a cabo en el gen específico de interés o mediante mutagénesis y selección de un organismo hospedero, de modo que la propiedad obtenida por técnicas de ingeniería se confiere a la proteína de interés. Preferiblemente, la termoestabilidad de los mutantes CocE se manipula por técnicas de ingeniería mediante una estrategia enfoque triple ("*through a three-prong approach*") de (i) diseño racional mediante mutagénesis dirigida al sitio, asistida por computador (véase a este respecto, Ejemplo 3; Ejemplo 4), (ii) mutagénesis aleatorio del gen CocE y detección selectiva de gran productividad (véase a este respecto, Ejemplo 4; Ejemplo 15); y (iii) mutagénesis del organismo hospedero de CocE, seguida por selección genética (véase a este respecto, Ejemplo 14).

[0078] Varios conceptos de diseño racional son conocidos por las personas de habilidad en el arte para reducir ΔG (desdoblado $\rightarrow$ plegado) y/o aumentar la energía libre de activación del proceso de desdoblamiento (véase a este respecto, Lehmann, 2001). Por ejemplo, se puede: disminuir la entropía del estado desdoblado introduciendo puentes adicionales disulfuro o mediante mutaciones X $\rightarrow$ Pro; aumentar una propensión α -hélice mediante sustituciones Gly $\rightarrow$ Ala o mediante estabilización de macrodipolos α -hélice; mejorar las interacciones electrostáticas entre residuos de superficie cargada introduciendo puentes sal adicionales o incluso redes de sal-puente, o mediante mutaciones termoestabilizadoras basadas en cálculos de potenciales electrostáticos.

[0079] Modelamiento molecular, basado en simulaciones de dinámica molecular apropiada (MD - *molecular dynamics*), puede guiar racionalmente la mutagénesis dirigida al sitio con el fin de diseñar polipéptidos CocE mutantes dotados de termoestabilidad creciente. La simulación MD clásica permite el estudio de la evolución en el tiempo de un sistema grande tomando muchos pequeños pasos de tiempo sucesivos pequeños bajo fuerzas atómicas determinadas por un conjunto de funciones de interacción parametrizadas (campo de fuerza), incluidas interacciones enlazadas (enlaces, ángulos, y ángulos diédricos), interacciones no enlazadas de van der Waals, e interacciones electrostáticas basadas o cargas atómicas netas. Debido a la forma simple de campo de fuerza, la simulación MD puede llevarse a cabo durante un tiempo de simulación suficientemente prolongado para producir propiedades significativas promediadas por el ensamble, incluso para un sistema muy grande que incluya más de cien mil átomos. En consecuencia, para CocE y cada mutante propuesto, la simulación MD puede llevar a una estructura 3D promediada dinámicamente, razonable, del polipéptido simulado en agua.

[0080] Una estrategia usada exitosamente en esta especificación se centra en el diseño racional de mutaciones termoestabilizadoras que disminuyen el valor ΔG (desdoblado $\rightarrow$ plegado) del polipéptido (véase a este respecto, Ejemplo 3). Tal estrategia requiere únicamente el cálculo de ΔG (desdoblado $\rightarrow$ plegado), sin realizar un cómputo más dispendioso en la estructura y la energética del estado de transición del desdoblamiento. Por lo tanto, para aumentar la termoestabilidad de un polipéptido antes de la pegilación, se puede emplear un método implementado en un programa de diseño racional (por ejemplo, RosettaDesign) que utiliza una función de energía para evaluar adaptabilidad de una secuencia particular para un pliegue dado y un algoritmo de búsqueda Monte Carlo para muestrear el espacio de la secuencia. Tal estrategia es conocida por producir una termoestabilidad incrementada de otras enzimas sin reducción en la

eficiencia catalítica (véase a este respecto, Korkegian, 2005). Por ejemplo, el pliegue usado en el cómputo puede corresponder al de la estructura cristalina de rayos X de la CocE disponible.

[0081] El programa de diseño racional permite la predicción de un conjunto de secuencias de aminoácidos modificadas, que potencialmente poseen energías más bajas (por ejemplo, valores ΔG (desdoblado $\rightarrow$ plegado)) y, por lo tanto, una mayor termoestabilidad. En consecuencia, se puede usar el diseño computacional descrito en esta especificación para predecir mutaciones en el núcleo del polipéptido CocE, que puedan llevar a la termoestabilización del polipéptido sin pérdida de la eficiencia catalítica. Esta estrategia minimiza el tiempo de prueba experimental y aumenta en gran medida el éxito de los resultados experimentales. Las mutaciones termoestabilizadoras predichas pueden examinarse individualmente por mutagénesis dirigida al sitio y luego en combinación, en un proceso iterativo (véase, por ejemplo, Ejemplo 15).

[0082] Puede usarse asimismo evolución dirigida para generar mutantes CocE termoestables. La evolución dirigida abarca una serie de técnicas experimentales que producen una diversidad y adaptación aceleradas mediante mutación y recombinación, seguidas o bien por detección selectiva del rasgo deseado o bien aplicando una presión selectiva para obtener el rasgo de interés (Lehmann & Wyss (2001) *Current Opinion in Biotechnology* 12, 371-375). Por consiguiente, la evolución dirigida involucra tanto un proceso para generar diversidad y una detección selectiva eficiente o método de selección para la detección o enriquecimiento del rasgo deseado. La evolución dirigida se ha aplicado previamente en forma exitosa a la producción de proteínas termoestables, y la generación de diversidad se ha alcanzado, por ejemplo mediante PCR propensa a error, mutagénesis de saturación, barajado de ADN, mutagénesis química, y combinaciones de las mismas. La PCR propensa a error amplifica el gen de interés con polimerasas no a prueba de lectura y

condiciones estresantes, diseñadas para generar aleatoriamente mutaciones de par de bases únicas. Luego de cada ronda se seleccionan los mejores mutantes y se usan como secuencias progenitoras en la siguiente ronda de mutagénesis. Esta técnica se ha utilizado para generar un número de variantes de proteínas termoestables, incluida propil endopeptidasa (Uchiyama ma., et al. 2000), betaglucuronidasa (Flores, H. and A. D. Ellington (2002) *Journal of Molecular Biology* 315, 325-337) y la familia de 10 xilanasas (Andrews et al. (2004) *J Biol Chem* 279, 54369-79). La mutagénesis de saturación amplifica también el gen de interés, pero incorpora bases universales durante la amplificación para generar un número mucho más alto de mutaciones. Esta técnica se ha utilizado para generar termoestabilidad en una enzima psicrófila (Miyazaki et al. (2000) *Journal of Molecular Biology* 297, 1015-1026). El barajado de ADN implica uno o más ciclos de recombinación entre un conjunto de secuencias homólogas para obtener variantes mejoradas de una enzima dada. Esta técnica puede usarse igualmente en tándem con PCR propensa a error, donde los mejores mutantes obtenidos por PCR propensa a error se combinan mediante barajado de ADN para generar un nuevo subconjunto de mutantes. El barajado de ADN se ha implementado para generar variantes termoestables de beta-glucuronidasa (Flores, H. and A.D. Ellington (2002) *Journal of Molecular Biology* 315, 325-337). La mutagénesis química implica tratar el ADN de un plásmido con químicos que introducen mutaciones puntuales en la secuencia tales como hidroxilamina, nitrosaminas o sulfato de dimetilo. El tratamiento del plásmido con hidroxilamina se ha usado para generar mutantes termoestables de la beta-glucosidasa A del *Bacillus polymyxa* (Lopez-Camacho et al. (1996) *Biochemistry Journal* 314, 833-838) y luciferasa de luciérnaga (White et al. (1996) *Biochemistry Journal* 319 (Pt 2), 343-350).

[0083] La mutagénesis del organismo hospedero de CocE puede emplearse igualmente para generar mutantes CocE termoestables. Un método sencillo y rápido para la producción de mutantes CocE

termoestables puede conseguirse utilizando la capacidad de la enzima para conferir metabolismo de cocaína como una fuente exclusiva de carbono a su organismo hospedero. El gen *CocE* se secuenció originalmente de *Rhodococcus* MB1 subclonando fragmentos del gen en *Rhodococcus erythropolis* CW25, una bacteria incapaz de metabolizar cocaína, pero capaz de crecer en los subproductos de cocaína esterasa ecgonina metil éster y benzoato (Bresler et al. (2000) Applied & Environmental Microbiology 66, 904-908). Otro organismo que demostró previamente metabolizar subproductos de cocaína es *Pseudomonas fluorescens* (MBER), que fue capaz de crecer en una relación simbiótica con otra bacteria capaz de metabolizar cocaína mediante la esterasa *Comamonas acidovorans* (MBLF).

[0084] Aunque difíciles de transformar con plásmidos con eficiencias incrementadas, estas cepas bacterianas pueden transformarse en forma relativamente fácil con el gen *CocE* tipo silvestre clonado en vectores lanzadera apropiados y, luego, puede realizarse mutagénesis bacteriana tradicional (véase a este respecto, Ejemplo 14). Debido a que estas bacterias, normalmente, sólo crecen a 25-30°C en productos de hidrólisis de la cocaína, la selección de mutantes capaces de metabolizar cocaína eficientemente a 37°C se esperaría seleccionar mutantes *CocE* altamente activos que son estables a 37°C.

[0085] La exposición de bacterias a radiación o agentes químicos para la producción de mutantes que portan nuevos fenotipos es bien conocida en el arte (véase a este respecto, Maron, D.M. and Ames, BN (1983) Mutation Research 113, 173-215). La mutagénesis por irradiación puede implicar tanto radiación ionizante como no ionizante; no obstante, la radiación no ionizante se usa ampliamente, y la radiación con UV a 260nm es la más efectiva como agente letal. La mutagénesis se causa por la inducción de dímeros de pirimidina, aumentando la probabilidad de incorporar errores de coincidencia ("*mismatches*") durante la replicación.

Las células se exponen a radiación con UV en una dosis predeterminada para eliminar 90-95% de la población celular, y luego se buscan mutantes entre los sobrevivientes. La mutagénesis química incluye el uso de análogos de base tales como 5-bromouracilo y 2-aminopurina que aumentan el error de copia durante la replicación, o el uso de agentes que reaccionan directamente con ADN tales como hidroxilamina o nitrosoguanidina, las cuales inducen mutaciones con una frecuencia mayor que los análogos de base.

GENERACIÓN DE POLIPÉPTIDOS CocE MUTANTES

[0086] Incorporaciones de la invención atañen además a métodos de producir los polipéptidos CocE mutantes. Por ejemplo, una célula hospedera, transfectada con un vector de ácido nucleico que dirige la expresión de una secuencia de nucleótidos que codifica los polipéptidos CocE mutantes, puede cultivarse bajo condiciones apropiadas para permitir que se produzca la expresión del péptido. Las células pueden recolectarse, lisarse y aislarse la proteína. Un polipéptido CocE mutante puede aislarse de células hospederas empleando técnicas conocidas en el arte para purificar proteínas, incluidas cromatografía de intercambio iónico, cromatografía de filtración en gel, ultrafiltración, electroforesis, y purificación por inmunoafinidad con anticuerpos específicos de tal proteína (véase a este respecto, Ejemplo 1).

[0087] Por ejemplo, luego que un polipéptido CocE mutante se ha expresado en una célula, puede aislarse usando cualquier cromatografía de inmunoafinidad. Más específicamente, un anticuerpo anti-CocE puede inmovilizarse en una matriz de cromatografía de columna, y la matriz puede usarse para cromatografía de inmunoafinidad para purificar el polipéptido CocE mutante de lisados de células por métodos estándar (véase, por ejemplo, Sambrook and Russel (2006) *Condensed Protocols from Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory

Press, ISBN 0879697717). Luego de la cromatografía de inmunoafinidad, el polipéptido CocE mutante puede purificarse posteriormente por otras técnicas estándar, por ejemplo, cromatografía líquida de alto rendimiento. En otra incorporación, un polipéptido CocE mutante se expresa en una proteína de fusión que contiene una marca de afinidad (por ejemplo, Hisx6) que facilita su purificación (véase a este respecto, Ejemplo 1).

PEGILACIÓN DE POLIPÉPTIDOS CocE MUTANTES

[0088] La CocE mutante puede pegilarse con el fin de aumentar la duración de la acción y la estabilidad al calor, y disminuir la inmunogenicidad. La pegilación puede aumentar aún más la termoestabilidad de la CocE mutante de la invención e incrementar la vida media sérica disminuyendo la depuración real, la proteólisis, la captura de macrófagos y la respuesta inmunológica.

[0089] La pegilación es el proceso de unir unidades repetitivas de etilen glicol (es decir, polietilen glicol, o PEG) a un polipéptido para reducir la inmunogenicidad del polipéptido y su tasa de depuración renal (véase generalmente (Veronese, FM and Harris, JM (2002b) *Advanced Drug Delivery Reviews* 54, 457-606, Veronese, FM and Harris, JM (2002c) *Advanced Drug Delivery Reviews* 55, 1259-1345, reviewing PEGylation technology). Cada unidad de etilen glicol puede unirse a dos o más moléculas de agua, lo cual aumenta efectivamente el tamaño del péptido, y puede proteger el péptido contra respuestas inmunitarias, degradación enzimática, y/o depuración renal rápida. El polietilen glicol puede estabilizar también contra variaciones en temperatura y pH. El resultado neto es que el polipéptido terapéutico puede mantenerse durante más tiempo en la sangre, e induce una respuesta inmunitaria menor (Harris, JM and Chess, RB (2003) *Nature Reviews. Drug Discovery* 2, 214-221). El PEG posee un conjunto único de propiedades, incluidas ausencia de toxicidad, antigenicidad e inmunogenicidad, una disminución, dependiente

de la masa, de la depuración renal, y una alta flexibilidad y solubilidad en agua. El PGE imparte estas características a las proteínas a las cuales se enlaza (Veronese, FM and Harris, JM (2002b) *Advanced Drug Delivery Reviews* 54, 457-606).

[0090] Un polímero PEG puede activarse primeramente con un grupo funcional que estimula la unión covalente a un aminoácido de la proteína. El grupo hidroxilo terminal del PEG puede modificarse por un carbonato activo, un éster activo, aldehído o derivado de tresilato. El PEG puede unirse a lisinas o a residuos introducidos de cisteína del a CocE mutante. Unidades repetitivas de óxido de etileno pueden construirse en muchas configuraciones con longitudes diferentes, con o sin ramificación, y con varios pesos moleculares. Medios de incorporación pueden incluir mutagénesis dirigida al sitio o el uso de derivados maleimida de transglutaminasa.

[0091] El PEG cuenta con la aprobación de la FDA para uso como vehículo o base en Productos Farmacéuticos, incluidas formulaciones inyectables, tópicas, rectales y nasales (Harris and Chess, 2003). Además, se han aprobado medicamentos PEGilados para uso clínico (véase a este respecto, PEG-interferón alpha-2a (Hamidi, M and Tajerzadeh, H (2003) *Drug Delivery* 10, 9-20; PEG-interferón alpha-2b (Reddy et al. (2002) *Advanced Drug Delivery Reviews* 54, 571-586).

[0092] Debido a la movilidad dinámica espacial sin paralelo del PEG, la modificación de la superficie de una CocE mutante por el polímero PEG puede blindar la enzima recubierta de acciones efectuadas por las enzimas proteolíticas y el reconocimiento por el sistema inmunitario; reduciendo en esa forma la inmunogenicidad y prolongando la vida media en circulación de la CocE mutante pegilada. El éxito de las estrategias de pegilación incluyen liposomas modificados con PEG (es decir, liposomas furtivos, así llamados debido a su capacidad de eludir la detección

inmunitaria y la depuración renal para proporcionar un tiempo de circulación significativamente ampliado (Lasic, DD (1997) *Journal of Controlled Release* 48, 203-222), así como asparaginasa pegilada, cuya vida media *in vivo* se ha mejorado drásticamente desde las 26 horas observadas para la asparaginasa libre hasta 15 días (Avramis et al. (2002) *Blood* 99, 1986-1994). Además, se reconoce en el arte que la pegilación puede mejorar significativamente la estabilidad térmica de una enzima (Kazan, D. and Erarslan, A. (1997) *Applied Biochemistry & Biotechnology* 62, 1-13; Efremova et al. (1998) *Biochemistry (Moscow)* 63, 441-447) y reducir la activación del sistema de complemento (Chang et al. (2005) *Bioconjugate Chemistry* 16, 147-155). En consecuencia, la tecnología de la PEGilación es bien adecuada para mejorar los efectos farmacológicos y farmacéuticos de la CocE mutante de la invención (véase a este respecto, Ejemplo 11). La pegilación de CocE mutante puede usarse igualmente en conjunción con encapsulación en RBC.

ENCAPSULACIÓN DE COCE MUTANTE EN RBC

[0093] La CocE mutante puede encapsularse en glóbulos rojos (RBC) con el fin de aumentar la duración de la acción y la estabilidad al calor, y disminuir la inmunogenicidad. La cocaína, justamente a medida que atraviesa rápidamente la barrera hematoencefálica, transita también rápidamente la membrana plasmática del RCB, y esta captura se ha demostrado en un estudio de administración de cocaína intravenosa en el hombre (Javaid et al. (1978) *Journal of Chromatography* 15, 105-113). Además, las concentraciones de cocaína en RBC excedieron las concentraciones en el plasma (Javaid et al. (1978) *Journal of Chromatography* 15, 105-113). Como tal, la encapsulación en RBC puede utilizarse para blindar formas termoestables de CocE contra la depuración.

[0094] Los eritrocitos se han aprovechado extensivamente por sus potenciales aplicaciones como sustancias portadoras de medicamentos

(Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). Siendo las células más abundantes del cuerpo humano, el RBC ofrece ventajas sin precedentes para funcionar como sustancia portadora de medicamentos. En primer lugar, los eritrocitos son completamente biocompatibles y biodegradables, particularmente cuando se utilizan células autólogas para carga de medicamentos. Además, la forma de disco bicóncavo de los eritrocitos los dota con la máxima relación de superficie a volumen (1.9×10^4 cm/g) (Guyton, AG & Hall, JE (1996) *Textbook of Medical Physiology*, 425-433) utilizable para encapsulación de medicamentos. De igual forma la encapsulación en RBC evita que el medicamento cargado se inactive por factores endógenos, así como protege los organismos de los efectos tóxicos del medicamento encapsulado (Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). Más aún, la encapsulación en RBC permite evitar respuestas inmunitarias indeseables (es decir, antigenicidad e inmunogenicidad) que pueden desencadenarse por cuerpos extraños (Wang et al. (2002) *Advanced Drug Delivery Reviews* 54, 547-570). Adicionalmente, los eritrocitos poseen una vida media en circulación más prolongada en comparación con otras sustancias portadoras sintéticas. Por ejemplo, se informa que la expectativa de vida normal de un eritrocito en la circulación sistémica es de alrededor de 120 días (Guyton, AG & Hall, JE (1996) *Textbook of Medical Physiology*, 425-433).

[0095] La encapsulación de CocE mutante en eritrocitos puede realizarse de acuerdo con varias técnicas conocidas en el arte, incluidas electroporación, endocitosis inducida (por ejemplo, por primaquina) del medicamento, y ósmosis (véase a este respecto, Green, R. and Widder, KJ (1987) *Methods In Enzymology*, Vol. 149). Estos métodos pueden incluir dilatar y romper la membrana celular, liberar el contenido interior incluyendo principalmente hemoglobina y el citoesqueleto, atrapar el medicamento y luego volver a sellar la membrana para producir el RBC en forma de copa, esférico, de color rosado o blanco (denominado generalmente "RBC-Ghost").

[0096] Alternativamente, pueden empelarse péptidos que penetran la membrana para encapsular CocE mutante en eritrocitos (véase a este respecto, Ejemplo 12). Péptidos que penetran la membrana, o péptidos de dominio de transducción de la proteína (PTD - *protein transduction domain*) son una familia de péptidos pequeños (que consisten normalmente de residuos de 10-15 aminoácidos) incluidos TAT, LMWP, y otros péptidos catiónicos ricos en arginina (véase generalmente, Dietz, GPH and Bahr, M (2004) *Molecular Cell Neurosciences* 27, 85-131). Se sabe en el arte que, enlazando covalentemente PTD a casi cualquier tipo de especie molecular, incluidas proteínas (MW>150 kDa; ya se han probado más de 60 diferentes proteínas (Dietz, GPH and Bahr, M (2004) *Molecular Cell Neurosciences* 27, 85-131) y nano-portadores (por ejemplo, liposomas), PTD puede transbordar la especie enlazada a través de la membrana celular de todos los tipos de órganos, incluido el cerebro (Schwarze et al. (1999) *Science* 285, 1569-1572). PTD no es tóxico ni inmunógeno (Schwarze et al. (1999) *Science* 285, 1569-1572), y la internalización de células mediada por PTD no induce perturbación o alteración de la membrana celular de los eritrocitos (Suzuki et al. (2002) *Journal of Biological Chemistry* 25, 2437-2443). El PTD se ha utilizado con éxito en cargar RBC con proteínas, y resulta en eritrocitos con atributos físicos y químicos inalterados (véase a este respecto, Li et al. (2003) *American Pharmaceutical Review* 6, 22-26). En consecuencia, la conjugación del péptido PTD a CocE mutante puede facilitar la encapsulación de RBC. La encapsulación de RBC puede llevarse igualmente a cabo usando CocE mutante pegilada.

ESTABILIZACIÓN DE COCE MUTANTE

[0097] Otro aspecto de la invención se dirige a la estabilización de polipéptidos CocE mutantes usando sustratos, productos, y/o inhibidores de cocaína. Sustratos y productos útiles en incorporaciones divulgadas en esta especificación incluyen, por ejemplo, pero no se limitan a, cocaína;

derivados de cocaína, tales como, por ejemplo, (-)-cocaína, (+)-cocaína, tropo cocaína, y similares; derivados de tio- cocaína, tales como, por ejemplo, Tiol-1, Tiol-2, y similares; derivados de amida-cocaína; derivados de provitamina-cocaína, tales como cocaína PABA, cocaína de niacina, y similares; ácido benzoico; 4-nitrofenil acetato (4NPA); 4-nitrofenol (4NP); y similares. Inhibidores ejemplares incluyen, pero no se limitan a, análogos de sustrato, tales como, fosfo-fluorococaína, O-fosfo-cocaína, O-metilfosfococaína, S-metilfosfococaína, y similares; análogos de producto, tales como, ecgonina y derivados de ecgonina, tales como, análogo de ecogonina metil éster de ácido bórico; ácido fenilbórico; derivados de ácido benzoico, tales como, ácido 4-terc-butil benzoico, ácido 1-naftoico, metil éster de ácido 2,3,4-trimetil-benzoico, y similares. Químicos adicionales incluyen, por ejemplo, pero no se limitan a, SDS, glicerol, PEG, y similares.

[0098] Preferiblemente, los sustratos, productos, y/o inhibidores estabilizan la desnaturalización térmica de los polipéptidos divulgados en esta especificación. En algunas incorporaciones, los sustratos, productos y/o inhibidores impiden también la agregación inducida térmicamente en electroforesis en gel. Generalmente, el uso de un sustrato, producto, y/o inhibidor resulta en por lo menos alrededor de un incremento del 10% en la estabilidad y/o inhibición, respectivamente. Por ejemplo el aumento puede ser de alrededor de 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 120%, 150%, 200%, 300%, o incluso 500% o mayor. Por consiguiente, sustratos, productos, y/o inhibidores son bien apropiados para estabilizar los polipéptidos CocE mutantes divulgados en esta especificación (véase a este respecto, Ejemplo 20).

[0099] En una incorporación, se utilizan moléculas pequeñas para termoestabilizar los polipéptidos CocE mutantes divulgados en esta especificación. En una incorporación preferida, tales moléculas no ocupan el sitio activo del polipéptido.

[0100] En algunas incorporaciones, los polipéptidos divulgados en esta especificación pueden co-infusionarse con una molécula estabilizadora. En otras incorporaciones, las moléculas estabilizadoras pueden usarse para estabilizar el polipéptido durante la elaboración. En todavía otras incorporaciones, las moléculas estabilizadoras pueden emplearse para estabilizar los polipéptidos CocE mutantes hasta que estén listos para uso.

MÉTODOS DE TRATAMIENTO

[0101] Otro aspecto de la invención se dirige a un enfoque, de degradación catalítica, a terapéuticos anti-cocaína. Se proveen tratamientos, tanto profilácticos como terapéuticos, de afecciones inducidas por la cocaína mediante la administración de polipéptidos CocE mutantes, termoestables, esterasa activos, a un sujeto necesitado de ello. Las variantes de cocaína esterasa de la invención poseen un valor clínico significativo debido a su termoestabilidad incrementada y vida media plasmática más prolongada, que la CocE de origen natural conocida. Es este aumento en la termoestabilidad y la vida media plasmática lo que permite una respuesta mucho más rápido a los síntomas mortales de la toxicidad de la cocaína que distinguen las variantes CocE de la invención de otras opciones de tratamiento.

[0102] Una determinación de la necesidad de tratamiento se evaluará típicamente por una historia y un examen físico consistente con la afección inducida por la cocaína. Las afecciones inducidas por la cocaína incluyen, pero no se limitan a, sobredosis de cocaína, toxicidad de la cocaína y dependencia de la cocaína y/o adicción. Por ejemplo, el diagnóstico de la toxicidad de la cocaína puede incluir convulsiones, convulsiones tónico-clónicas generalizadas ("*grand-mal seizures*"), paro cardíaco, infarto del miocardio, arritmias cardíacas, presión sanguínea

elevada, apoplejía, psicosis inducida por drogas, aneurisma disecante, y elevada demanda de oxígeno del miocardio. Como otro ejemplo, en el caso de dependencia de la cocaína y/o adicción, los síntomas de abstinencia incluyen sensaciones subjetivas de disforia moderada a severa, depresión, ansiedad, o irritabilidad. Los sujetos con una necesidad identificada de terapia incluyen aquellos con una afección diagnosticada, inducido por la cocaína, un indicio de afección inducida por la cocaína, y sujetos que han sido tratados, están siendo tratados, o serán tratados por una afección inducida por la cocaína. Preferiblemente, el sujeto es un animal, incluidos, pero no limitados a, mamíferos, reptiles, y aves, más preferiblemente caballos, vacas, perros, gatos, ovejas, cerdos, y gallinas y, muy preferiblemente, un humano.

[0103] Una cantidad efectiva de los polipéptidos CocE mutantes descritos en esta especificación es generalmente aquella que puede reducir la toxicidad de la cocaína o la gravedad de una afección inducida por la cocaína. La reducción en la gravedad incluye, por ejemplo, una detención o una disminución en los síntomas, indicadores fisiológicos, marcadores biológicos, o indicadores metabólicos. Cuando se usa en los métodos de la invención, una cantidad terapéuticamente efectiva del polipéptido CocE mutante descrito en esta especificación puede emplearse en forma pura o, donde tales formas existen, en forma de sal farmacéuticamente aceptable y con o sin un excipiente farmacéuticamente aceptable. Por ejemplo, los polipéptidos CocE mutantes de la invención pueden administrarse con una relación razonable riesgo/beneficio aplicable a cualquier tratamiento médico, en una cantidad suficiente para reducir sustancialmente la concentración de cocaína en la sangre y/o tejidos del sujeto.

[0104] La toxicidad y eficacia terapéutica de los polipéptidos CocE mutantes pueden determinarse por procedimientos farmacéuticos estándar en cultivos de células y/o animales experimentales para determinar

la LD₅₀ (la dosis letal al 50% de la población) y la ED₅₀, (la dosis terapéuticamente efectiva en 50% de la población). La relación de la dosis entre efectos tóxicos y terapéuticos es el índice terapéutico que puede expresarse como la relación LD₅₀/ED₅₀, prefiriéndose índices terapéuticos elevados.

[0105] La cantidad de polipéptido CocE mutante que puede combinarse con una sustancia portadora farmacéuticamente aceptable para producir una presentación farmacéutica única variará dependiendo del hospedero tratado y del modo de administración. Se observará por los expertos en el arte que el contenido unitario de agente contenido en una dosis individual de cada presentación farmacéutica no necesita constituir en sí mismo una cantidad terapéuticamente efectiva, ya que la cantidad terapéuticamente efectiva necesaria podría alcanzarse mediante administración de un número de dosis individuales. La administración del agente puede ocurrir como un episodio único o durante el tiempo de ciclo de tratamiento. Por ejemplo, un agente puede administrarse diaria, semanal, bi-semanal, o mensualmente. Para algunas afecciones, el tratamiento puede prolongarse desde varias semanas a varios meses, o incluso un año o más.

[0106] El nivel específico de dosis terapéuticamente efectiva para cualquier sujeto particular dependerá de una variedad de factores, incluida la afección inducida por la cocaína que se está tratando y la gravedad de la afección inducida por la cocaína; la actividad del polipéptido CocE mutante empleado; la composición específica empleada; la edad, el peso corporal, el estado general de salud, el sexo y la dieta del paciente; el tiempo de administración; la vía de administración; la vida media plasmática del polipéptido CocE mutante; la velocidad de excreción del polipéptido CocE mutante empleado; la duración del tratamiento; los medicamentos utilizados en combinación o coincidentes con el polipéptido CocE mutante empleado; y factores similares bien conocidos en las artes médicas (véase a este respecto, Koda-Kimble et al. (2004) Applied

Therapeutics: The Clinical Use of Drugs, Lippincott Williams & Wilkins, ISBN 0781748453; Winter (2003) Basic Clinical Pharmacokinetics, 4th ed., Lippincott Williams & Wilkins, ISBN 0781741475; Sharqel (2004) Applied Biopharmaceutics & Pharmacokinetics, McGraw-Hill/Appleton & Lange, ISBN 0071375503). Se entenderá por un facultativo experto que la utilización diaria total del polipéptido CocE mutante para uso en incorporaciones de la invención, divulgada en esta especificación, se decidirá por el médico especialista dentro del ámbito de un juicio médico acertado.

[0107] Los polipéptidos CocE mutantes descritos en esta especificación pueden usarse igualmente en combinación con otras modalidades terapéuticas. Por consiguiente, además de las terapias descritas en esta especificación, se le pueden aplicar también al sujeto otras terapias conocidas por su eficacia en afecciones particulares inducidas por la cocaína.

[0108] Los polipéptidos CocE mutantes descritos en esta especificación pueden formularse en cualquier manera convencional usando una o más sustancias portadoras farmacéuticamente aceptables y/o excipientes (véase a este respecto, Gennaro (2005) Remington the Science and Practice of Pharmacy 21st ed. Lippincott Williams & Wilkins, ISBN 0781746736). Tales formulaciones contendrán una cantidad terapéuticamente efectiva del polipéptido CocE mutante, preferiblemente en forma purificada, junto con una cantidad apropiada de una sustancia portadora, con el fin de proveer la forma de administración adecuada al sujeto. La formulación debe adaptarse al modo de administración. Los polipéptidos CocE mutantes de uso con la actual invención pueden formularse por métodos conocidos para la administración a un sujeto utilizando varias vías que incluyen, pero no se limitan a, la vía parenteral, pulmonar, oral, tópica, intradérmica, intramuscular, intraperitoneal, intravenosa, subcutánea, intranasal, epidural, oftálmica, bucal y rectal. El

polipéptido CocE mutante puede administrarse además en combinación con uno o más agentes adicionales divulgados en esta especificación y/o junto con otros agentes inertes, biológicamente activos o biológicamente inertes. Tales agentes biológicamente activos o inertes pueden estar en comunicación fluida o mecánica con el agente(s) o unirse al agente(s) por fuerzas iónicas, covalentes, de Van der Waals, hidrofóbas, hidrófilas u otras fuerzas físicas.

[0109] Los polipéptidos CocE mutantes descritos en esta especificación pueden administrarse parenteralmente, incluidas las inyecciones intravenosas, intramusculares, subcutáneas e intraperitoneales. Excipientes usados comúnmente en la aplicación parenteral de moléculas pequeñas de medicamentos, incluidos potenciadores de solubilidad, agentes osmóticos, soluciones amortiguadoras y preservantes, pueden incluirse igualmente en formulaciones de biomoléculas. La inclusión de agentes de anti-agregación y anti-adsorción, tales como tensioactivos y albúmina, cuando se formulan y aplican biomoléculas, puede agregar una estabilidad incrementada y disminuir el riesgo de que la biomolécula interactúe con una interfase, la cual puede llevar al desdoblamiento, agregación y/o precipitación. El polipéptido CocE mutante puede liofilizarse para impartirle estabilidad adicional durante el almacenamiento, y re-procesarse antes de la administración parenteral.

[0110] Preferiblemente, el polipéptido CocE mutante se pega, proporcionando en esta forma una estabilidad incrementada y una inmunogenicidad disminuída (véase más arriba).

[0111] La aplicación pulmonar de macromoléculas, tales como polipéptidos CocE mutantes, permite una administración no invasiva, relativamente fácil, al sistema circulatorio para distribución sistémica (véase a este respecto, Cryan (2004) AAPS J. 7(1) article 4, E20-41, que ofrece una revisión de la tecnología de aplicación pulmonar). Las ventajas

de la aplicación pulmonar incluyen no-invasividad, un área superficial grande para absorción ($\sim 75 \text{ m}^2$), epitelio alveolar delgado (~ 0.1 a $0.5 \text{ }\mu\text{m}$) que permite una rápida absorción, ausencia de metabolismo de primer paso, actividad proteolítica disminuída, iniciación rápida de la acción y alta biodisponibilidad. Varios dispositivos de aplicación por inhalación, tales como inhaladores de dosis medida, nebulizadores e inhaladores de polvo seco, que pueden usarse para aplicar las biomoléculas descritas en esta especificación son conocidos en el arte (por ejemplo, AERx (Aradigm, CA); Respimat (Boehringer, Alemania); AeroDose (Aerogen Inc., CA)). Los dispositivos de inhalación de polvo seco son particularmente preferidos para aplicación pulmonar de agentes a base de proteínas (por ejemplo, Spinhaler (Fisons Pharmaceuticals, NY); Rotohaler (GSK, NC); Diskhaler (GSK, NC); Spiros (Dura Pharmaceuticals, CA); Nektar (Nektar Pharmaceuticals, CA)).

[0112] Preparaciones de liberación controlada (o liberación sostenida) pueden formularse para extender la actividad del polipéptido CocE mutante y reducir la frecuencia de dosificación. Las preparaciones de liberación controlada pueden usarse igualmente para concretar el tiempo de iniciación de la acción u otras características, tales como niveles en la sangre del agente y, en consecuencia, afectar la ocurrencia de efectos secundarios. Las preparaciones de liberación controlada puede diseñarse para liberar inicialmente una cantidad del polipéptido CocE mutante, que produce el efecto terapéutico deseado, y liberar en forma gradual y continua otras cantidades para mantener el nivel de efecto terapéutico durante un período extendido de tiempo. Con el fin de mantener un nivel casi constante del polipéptido CocE mutante en el cuerpo, el agente puede liberarse de la forma farmacéutica a una velocidad que reemplace la cantidad de agente que se está metabolizando y/o excretando del cuerpo. La liberación controlada de un agente puede estimularse por varios inductores, por ejemplo, cambio en el pH, cambio en la temperatura, enzimas, agua, u otras condiciones fisiológicas o moléculas.

[0113] Los sistemas de liberación controlada pueden incluir, por ejemplo, una bomba de infusión, que puede usarse para administrar el agente en una manera similar a la empleada para aplicar insulina o quimioterapia a órganos o tumores específicos. Los agentes de la invención pueden administrarse por otros medios de liberación controlada o aplicación, que son bien conocidos por las personas de habilidad ordinaria en el arte, incluidos, por ejemplo, hidropropilmetilcelulosa, otras matrices poliméricas, geles, membranas permeables, sistemas osmóticos, medicamentos de absorción ("*depots*"), cubiertas multi-capa, micropartículas, liposomas, microesferas, o similares, o una combinación de cualesquiera de los anteriores, para ofrecer el perfil de liberación deseado en proporciones variables. Otros métodos de aplicación con liberación controlada de agentes serán bien conocidos por el profesional experto y se encuentran dentro del ámbito de la invención.

[0114] Los polipéptidos CocE mutantes pueden encapsularse y administrarse en una variedad de sistemas de aplicación de sustancias portadoras. Sistemas a base de sustancias portadoras para aplicación de agentes biomoleculares pueden: posibilitar la aplicación intracelular; adaptar las velocidades de liberación de biomoléculas/agentes; aumentar la proporción de biomolécula que alcanza su sitio de acción; mejorar el transporte del medicamento a su sitio de acción; permitir la deposición co-localizada con otros agentes o excipientes; mejorar la estabilidad del agente *in vivo*; prolongar el tiempo de permanencia del agente en su sitio de acción reduciendo la depuración; disminuir la aplicación no específica del agente a tejidos distintos al tejido diana; disminuir la irritación causada por el agente; disminuir la toxicidad debida a dosis iniciales elevadas del agente; alterar la inmunogenicidad del agente; disminuir la frecuencia de la dosificación, mejorar el sabor del producto; y/o mejorar la vida útil del producto.

[0115] Ejemplos de sistemas de aplicación con sustancias portadoras para polipéptidos CocE mutantes descritos en esta especificación incluyen microesferas (véase a este respecto, Varde & Pack (2004) Expert Opin. Biol. 4(1) 35-51), hidrogeles (véase generalmente, Sakiyama et al (2001). FASEB J. 15, 1300-1302), implantes poliméricos (véase generalmente, Teng et al (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 3024-3029), sustancias portadoras poliméricas inteligentes (véase generalmente, Stayton et al. (2005) Orthod Craniofacial Res 8, 219-225; Wu et al. (2005) Nature Biotech (2005) 23(9), 1137-1146), y liposomas (véase a este respecto, Galovic et al. (2002) Eur. J. Pharm. Sci. 15, 441-448; Wagner et al. (2002) J. Liposome Res. 12, 259-270). Preferiblemente, el polipéptido CocE mutante se encapsula en RBC (véase más arriba; Ejemplo 12).

MÉTODOS DE DETECCIÓN SELECTIVA

[0116] Otro aspecto de la invención se dirige a métodos de detección selectiva para la generación, identificación y purificación de los polipéptidos CocE mutantes termoestables. Generalmente, los polipéptidos CocE mutantes pueden diseñarse inicialmente de acuerdo con los métodos ya descritos. Tales polipéptidos diseñados pueden detectarse entonces selectivamente tomando en cuenta características preferidas, tales como retención de eficiencia hidrolítica, termoestabilidad incrementada, vida media plasmática incrementada, y/o antigenicidad reducida. Además, los polipéptidos CocE mutantes aleatorios pueden detectarse selectivamente tomando en cuenta las características deseadas.

[0117] Los métodos de detección para identificar selectivamente mutantes termoestables abarcan una amplia variedad de técnicas. El siguiente es un resumen ejemplar de un protocolo genérico. Ácido nucleico que codifica los polipéptidos CocE mutantes (generados, por ejemplo, mediante diseño racional, mutagénesis aleatoria, o mutagénesis de hospedero) se transforma en un hospedero de expresión

apropiado (por ejemplo, células de *E. coli* tales como BL21 Gold de *E. coli* (Stratagene)), y se induce expresión del polipéptido mutante de acuerdo con protocolos estándar (por ejemplo, mediante IPTG). La expresión se lleva a cabo a temperaturas para producir expresión óptima de la proteína (por ejemplo, 16°C para CocE, véase a este respecto, Ejemplo 1) durante un período de tiempo predeterminado (por ejemplo, donde sea desde 30 minutos a 24 horas o más). Alternativamente, la expresión se realiza a una temperatura incrementada (por ejemplo, la temperatura incrementada puede ser por lo menos alrededor de 35°C, por lo menos alrededor de 36°C, por lo menos alrededor de 37°C, por lo menos alrededor de 38°C, por lo menos alrededor de 39°C, por lo menos alrededor de 40°C, o incluso mayor). Preferiblemente, la temperatura incrementada a la cual se expresa el polipéptido CocE mutado es 37°C. Alrededor de esta temperatura, el polipéptido CocE tipo silvestre se escinde casi exclusivamente en cuerpos de inclusión. Células que contienen los polipéptidos mutantes expresados se detectan selectivamente tomando en cuenta la presencia de variantes termoestables de CocE.

[0118] La detección selectiva de la presencia de variantes termoestables de CocE implica generalmente la medición directa en células cultivadas, en lisados de células, o luego del rompimiento celular y aislamiento del polipéptido CocE mutante. El rompimiento celular puede incluir choque osmótico, lisis química, sonicación, y/o homogeneización, y el aislamiento del polipéptido mutante puede obtenerse mediante numerosos métodos, incluidos absorción directa a una matriz o absorción por afinidad a través del uso de anticuerpos anti-cocaína o sistemas de captura específicos de proteína de fusión. Una matriz apropiada para absorción incluye papel de nitrocelulosa, filtros, placas de microvaloración tratadas por afinidad o no tratadas, resinas de agarosa o sefarosa, y o puntas recubiertas con afinidad.

[0119] La actividad esterasa de las células cultivadas o un

polipéptido mutante aislado puede medirse posteriormente a una o más temperaturas para determinar la termoestabilidad de los mutantes. La temperatura a la cual se realiza el análisis de la actividad determina el grado de detección de la termoestabilidad. Por consiguiente, aunque los mutantes finales tendrán preferiblemente una temperatura de fusión de 45°C o más (determinada, por ejemplo, mediante dicroísmo circular), a menudo la detección selectiva inicial a 45°C no encontrará enzimas activas. Más bien, pueden llevarse a cabo varios ciclos de mutagénesis y detección selectiva, a temperaturas consecutivamente crecientes, para obtener mutantes termoestables. Por consiguiente, la detección selectiva inicial puede realizarse a 30°C y, luego de ciclos adicionales de mutagénesis, puede llevarse a cabo una detección selectiva con temperaturas incrementalmente crecientes (por ejemplo, 34°C, 37°C, 40°C, 42.5°C, 45°C, etc.), hasta obtener un mutante de termoestabilidad apropiada. Los aumentos de temperatura incremental se determinan empíricamente durante el procedimiento, y se afectan por el número de aciertos a temperaturas particulares, y la T_m determinada de los mutantes generados.

[0120] Aunque sin obligación de hacerlo, y sin querer entrar en limitaciones teóricas, sigue en esta especificación lo que se cree ser una explicación mecánica de la fusión de varias incorporaciones de los polipéptidos CocE mutantes descritos en esta especificación. Los datos de espectros CD ilustran que la fusión CD de cocaína esterasa y mutantes es irreversible, ya que enfriar a 0 grados no reforman los espectros originales (véase, por ejemplo, Ejemplo 19). Aunque no pueden verificarse parámetros termodinámicos, los espectros CD pueden utilizarse para determinar comparativamente si los mutantes son más o menos estables, si tienen estructuras secundarias diferentes o propiedades de agregación. Se cree que los polipéptidos CocE descritos en esta especificación se funden mediante un paso intermedio, es decir, los polipéptidos experimentan un proceso de fusión de 2 pasos.

[0121] La detección de la actividad esterasa puede realizarse usando una variedad de métodos, donde sustratos se acoplan a un sistema de detección específico. Sustratos apropiados para uso en determinar la actividad esterasa pueden incluir cocaína, cocaína tritiada (3H), derivados de sustrato de cocaína tales como un derivado de tio-cocaína (véase a este respecto, Figura 6), y/o sustratos que dan cuenta de actividad esterasa general tales como 4-nitrofenil acetato. El sistema de detección puede acoplarse directamente a las especificidades del sustrato, por ejemplo: escisión de cocaína no modificada puede detectarse monitorizando cambios e la absorbencia de la cocaína a 240nm (véase a este respecto, Ejemplo 4), o monitorizando cambios de pH que resultan de la acumulación del producto ácido benzoico ácido (véase a este respecto, Ejemplo 15), o mediante el uso de aptámeros de cocaína (véase a este respecto, Stojanovic, M. N., de Prada, P. & Landry, D. W. (2001) J Am Chem Soc 123, 4928-4931; Stojanovic, M. N. & Landry, D. W. (2002) J Am Chem Soc 124, 9678-9679) monitorizando cambios en fluorescencia luego de la degradación de la cocaína (véase ejemplo 15); la escisión de cocaína tritiada (3H) puede detectarse mediante acidificación y detección de producto de ácido benzoico tritiado mediante la separación por cromatografía (véase Ejemplo 1 y 15); la escisión de derivados de cocaína tales como tio-cocaína puede monitorizarse mediante la detección de grupos sulfhidrilo reactivos, mediante la adición del reactivo de Ellmany la determinación de cambios de absorbencia a 412nm (véase a este respecto, Ejemplo 15), o mediante la adición y visualización de metales pesados que reaccionan con sulfhidrilo de precipitación; la escisión de 4-nitrofenil acetato puede detectarse monitorizando cambios en la absorbencia a 420nm (véase a este respecto, Halgasova, N. et al. (1994) Biochem J 298 Pt 3, 751-755; O'Conner, C.J. & Manuel, R.D. (1993) J Dairy Sci. 76, 3674-3682).

[0122] Los polipéptidos CocE mutantes identificados a través de los procedimientos anteriores, o un análisis similar de gran productividad, pueden evaluarse además empelando procedimientos *in vitro*

descritos en esta especificación (por ejemplo, valores K_{cat} y K_m , estabilidad a 37°, temperatura de fusión (T_m), niveles de endotoxina, capacidad de degradar cocaína en plasma). Los polipéptidos CocE mutantes con actividad esterasa termoestable y/o inmunogenicidad reducida, pueden evaluarse además utilizando procedimientos *in vivo* descritos en esta especificación (por ejemplo, potencia, duración de la acción, efectos con dosificación repetida, y/o evaluación inmunológica). Preferiblemente, en los polipéptidos CocE mutantes se examina primero la disminución en magnitud de la toxicidad de la cocaína (véase a este respecto, Ejemplos 5, 7 y 8), y aquellos mutantes que reducen la toxicidad en por lo menos alrededor de 5-10 veces pueden evaluarse adicionalmente con respecto al ciclo de tiempo de acción (véase a este respecto, Ejemplo 6). Los polipéptidos CocE mutantes candidatos pueden estabilizarse adicionalmente mediante, por ejemplo, pegilación y/o encapsulación en RBC y re-evaluarse en los procedimientos ya descritos.

[0123] Habiendo descrito la invención en detalle, será evidente que son posibles modificaciones, variaciones e incorporaciones equivalentes sin apartarse del ámbito de la invención definida en las reivindicaciones adjuntas. Más aún, se observará que todos los ejemplos en la presente divulgación se proveen como ejemplos no limitantes.

EJEMPLOS

[0124] Los siguientes ejemplos no limitantes se proveen para ilustrar aún más varias incorporaciones de la invención divulgada en esta especificación. Debe observarse por los expertos en el arte que las técnicas divulgadas en los ejemplos que siguen representan métodos que los inventores han descubierto que funcionan bien en la práctica de la invención y, por consiguiente, pueden considerarse que constituyen ejemplos de modos para su práctica. No obstante, a la luz de la presente divulgación, los expertos en el arte deben observar que pueden introducirse

muchos cambios en las incorporaciones específicas que se divulgan y obtener aún un resultado parecido o similar sin apartarse del espíritu y el ámbito de la invención.

EJEMPLO 1: EXPRESIÓN DE CocE

[0125] Se estableció un método para la expresión y purificación de CocE, donde CocE se expresa en *E. Coli* como una proteína de fusión con una marca-Hisx6 del término carboxilo. El gen de la cocaína esterasa se subclonó en el vector de expresión de *E. coli* pET-22b(+) y se indujeron altos niveles de la enzima cocaína esterasa que contenía una marca histidina C-terminal luego de la adición de IPTG a 23°C. La proteína recombinante se acumuló a aproximadamente 10-15% de la proteína total. CocE se enriqueció en una columna de cobalto-quelato (Talon™ Clontech) o una columna de agarosa quelada con níquel (Pierce) en virtud de la marca Hisx6. La proteína eluida fue aproximadamente 95% pura por SDS-PAGE y coloración con azul Coomassie y, posteriormente, se resolvió por columna FPLC de cromatografía de intercambio iónico (Q-Sepharose) usando un gradiente NaCl. La proteína eluida como un pico simple fue aproximadamente 99% pura, como se indicó por SDS-PAGE y coloración con azul Coomassie (véase a este respecto, Figura 3).

[0126] La actividad enzimática se determinó mediante dos análisis: un análisis de actividad de radio-ligando en el cual cocaína tritiada se hidrolizó y luego, después de la acidificación, se separó el producto ácido benzoico tritiado a partir del clorhidrato de cocaína tritiado mediante cromatografía; y un análisis espectrofotométrico bajo condiciones similares, como se describe por Turner et al. (2002) *Biochemistry* 41, 12297-12307. Los espectros de absorción únicos de cocaína (coeficiente de extinción 6.7 L/mmol/cm, a 240 nm) permiten la observación de cocaína remanente luego de la escisión enzimática. Los porcentajes lineales ideales de descenso de la cocaína, representando velocidad, se determinaron en un lector de placas

SpectraMax 190 (Molecular Devices) usando software SOFTmax Pro (v1.13). La reacción se inició agregando 150 μL de una solución enzimática 2x a los 150 μL de una solución de cocaína 2x. Las concentraciones finales de CocE variaron desde 100 ng/mL a 20 ng/mL. Las concentraciones finales de cocaína fueron las siguientes: 250, 126, 62.5, 31.25, 15.63, 7.81, 3.91 y 1.95 μM . Para la cinética de todas las enzimas, el amortiguador fue solución salina amortiguada con fosfato, pH 7.4. Los porcentajes iniciales se ajustaron a la ecuación de Michaelis-Menten, con Kcat y Km como parámetros ajustables (GraphPad; PRISM, v4).

[0127] Como se determinó utilizando el análisis espectrofotométrico de cocaína, el polipéptido CcoE tipo silvestre purificado hidroliza la cocaína con un Kcat de aproximadamente 500 min^{-1} y un Km de aproximadamente 2 μM , lo cual es consistente con valores informados anteriormente (véase a este respecto, Turner et al. 2000).

[0128] Tales procedimientos de expresión pueden utilizarse para los polipéptidos CocE mutantes descritos en esta especificación.

EJEMPLO 2: ACTIVIDAD PLASMÁTICA DE CócE EX VIVO

[0129] La determinación *ex vivo* de los niveles de cocaína luego de la cocaína esterasa se examinó en plasma humano (University of Michigan Hospital blood bank). Se obtuvo cocaína de The National Institute of Drug Abuse (Bethesda, MD, USA). La cocaína se disolvió en agua esterilizada. Alícuotas (3 ml) de plasma humano se mantuvieron a 37°C en un baño maría durante 10 minutos antes del inicio y durante el experimento. Luego de equilibrar el plasma en un baño maría, se agregó la cocaína a una concentración final de 300 μM y se homogeneizó durante 30 segundos. Se retiraron muestras del plasma y se pusieron en un tubo de microcentrífuga que contenía el estándar interno y una solución de fluoruro de sodio saturada para impedir el metabolismo posterior de la cocaína.

Inmediatamente después de tomar la primera muestra de plasma (cocaína sola), se agregó 0.05 mg/ml CocE o vehículo CocE y se homogeneizó. Se recogieron las muestras de plasma a 1, 2, 4, 6, 8, 10, 15, 30, 45, 60 y 120 minutos después de agregar CocE. Se midieron los niveles de cocaína usando cromatografía líquida de alto rendimiento en tándem con espectrometría de masas.

[0130] Se realizó cromatografía líquida usando un sistema HPLC Surveyor (ThermoElectron Corp., Franklin, MA) con una bomba cuaternaria y autosampler configurado con un ciclo de inyección de 10 µl. La separación se logró empleando una columna Phenomex C18 3 µm 30 x 4.6 mm con una columna de guarda correspondiente (Waters Corp., Milford, MA) con un caudal de 600 µl/min. El disolvente A consistía de una solución de ácido fórmico 0.1%, y el disolvente B ácido fórmico 0.1% en acetonitrilo (alto grado de pureza; Burdick and Jackson, Muskegon, MI). Se empleó un gradiente balístico de 3 minutos con cocaína y el estándar interno co-eluyendo a 2.3 minutos.

[0131] Para la detección y cuantificación por espectrometría de masas, se usó un espectrómetro de masas triple quadrapole Finnigan TSQ Quantum Ultra AM, equipado con una fuente de ionización electrospray IonMax (ThermoElectron Corp., Franklin, MA), en ión positivo, en modo de monitorización de reacción seleccionado. Nitrógeno sirvió como el gas nebulizador y argón como el gas de colisión. Se optimizaron los caudales de gas, voltajes de aspersión y energías de colisión. Se determinaron las curvas de calibración para la cocaína con 50 nM de cocaína deuterada (cocaína D₃) como el estándar interno en muestras de plasma no tratado. Las muestras no conocidas se fijaron también con cocaína D₃. La evaluación de todas las muestras se realizó en triplicado. Las curvas estándar y las no conocidas se analizaron mediante el programa Quan Browser en software versión Xcalibur 1.4 (ThermoElectron Corp., Franklin, MA). Se construyeron curvas de calibración empleando regresión lineal de la relación área de

pico/área del estándar interno de la cocaína como una función de la concentración estándar con un factor de ponderación de $1/x$. Se aceptaron valores estándar de ajuste de curva con un valor mayor que 0.99, y los valores RSD para muestras replicadas están entre 0-10%.

[0132] Los resultados demuestran que la CocE es capaz de degradar cocaína muy rápidamente cuando las dos se mezclan brevemente en plasma humano (véase a este respecto, Figura 4). El primer punto de tiempo indica la concentración de cocaína antes de la adición de cocaína esteras o vehículo esterasa. Antes del tratamiento con esterasa, los niveles de cocaína fueron similares, pero en el término de 1 minuto de la administración de la cocaína esterasa, los niveles de cocaína disminuyeron por lo menos 100 veces a aproximadamente $2 \mu\text{M}$ comparados con la muestra de plasma tratada con vehículo. Los niveles de cocaína siguieron disminuyendo en las muestras de plasma tratadas con esterasa, cayendo por debajo de 1 microM en el punto de tiempo de 2 minutos.

[0133] Se demostró la eficiencia terapéutica de la enzima aumentando la dosis de cocaína requerida para producir efectos tóxicos luego de una inyección intravenosa única de CocE. La enzima tipo silvestre demostró una cinética rápida para la degradación de la cocaína *ex vivo* en suero de rata y suero humano. Dos mutantes inactivos de CocE no protegieron las ratas de los efectos tóxicos de la cocaína, confirmando que los efectos protectores son debidos a la actividad hidrolítica. Además, la CocE no cambió la mortalidad de WIN-35065-2, un análogo de cocaína que carece de la fracción éster de benzoilo dianizada por CocE. La caracterización *in vivo* y *ex vivo* de CocE sustenta el papel de la enzima como un antídoto apropiado a la toxicidad en humanos.

EJEMPLO 3: CocE MUTANTE TERMOESTABLE PREVISTA

[0134] El diseño racional de la CocE mutante descrita en esta

especificación se basó en simulaciones de dinámica molecular (MD). Se construyó un modelo computacional de CocE usando la estructura cristalina publicada de CocE tipo silvestre (véase a este respecto, Figura 2). Tales modelos pueden emplearse para identificar ciertas modificaciones de aminoácidos que aumentan la temperatura de fusión teórica de la proteína sin alterar la estructura en el sitio activo. La simulación MD clásica permite el estudio de la evolución en el tiempo de un sistema grande, tomando muchos pasos de tiempo sucesivos pequeños bajo fuerzas atómicas determinadas por un conjunto de funciones de interacción parametrizadas (campo de fuerza), incluidas interacciones enlazadas (enlaces, ángulos y ángulos diédricos), interacciones van der Waals no enlazadas e interacciones electrostáticas basadas o cargas atómicas netas. Debido a la forma simple de campo de fuerza la simulación MD puede llevarse a cabo durante un tiempo de simulación suficientemente largo para ofrecer propiedades significativas promediadas por el ensamble, incluso para un sistema muy grande que implica más de cien mil átomos. Así, para CocE y cada mutante propuesto, la simulación MD llevará a una estructura 3D dinámicamente promediada, razonable, del polipéptido simulado en agua.

[0135] Basado en la estructura cristalina de rayos X (código PDB 1JU3) de la cocaína esterasa bacteriana (CocE) (Larson et al. (2002) Nature 9, 17), se construyó un modelo 3D completo de CocE que se une con (-)-cocaína, apropiado para modelación computacional de su estabilidad termodinámica. Para aumentar la termoestabilidad de CocE, se implementó un método computacional en el programa RosettaDesign (Kuhlman and Baker (2000) PNAS 97, 10383) capaz de predecir mutaciones termoestabilizadoras en un múltiplo dado, minimizando al mismo tiempo cualquier cambio en el esqueleto que pudiera alterar estructuralmente la estructura del sitio activo o extinguir su flexibilidad. El método implementado en el programa RosettaDesign usa una función de energía para evaluar la idoneidad de una secuencia particular para un múltiplo dado ("*a given fold*") y un algoritmo de búsqueda Monte Carlo para muestrear el

espacio de la secuencia. Un método similar se ha empleado exitosamente por otros investigadores para aumentar la termoestabilidad de una enzima sin reducción en la eficiencia catalítica (Korkegian et al. (2005) Science 308, 857). Las cargas atómicas parciales para los átomos residuales no estándar se calcularon usando el protocolo RESP estándar implementado en el módulo Antechamber del paquete del programa Amber7 (u 8) (Case, 2002). El modelamiento computacional que utiliza el programa RosettaDesign permitió la predicción de un conjunto de mutaciones CocE calculadas como poseedoras de energía más baja y, por lo tanto, termoestabilidad incrementada (véase a este respecto, Tabla 2). Para este ejemplo, la computación consideró únicamente las mutaciones posibles en los residuos de aminoácidos con una distancia de entre 6-25 Å de la molécula de sustrato de cocaína.

[0136] Los polipéptidos CocE de mutación única identificados, calculados para estabilizar CocE en alrededor de 2.1 a alrededor de 4.5 kcal/mol incluían: L163V (SEQ ID NO: 3); V121D (SEQ ID NO: 4); SWA (SEQ ID NO: 5); Q123E (SEQ ID NO: 6); V225I (SEQ ID NO: 7); I218L (SEQ ID NO: 8); A310D (SEQ ID NO: 9); A149S (SEQ ID NO: 10); S159A (SEQ ID NO: 11); S265A (SEQ ID NO: 12); S56G (SEQ ID NO: 13); W220A (SEQ ID NO: 14); T122A (SEQ ID NO: 15); S140A (SEQ ID NO: 16); F189L (SEQ ID NO: 17); A193D (SEQ ID NO: 18); T254R (SEQ ID NO: 19); N42V (SEQ ID NO: 20); V262L (SEQ ID NO: 21); L508G (SEQ ID NO: 22); Y152H (SEQ ID NO: 23); V160A (SEQ ID NO: 24); T172R (SEQ ID NO: 25); Y532F (SEQ ID NO: 26); T74S (SEQ ID NO: 27); W285T (SEQ ID NO: 28); L146P (SEQ ID NO: 29); D533S (SEQ ID NO: 30); A194R (SEQ ID NO: 31); G173Q (SEQ ID NO: 32); C477T (SEQ ID NO: 33); K531A (SEQ ID NO: 34); R41I (SEQ ID NO: 35); L119A (SEQ ID NO: 36); K46A (SEQ ID NO: 37); y F84Y (SEQ ID NO: 38)

Tabla 2: Resumen del modelamiento computacional usando el

programa RosettaDesign con estrategia de consenso

R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Mutación de Consenso	Cambio de Energía (kcal/mol)
163	163	163	163	163	163	163	163	163	163	L163V	-4.5
121	121	121	121	121	121	121	121	121	121	V121D	-3.9
167	167	167	167	167	167	167	167	167	167	S167A	-3.9
123	123	123	123	123	123	123	123	123	123	Q123E	-3.8
225	310	310	225	225	225	225	310	225	218	V225I	-3.6
218	225	218	218	218	218	218	225	218	225	I218L	-3.5
310	218	225	310	310	310	310	218	310	152	A310D	-3.4
149	149	152	149	149	149	149	149	149	310	A149S	-3.3
159	159	140	159	159	159	159	159	159	149	S159A	-3.3
189	265	149	265	265	265	265	265	189	265	S265A	-3.3
265	140	265	56	56	56	56	220	265	159	S58G	-3.2
56	220	159	220	220	220	220	122	56	220	W220A	-3.2
220	122	220	122	122	122	122	140	220	56	T122A	-3.1
122	189	122	140	140	140	140	189	122	122	S140A	-3.1
140	193	189	189	189	189	189	193	140	140	F189L	-3.1
254	42	193	193	193	193	193	42	254	189	A193O	-3.1
262	262	42	254	254	254	254	254	42	193	T254R	-3.1
508	508	262	42	42	42	42	262	262	262	N42V	-3.0
152	152	508	262	282	262	262	508	508	508	V262L	-3.0
160	160	198	508	508	508	508	152	152	198	L508G	-2.9
74	198	160	152	152	152	152	160	160	254	Y152H	-2.9
172	74	74	160	160	160	160	198	198	74	V160A	-2.8
193	172	172	198	198	74	198	74	172	160	T172R	-2.8
532	532	532	532	74	172	74	172	74	172	Y532F	-2.7
146	146	56	74	172	532	172	532	193	532	T74S	-2.7
285	285	285	172	532	146	532	146	532	285	W285T	-2.6
533	290	146	285	146	285	146	285	285	290	L146P	-2.6
173	254	533	146	285	533	285	533	146	146		
194	533	173	533	533	173	533	173	533	533	D533S	-2.5
477	56	194	173	194	194	173	194	173	173	A194R	-2.4

531	173	200	194	200	290	194	200	194	194	G173Q	-2.4
42	194	477	200	290	477	200	290	200	200	C477T	-2.4
119	200	531	290	477	531	477	477	290	477	K531A	-2.4
200	477	305	477	531	200	531	531	477	531		
41	531	41	41	41	41	41	41	531	42	R41I	-2.2
46	41	119	119	119	119	119	119	41	119	L119A	-2.2
84	119	46	46	173	46	46	46	119	41	K46A	-2.1
305	46	84	84	46	84	84	56	46	46	F84Y	-2.1
478	57	158	478	84	478	305	84	84	57		
57	84	307	57	478	57	478	307	478	84		
87	478	478	142	57	87	57	478	57	158		
142	87	57	263	87	142	87	57	142	478		
263	142	142	307	142	263	142	142	263	142		
307	263	263	78	263	307	263	263	307	263		
78	307	78	257	307	78	307	78	78	78		
257	78	257	531	48	257	78	257	257	257		
290	257	290	49	78	49	257	49	49	49		
291	201	49	201	257	305	201	201	201	201		
49	49	201	305	45	291	290	412	305	307		
176	291	291	412	49	176	291	291	412	291		
45	305	412	291	201	45	49	176	291	176		
54	176	176	176	305	54	176	45	45	305		
406	45	254	45	291	406	45	413	176	45		
50	54	45	413	176	50	54	54	413	54		
	406	54	54	54		406	305	54	406		
	50	413	50	406		50	406	406	50		
		406	406	50			50	50			
		50									

**EJEMPLO 4: PARÁMETROS CINÉTICOS DE CocE MUTANTE
T172R Y S159A**

[0137] CocE tipo silvestre y polipéptidos CocE mutantes T172R y S159A se analizaron con respecto a eficiencia catalítica.

[0138] Se realizó mutagénesis dirigida al sitio (QuickChange™, Invitrogen) de CocE para generar el polipéptido CocE mutante S159A (SEQ ID NO: 11). Las técnicas de clonación y expresión utilizadas para producir el S159A fueron las mismas del Ejemplo 1, excepto lo indicado en contrario. Se amplificó el gen CocE a través de Reacción en Cadena de Polimerasa (PCR - *Polymerase Chain Reaction*) en la presencia de cebadores que contienen la mutación requerida (Integrated DNA Technologies, Inc.). La mutación específica se volvió a clonar en el plásmido de expresión y se determinó la secuencia nucleotídica de estos plásmidos para verificar la presencia de la mutación.

[0139] El mutante T172R se generó por PCR traslapante utilizando cebadores 5' y 3' que contenían la mutación T172R específica, así como un sitio de enzima de restricción Sac II para fácil detección del gen mutado. Las parejas de cebadores CocE 20-5'F-Nde I (5' GATATACATATGGTGGACGGGAATTAC 3') y T172R-3'R (5' CAGACCTCGACGTGATGAGCCCGCGGCCTATGAGAGCTGACCAG C 3') así como CocE-1800-3'R (5' GTGGTGCTCGAGTCGCTTGATAATCG 3') y T172R-5'F (5' GCTGGTCAGCTCTCATAGGCCGCGGGCTCATCACGTCGAGGTCT G 3') se amplificaron mediante PCR empleando la enzima Pfu de alta fidelidad (Stratagene) con una temperatura de hibridación de 55°C. Los productos PCR resultantes se combinaron y se re-amplificaron, generando un gen CocE de longitud total que codificaba la mutación T172R. El gen se digirió con Nde I y Xho I; se subclonó en el vector de expresión y se secuenció en su totalidad para verificar tanto la presencia de la mutación como la ausencia de mutaciones adicionales por error de copia de PCR.

[0140] Plásmidos que contenían las mutaciones se transformaron en células BL21 de *E. coli* y la enzima inducida por IPTG se purificó en Ni-Agarosa. Las proteínas expresadas se probaron luego con respecto a actividad enzimática y termoestabilidad a 37°C. Se cuantificó la

actividad enzimática empleando el análisis espectrofotométrico como se describe en el Ejemplo 1. Igualmente se probó la termoestabilidad mediante el análisis espectrofotométrico por pre-incubación de la enzima tipo silvestre y de los mutantes a 37° C durante varios tiempos. Adicionalmente, se analizó la naturaleza de la termoinestabilidad de la enzima tipo silvestre y del mutante T172R por electroforesis en gel de poliacrilamida bajo condiciones desnaturalizantes y no desnaturalizantes. Concretamente, mutante y enzima tipo silvestre a 0.1mg/ml se incubaron a 37°C durante varios puntos de tiempo, se enfriaron a 4°C, se mezclaron con colorante de carga SDS que contenía β -mercaptoetanol y se corrieron en geles 10% SDS-PAGE (condiciones desnaturalizantes) o se corrieron en geles de 10% de poliacrilamida nativa (condiciones no desnaturalizantes) a 4°C. Los geles se fijaron con 10% metanol, 7% ácido acético durante 30 minutos, y luego se colorearon con colorante de gel de proteína Sypro-Ruby (Molecular Probes, Invitrogen) durante 3 horas. La coloración de la proteína se visualizó bajo luz UV utilizando un Alphamager™ 3400 (Alpha Innotech). Finalmente, se determinó la temperatura exacta de fusión de la enzima tipo silvestre y del mutante T172R mediante dicroísmo circular usando un espectropolarímetro JASCO-810 operado por un programa de análisis JASCO V500/FP-750 para Windows. Los espectros CD se cuantificaron en miligrados y se normalizaron contra amortiguador PBS.

[0141] Los resultados del análisis espectrofotométrico mostraron que T172R tenía V_{max} y K_m elevados a temperatura de la sala y un V_{max} y K_m sumamente elevados a 37°C, comparado con CocE tipo silvestre (véase a este respecto, Tabla 3; Figura 5). En efecto, el V_{max} y K_m de T172R a 37° C fue comparable con el V_{max} y K_m de CocE tipo silvestre a temperatura de la sala. La CocE mutante S159A mostró un V_{max} y K_m ligeramente elevados a 37°C, pero un V_{max} y K_m disminuidos a temperatura de la sala, comparada con la CocE tipo silvestre. La electroforesis bajo condiciones desnaturalizantes (Figura 6a) indicó una banda de proteína única para la enzima tipo silvestre y T172R de

aproximadamente 65000 Da, sin importar la temperatura de incubación, indicando que la degradación proteolítica no describe adecuadamente el mecanismo de la inestabilidad térmica. Los geles no desnaturizantes (Figura 6b) mostraron una banda de proteína única para la enzima tipo silvestre antes de la incubación a 37°C; sin embargo, luego de la incubación a 37°C, se observa aparecer especies de peso molecular más alto a medida que la banda original se desdibuja. Estos supuestos agregados de proteína pueden observarse igualmente para el mutante T172R; no obstante, el tiempo hasta la agregación luego de la incubación a 37°C es mayor, y en este procedimiento el mutante T172R se aproximó a tener una vida media 8x más prolongada a 37°C que la enzima tipo silvestre. El análisis de la temperatura de fusión de la proteína mediante dicroísmo circular (Figura 7) indicó que cambios sensibles a la temperatura en la estructura terciaria de la proteína estaban ocurriendo en el rango cercano a UV del espectro (entre 260nm y 320nm). El ajuste de curva de los cambios sensibles a la temperatura en esta región del espectro indicó que la CocE tipo silvestre tiene una temperatura de fusión de 36.15°C, comenzando la desnaturalización detectable a aproximadamente 30°C. Se determinó que el mutante T172R tenía una temperatura de fusión de 41.43°C, empezando la desnaturalización detectable a aproximadamente 28°C. En consecuencia, se determinó el cambio único de aminoácido de tirosina a arginina en el aminoácido 172, con un aumento 2.8kCa/mol estimado en termoestabilidad tenía una temperatura incrementada en 5 grados completos, comparado con la CocE tipo silvestre.

TABLA 3: Parámetros cinéticos para T172R, S159A y CocE tipo silvestre.

	S159A	S159A (40min @ 37°C	T172R	T172R (40min @ 37°C	CocE tipo silvestre	CocE tipo silvestre (40 min@ 37°C
Vmax	876.6	130.7	1466	1267	1264	94.06

Km	43.65	15.69	88.20	78.80	71.81	12.30
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EJEMPLO 5: PREVENCIÓN POR COCE TIPO SILVESTRE IN VIVO DE LA MORTALIDAD DE LA COCAÍNA EN RATAS

[0142] Para determinar la actividad esterática de la CocE *in vivo* se implementó un modelo de roedores de la toxicidad aguda de la cocaína. Cuando se trataron con altas dosis de cocaína, las ratas exhibieron primeramente convulsiones seguidas por cesación de la respiración y el movimiento. La dosis tóxica mínima de cocaína, cuando se administró intraperitonealmente, producirá una fatalidad en el término de 15 minutos del tratamiento.

[0143] La protección contra la mortalidad, inducida por la cocaína, mediante la CocE tipo silvestre se determinó y comparó con los efectos protectores de BChE humana. La actividad esterática de la CocE se estableció evaluando la actividad de dos enzimas mutantes, cada una de las cuales carecía de uno de tres aminoácidos en el sitio activo. Adicionalmente, se determinó la actividad de una enzima tipo silvestre, modificada por una modificación covalente de Ser17 en el sitio activo, con fluoruro de fenilmetil sulfonato (PMSF - *phenylmethyl sulphonate fluoride*). Se demostró que la degradación esteárica de la cocaína era el mecanismo de los efectos protectores de la CocE verificando si la enzima protegía contra la toxicidad inducida por WIN 35065-2 (Madras et al. (1989) J Pharmacol Exp Ther 251, 13-141), un análogo de cocaína que carece del puente éster en el sitio propuesto de hidrólisis enzimática.

[0144] Ratas machos Sprague-Dawley (300 gramos) (Harlan Sprague Dawley, Indianapolis, IN) se alojaron en jaulas de tres animales. Luego de la implantación quirúrgica de un catéter yugular, todas las ratas se alojaron individualmente hasta la terminación del experimento. Las ratas se mantuvieron en un ciclo de 12 horas de luz/oscuridad, encendiendo las

luces a 7:30 a.m. y disponiendo de alimento y agua *ad libitum*. Luego de anestesiar las ratas con clorhidrato de ketamina (100mg/kg, i.p.) y xilazina (10 mg/kg, i.p.), se implantaron catéteres intravenosos (tubos Micro-renetano, 15 cm, MRE-040, Braintree Scientific Inc., Braintree, MA) en la vena yugular derecha. Aproximadamente 3 cm del catéter se insertaron en la vena; el tubo restante se pasó subcutáneamente a la espalda, donde salió de una incisión practicada entre las paletillas. El tubo expuesto se tapó con una pieza de 1 cm de acero inoxidable (0.28 de diámetro, Small Parts Inc., Miami, FL). Los catéteres se lavaron diariamente con 0.5 ml de solución salina heparinizada (50 U/ml) para mantener la patencia del catéter. Luego de la cirugía, se dejó que las ratas se recuperaran durante una semana. Cada rata se utilizó para un solo experimento, y todos los grupos experimentales constaban de 6-8 ratas.

[0145] Para determinar la dosis efectiva mínima de CocE bloqueaba las convulsiones inducidas por la cocaína y la muerte, se administraron 0.1, 0.32, o 1.0 mg de CocE o vehículo (solución salina amortiguada con fosfato, PBS - *phosphate buffered saline*) intravenosamente un minuto después de 180 mg/kg de cocaína (i.p.). Para determinar los límites catalíticos de la CocE, se administraron dosis crecientes de cocaína (100, 560, 1000 mg/kg, i.p.) un minuto antes de 1.0 mg CocE (i.v.). Se administraron mutantes y CocE bloqueada en PMSF (1mg, i.v.) un minuto antes de 180 mg/kg de cocaína (i.p.). Se administró igualmente CocE (1.0 mg, i.v.) un minuto después de la dosis mínima de WIN-35065-2 (560 mg/kg, i.p.). CocE (1.0 mg, i.v.) se aplicó antes y después de la cocaína (100 mg/kg, i.p.) para determinar la vida media *in vivo* de la esterasa. Todas las inyecciones intravenosas fueron seguidas por un lavado con solución salina heparinizada (0.5 ml). Luego del tratamiento, se observaron las ratas en cuanto a convulsiones y muerte. Se registraron el número de episodios convulsivos, la duración de cada episodio y el tipo de convulsión. La muerte se definió como la cesación de movimiento y respiración observados. Se calculó el porcentaje de animales en cada grupo

experimental que exhibían convulsiones y mortalidad. Seguidamente se calculó la media de error estándar porcentual para cada punto de datos.

[0146] Los resultados mostraron que, en el modelo con roedores de toxicidad aguda, la cocaína indujo convulsiones, dependientes de la dosis, y muerte en las ratas; la muerte se observó en menos de 15 minutos después de la en 100% de los animales a los que se administraron 100 mg/kg de cocaína (véase a este respecto, Figura 9). CocE (1.0 mg) infundida después de la administración de la cocaína produjo un cambio de 10 veces en la curva de efecto de la dosis de la cocaína sobre la toxicidad (véase a este respecto, Figura 9), de modo que se requirieron 1000 mg/kg de cocaína para superar las propiedades catalíticas protectoras de la CocE. Este régimen de tratamiento se asemeja muy de cerca a situaciones de toxicidad humana, donde el antídoto a sobredosis se da únicamente después que la cocaína se ha ingerido, inhalado, o inyectado.

[0147] La CocE tipo silvestre mostró eficiencia catalítica superior sobre la BChE humana. Aplicada un minuto antes de 180 mg/kg de cocaína, 1 mg de CocE ofreció una protección del 100% contra la mortalidad inducida por la cocaína (véase a este respecto, Figura 10), mientras que una dosis equivalente molar de 10 veces de BChE humana (13 mg) no ofreció protección, similar a una dosis 10 veces más baja de CocE (0.1 mg) (véase a este respecto, Figura 10).

[0148] Ambas mutaciones de la CocE (Ser117Ala o Tyr44Phe) carecieron de actividad *in vivo* y, por lo tanto, no tuvieron efectos protectores (Figura 11b). Más aún, la enzima tratada con PMSF eliminó igualmente el efecto protector de la CocE contra la cocaína (Figura 11b). Adicionalmente, el efecto letal del análogo de cocaína no hidrolizable, WIN 35065-2, no fue superado por el tratamiento con CocE (Figura 12). Basándose en estudios de protección *in vivo* realizados con preparaciones catalíticamente inactivadas de la enzima (mutantes tratados con PMSF e

inactivos de CocE), es claro que los efectos protectores de la enzima son debidos a su habilidad de hidrolizar cocaína. Tomados en conjunto, estos datos satisfacen las evaluaciones *in vitro* de la actividad esteárica de la CocE y confirman el mecanismo de protección de la enzima contra la mortalidad inducida por la cocaína *in vivo*.

EJEMPLO 6: EFECTOS DEPENDIENTES DEL TIEMPO DE LA CocE TIPO SILVESTRE

[0149] Se examinaron los efectos de administrar CocE tipo silvestre antes de la dosificación de cocaína. El modelo de toxicidad en ratas fue como se describe en Ejemplo 5. La CocE tipo silvestre se aplicó 100, 30, 10, 3 y 1 minutos antes y 1 y 6 minutos después de la administración de la cocaína. Se realizaron extracciones compuestas de muestra de plasma humano en 100% de acetronitrilo (volumen 3x), se incubaron durante aproximadamente 15 minutos, se centrifugaron a 13.000 rpm durante 4.5 minutos, y se recogió el sobrenadante resultante. Los extractos se concentraron en un Savant Speed Vac (ThermoElectron Corp., Franklin, MA) para retirar el acetronitrilo. Las muestras extraídas se reconstituyeron en agua y se diluyeron 10-1.000 veces.

[0150] Muestras de plasma humano se fijaron con 300 uM de cocaína y se mantuvieron a 37°C. Se tomó una alícuota de plasma antes de la adición de cocaína esterasa o vehículo de esterasa, y otra alícuota se recogió 1 minuto luego de la administración de esterasa. Las alícuotas de plasma se mezclaron inmediatamente con el estándar interno (cocaína-D3) y una solución de fluoruro de sodio saturado para impedir el metabolismo posterior de la cocaína. Se practicaron extracciones de tejido y se cuantificaron los niveles de cocaína y estándar interno por HPLC con espectrometría de masas en tándem.

[0151] Para la inactivación dependiente del tiempo de CocE *in*

vitro, se incubó enzima CocE purificada (a 250 ng/ml) en solución amortiguadora de análisis en la ausencia de cocaína a 37°C durante varios tiempos. Luego de la incubación a 37°C las muestras se pusieron en hielo. Para evaluar el efecto de la temperatura sobre la actividad de la CocE, las muestras se incubaron con (-)cocaína a diferentes concentraciones como se indica con una concentración enzimática final de 125 ng/ml. El porcentaje de decrecimiento de la (-)-cocaína a A240 se cuantificó en un lector multiplacas. Los datos se ajustaron a un decrecimiento exponencial único usando Kaleidagraph™ (software Synergy) produciendo un $t_{1/2}$ de 13.2.

[0152] Se descubrió que la CocE tipo silvestre tenía efectos protectores dependientes del tiempo; 100% de las ratas se salvaron cuando se trataron con CocE (1 mg) 1 minuto antes de la cocaína, sobreviviendo únicamente el 66% y el 32% de las ratas cuando se trataron con CocE 30 y 1 minutos antes de la cocaína, respectivamente (véase a este respecto, Figura 14). Los efectos protectores de la CocE se eliminaron cuando las ratas se trataron 100 minutos antes de la cocaína. Este efecto dependiente del tiempo se debe muy probablemente a la desactivación térmica de la enzima *in vivo*. En el plasma de rata, se encontró que la CocE tenía una vida media notablemente corta (alrededor de 10 minutos, Figura 13), muy probablemente debido a la sensibilidad a cambios en pH y temperatura. Adicionalmente, datos preliminares *in vitro* con la enzima purificada sugieren que la CocE experimenta una inactivación dependiente de la temperatura con un $t_{1/2}$ de aproximadamente 15 minutos a 37° C.

[0153] Dados estos datos, se puede que un 1 mg de dosis de CocE administrada 30 minutos antes de la cocaína, decrecerá 3 vidas medias, dejando 0.25 mg de enzima tipo silvestre en la circulación natural cuando se administra cocaína.

[0154] Estudios previos han sugerido que concentraciones letales en la sangre de cocaína en la rata varían entre 50-128 μ M (Mets B

and Virag L(1995) *AnesthAnalg* 81, 1033-1038; Mets et al. (1999) *Life Sci* 65, 1317-1328), y niveles pico en el plasma de cocaína ocurren alrededor de 13 minutos después de una inyección intraperitoneal (Sun et al. (2002) *J Pharmacol Exp Ther* 302, 710-716). Con base en informes de la cinética de la administración de cocaína intraperitoneal (Sun et al. (2002) *J Pharmacol Exp Ther* 302, 710-716), se calcula que 100 y 320 mg/kg de cocaína producen una concentración pico de cocaína en la sangre de 35 μM y 113 μM , respectivamente. Concentraciones letales de cocaína son de una magnitud similar en humanos (20-200 μM) (Finkle BS and McCloskey KL (1978) *J Forensic Sci* 23, 173-189; Wetli and Wright (1979) *J Am Med Assoc* 241, 2519-2522). Como 1 mg de CocE tipo silvestre salvó las ratas tratadas con estas dosis de cocaína (Figura 9), puede predecirse justificadamente que la enzima protegerá contra la toxicidad de la cocaína en el humano. Adicionalmente, los niveles de cocaína medidos usando espectrometría de masas en tándem con cromatografía líquida de alto rendimiento en plasma humano fijado con 300 μM de cocaína, una concentración que excede los niveles tóxicos de cocaína informados, y luego tratado con CocE (un equivalente molar de nuestra dosis *in vivo* de 1.0 mg), redujo la concentración de cocaína a aproximadamente 2 μM en menos de un minuto (Figura 10).

[0155] La protección contra una dosis LD_{50} de cocaína en ratas requiere un tratamiento de 10 mg/kg de BChE (Lynch et al. (1997) *Toxicol Appl Pharmacol* 145, 363-371), asumiendo que la enzima se distribuya análogamente en el humano, un individuo de 70 kg necesitaría una dosis de 700 mg de BChE exógena para protegerlo contra una sobredosis. Además, no existe evidencia de que la BChE pueda actuar para revertir la toxicidad de la cocaína cuando se administra después de la cocaína, una característica necesaria de un antídoto para la toxicidad de la cocaína.

[0156] Queda así demostrado que una dosis de 1 mg de CocE tipo silvestre en una rata de 300 gramos es suficiente para proteger contra

una dosis de cocaína que excede la LD₁₀₀ (Figura 9). Adicionalmente, la enzima administrada antes, y más importante aún, hasta 6 minutos después de la dosis LD₁₀₀ de cocaína, brindó protección de la toxicidad (Figura 14). La CocE metabolizó concentraciones de cocaína en el suero en 150 veces en menos de un minuto (Figura 15).

[0157] Dados estos datos, se predice que 250 mg de CocE administrados a un humano de 70 kg luego de la ingestión tóxica de cocaína liberarán al individuo de una muerte cierta. Lo anterior demuestra que la CocE tipo silvestre es una molécula eficiente anti-cocaína pero que el corto tiempo de actividad de la enzima bajo condiciones fisiológicas limita su valor terapéutico. Tales resultados señalan la importancia de ampliar la termoestabilidad de la CocE tipo silvestre.

EJEMPLO 7: PREVENCIÓN POR CocE MUTANTE T172R IN VIVO DE LA LETALIDAD DE LA COCAÍNA EN RATAS

[0158] La actividad hidrolítica de la CocE tipo silvestre y la CocE mutante T172R se caracterizó y confirmó *in vivo* evaluando su capacidad de prevenir la mortalidad inducida por la cocaína en ratas.

[0159] El tratamiento de los animales fue como se describe en Ejemplo 5. Se administraron dosis crecientes de cocaína (i.p.) a las ratas y, un minuto después, se administraron por vía intravenosa CocE tipo silvestre (0.32 mg), CocE T172R mutante (0.32 mg), o vehículo. Todas las inyecciones intravenosas fueron seguidas por un lavado heparinizado (0.5 ml). Después del tratamiento, se observó la muerte de las ratas. El tiempo hasta la muerte se registró para la dosificación de 1g/kg de cocaína. CocE tipo silvestre y CocE T172R (0.32 mg) se aplicaron en varios tiempos precedentes (1, 10, 30 y 60 minutos) a la administración de 320 mg/kg i.p. de cocaína, monitorizando entonces las ratas en cuanto a muerte.

[0160] Los resultados demuestran la capacidad de la CocE intravenosa y, especialmente, la CocE mutante de revertir o prevenir los efectos letales producidos por la cocaína. La CocE tipo silvestre aplicada un minuto después de la administración de la cocaína evitó la mortalidad con dosificaciones de cocaína, que mataron las ratas de control, y no ocurrió mortalidad del 100% hasta las dosificaciones de cocaína de 1g/kg (véase a este respecto, Figura 16). Las ratas tratadas con CocE mutante T172R fueron capaces de tolerar dosificaciones incluso mayores, resultando el 1g/kg de cocaína en únicamente alrededor de 70% de mortalidad. Además, los efectos profilácticos de la CocE mutante T172R fueron más prolongados que la CocE tipo silvestre (véase a este respecto, Figura 17).

EJEMPLO 8: DOSIS REPETIDA DE CocE IN VIVO EN RATONES

[0161] Se realizaron estudios adicionales del efecto de la CocE sobre la toxicidad en el ratón, particularmente con respecto a dosis repetidas.

[0162] El modelo de toxicidad animal fue similar al descrito previamente, excepto como se describe. Se emplearon ratones machos NIH Swiss. Para inyección intravenosa en la cola, los ratones se ponen en una pequeña cámara de reclusión que deja libre su cola. Se ubica una lámpara de calor con una bombilla infrarroja de 250w en una distancia de alrededor de 4 pulgadas de la cola, y se dejó durante un par de minutos. A continuación la cola se limpia frotándola con alcohol y se inserta una aguja deslizando de precisión 30G 1/2 (Fisher Scientific) en una de las venas laterales para infusión. Para verificar si la aguja está en la vena se infunde una pequeña cantidad de droga, [y] si está en la ubicación correcta, la solución se difundirá fácilmente sin ninguna indicación de localización subcutánea incorrecta, que aparece en color blanco en el sitio de inyección. Para caterización intravenosa, ratones machos NIH Swiss se anestesiaron

con 100 mg/kg de ketamina y 10 mg/kg de xilazina, co-administradas i.p. Cuando los ratones ya no responden más a la presión en la pata, se rasura el cuello y se prepara frotándolo alternadamente con betadina y alcohol. Bajo condiciones de limpieza, se practica una incisión en el cuello transversal derecho y se aísla la vena yugular externa. Se inserta un catéter en la vena con la ayuda de un microscopio disecante al nivel de la aurícula derecha, y se asegura en la vena con nylon, 4-0 suturas y adhesivo de tejido, 3M Vetbond (3M Animal Care Products, St. Paul, MN). Los catéteres son un tramo corto de tubo Tygon con un diámetro interno de 0.010 pulgadas y un diámetro exterior de 0.030 pulgadas. (Small Parts, Inc., Miami Lakes, FL). Se practica una pequeña incisión en el centro de la espalda del animal, y se inserta subcutáneamente un trocar con salida en el sitio de incisión ventral. El catéter se hala entonces a través del trocar y se saca de la espalda del animal donde se mantiene en su sitio con material de sutura de nylon y el adhesivo de tejido. Una corta pieza de alambre de acero con un diámetro de 0.011 pulgadas (Small Parts, Inc., Miami Lakes, FL) se inserta en el extremo del catéter. Se cierra la incisión ventral con material de sutura 4-0 Vicryl y el ratón se pone bajo una lámpara de calor para su recuperación. Aproximadamente una hora después, el ratón se devuelve a su jaula de residencia.

[0163] A los ratones que sobrevivieron una primera administración de la combinación de 0.32 mg CocE y 320 mg/kg de cocaína se les aplicó esta combinación una vez más, 14 días después de la administración inicial. Todos los ratones sobrevivieron esta segunda administración. Todos los ratones sobrevivieron también una tercera administración 21 después de la primera combinación de dosis. La efectividad de la administración repetida sugiere que no se está montando una respuesta inmunitaria fuerte a la CocE en esta preparación, quizá debido a su rápida depuración.

[0164] Los resultados mostraron que un cambio dramático a la

derecha en la potencia de la cocaína en producir la muerte fue facilitado por 0.32 y 1.0 mg/kg de cocaína (véase a este respecto, Figura 18). La latencia a la muerte se amplió apreciablemente por la CocE: luego de la administración de 180 mg/kg i.p. de cocaína, los ratones murieron generalmente en alrededor de 3 minutos. Luego de una dosis de 1.0 gm/kg de cocaína más 1.0 mg de CocE, la muerte ocurrió en 28 minutos en promedio. Los datos se examinaron asimismo en cuanto a la capacidad de la hBChE de bloquear la toxicidad de la cocaína. Con una dosis de 1 mg, la hBChE produjo un ligero aumento en la dosis de cocaína necesaria para matar los ratones (véase a este respecto, Figura 18). La enzima es notablemente menos efectiva que la CocE. Aunque dosis mayores de cocaína fueron necesarias para matar los ratones luego de la administración de la hBChE, el tiempo hasta la muerte no se modificó por esta enzima.

EJEMPLO 9: VIDA MEDIA IN VIVO Y PRUEBAS DE BIODISTRIBUCIÓN

[0165] La vida media *in vivo* y la biodistribución de los polipéptidos CocE mutantes se examinan en ratones BALB/c. Concretamente, ^{125}I se etiqueta a los residuos tirosina de CocE mutante utilizando un método bien establecido de yodinación con cloramina-T (Hunter, WM and Greenwood, FC (1962) Nature 194, 495-496). Los ratones BALB/c se inyectan a través de las venas de su cola con 0.1 mL de CocE mutante etiquetada con 0.5 μCi ^{125}I o los conjugados CocE mutante-PEG. Cada grupo experimental se componía de 24 ratones. Se sacrifican tres ratones por dislocación cervical en intervalos de tiempo de 15, 30, 90 minutos, 3, 12, 24, 48 y 72 horas luego de la inyección de la droga. Se toman muestras de sangre, así como muestras de tejido de hígado, pulmón, corazón, riñón y bazo, se pesan y se miden en cuanto a radioactividad usando un contador gamma. Las muestras de sangre se centrifugan también, y se recogerán los sobrenadantes y se someten a conteo para calcular las radiomarcas asociadas con el plasma. La relación del pico a la del estándar interno se emplea como parámetro de análisis. Se calculan los

parámetros PK usando el programa de computador de mínimos cuadrados no lineales KINFT (Kaltenbach, ML and Vistelle, R (1994) Anticancer Research 14, 2375-2377) ajustando los datos de radioactividad plasmática a una ecuación bi-exponencial (Gibaldi, M. and Perrier, D. (1982) Pharmacokinetics):

$$A(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

[0166] Donde $A(t) = \%ID/mL$ plasma e $ID =$ dosis inyectada. k_2 se usará para calcular el tiempo de eliminación de primer orden $t_{1/2}$. El área bajo la curva (AUC - *area under the curve*) de la curva concentración del plasma-tiempo, el volumen en estado de equilibrio de la distribución (V_{ss}), depuración del plasma total (CI) y el tiempo medio de residencia (MRT - *mean residence time*) se calculan de A_1 , A_2 , k_1 , k_2 y el peso corporal (kg) del ratón, como se describe por Gibaldi and Perrier, 1982). El producto permeabilidad-área superficial del órgano (PS - *permeability-surface*) se calcula como:

$$PS = [V_d - V_o] C_{p(60min)} / AUC_{(0-60min)}$$

[0167] Donde $CP_{(60min)}$ es la concentración plasmática terminal (dpm/ μL) a 60 minutos después de la inyección, V_d es el volumen de tejido de distribución determinado de la relación de desintegraciones por minuto por gramo de tejido a $CP_{(60 min)}$, y V_o es el volumen plasmático del órgano. El suministro al órgano de las muestras se determina como:

$$\%ID/g = PS \times AUC_{(0-60min)}$$

[0168] Donde $\%ID/g$ es el porcentaje de dosis inyectada absorbida por gramo de órgano.

EJEMPLO 10: INMUNOLOGÍA

[0169] La CocE puede usarse en adyuvante incompleto de

Freund (IFA – *incomplete Freund's adjuvant*) para inmunizar los ratones (véase Tabla 4). Se estableció un ELISA directo específico de anticuerpos CocE por un protocolo estándar. CocE (1 ug/ml) se utilizó para recubrir una placa de micro-valoración de 96 pocillos empleando una solución amortiguada con borato (1.5 M NaCl, 0.5 M H₃BO₃, 1.0 M NaOH) para resuspender la cocaína esterasa (50 uL/pocillo). Las placas recubiertas se dejaron de un día para otro a 4°C. El amortiguador de recubrimiento se retiró a la mañana siguiente y las placas se bloquearon con 2% suero de cabra normal en PBS durante 1 hora a 37°C y se lavaron 3 veces. Se diluyó en serie suero de los distintos grupos de ratones en 50 µL de PBS en los pocillos en un rango de 10² a 10⁷ y se corrieron en duplicado. Las placas se cubrieron e incubaron durante 30 minutos a 37°C. Posteriormente, las placas se lavaron 3 veces y [se agregaron] 50 µL/pocillo de anticuerpo etiquetado con peroxidasa IgG anti-ratón en cabra diluido a 1:400. Las placas se lavaron entonces 3 veces y se agregaron a cada pocillo 100 µL de solución de sustrato de peroxidasa (OPD disuelto en amortiguador de citratelfosfato). Luego de una incubación de 5-10 minutos (basándose en el desarrollo de color en los controles positivos), se detuvo la reacción usando 3M H₂SO₄ (50 µL/pocillo). Las placas se leyeron a 490 nm y se determinó la valoración por la dilución máxima que mostró incrementos sobre la absorbencia de fondo. Se derivaron controles positivos inmunizando ratones Balb/c con 100 µg en 100 µL de cocaína esterasa emulsionada en adyuvante incompleto de Freund (IPA) mediante inyección intraperitoneal (IP). En el grupo positivo 1 se aisló el suero de los ratones inmunizados 2 semanas. En el grupo positivo 2 los ratones inmunizados se reforzaron usando 100 µg en 100 µL mediante inyección IP 2 semanas de la inmunización pos-primaria y se recolectó el suero luego de una semana adicional (3 semanas pos-primaria).

[0170] Se derivaron valoraciones elevadas de los dos grupos de control positivos inmunizados con CocE, 10⁵ y 10⁶, respectivamente. Las valoraciones de anticuerpo de los animales a los que se administró CocE i.v.

durante exposiciones a cocaína demostraron valoraciones detectables pero relativamente bajas, comparadas con los animales de control positivo inmunizados por CocE más IFA. El suero recogido de animales inmunizados una vez con CocE/IFA demostró una alta valoración de 10⁵, mientras que el suero de animales a los que se aplicó un refuerzo adicional tuvo una valoración más alta de 10⁸ (3 ratones/grupo). El suero recogido de estos animales servirá como controles positivos para todos los experimentos de valoración subsiguientes.

TABLA 4: Valoraciones CocE de ratones inmunizados

Grupo	Protocolo	Valoración (dilución log10 +/-SE
Positivo 1 (n=3) 2 semanas	Inmunizados (IFA/IP)	5
Positivo 2 (n=3) 3 semanas	Inmunizados / refuerzo (IFA/IP)	6
Expuestos y tratados (3x)	CocE administrada con cocaína	3.33 +/- 0.333
Expuestos y tratados (4x)	CocE administrada con cocaína	3.5 +/- 0.5

EJEMPLO 11: PEGILACIÓN DE CocE

[0171] La conjugación de uno a dos polímeros PEG por molécula de enzima es generalmente suficiente para proporcionar los efectos protectores deseados (Avramis et al. (2002) Blood 99, 1986-1994). Debido a que se informa que cada molécula de CocE tipo silvestre contiene 8 residuos de lisina, sin ninguna en el sitio activo sino únicamente 2 estando cerca del sitio activo (Turner et al. (2002) Biochemistry 41, 12297-12307), dianizar lisina para pegilación es improbable que inactive la enzima. La CocE mutante se mezcla con varios polímeros monometoxi-PEG (m-PEG) (con un peso molecular (MW) que varía desde 3-12 KDa); todos contienen un grupo hidroxisuccinil éster funcional N-terminal activado (mPEG-NHS; from Shear Water Inc., Birmingham, AL). Se ensayará primeramente un PEG con un peso molecular de 5.5 KDa, porque los resultados de los

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inventores y aquellos de otros investigadores (véase a este respecto, Veronese, FM and Harris, JM (2002) *Advanced Drug Delivery Reviews* 54, 453-456; Avramis et al. (2002) *Blood* 99, 1986-1994) demostraron que este peso molecular ofrece protección benéfica. Relaciones molares diferentes de $[NH_2]:[mPEG]$ (la primera se calcula con base en las moles totales de residuos de lisina de CocE mutante) que varían de 1:2 a 1:10 se prueban para obtener condiciones óptimas. La conjugación prosiguió durante alrededor de 40 minutos a 4°C agitando suavemente. Los productos de reacción se purifican entonces por ultrafiltración (MWCO 10.000) a 4°C. Se determinan las actividades de los productos de CocE mutante pegilada cuantificando las tasas iniciales de hidrólisis de cocaína utilizando el procedimiento previamente establecido (Turner et al. (2002) *Biochemistry* 41, 12297-12307). Adicionalmente, se realiza espectrofotometría MALDI-TOF en estos productos para analizar el grado de pegilación y su peso molecular. Los productos pegilados se almacenan a -40°C y se descongelan inmediatamente antes de sus usos.

[0172] La caracterización estándar, incluida la determinación del pH óptimo, temperatura, resistencia iónica, así como los parámetros cinéticos (por ejemplo, K_m , V_m) de los conjugados CocE mutante-PEG se llevan a cabo en PBS. Más aún, se examinan la estabilidad térmica de los productos pegilados y su estabilidad contra degradación proteolítica en la presencia de plasma o sangre humanos. Pruebas funcionales *in vivo* de los productos CocE mutante-PEG se conducen como se describe anteriormente. La vida media *in vivo* y la biodistribución de los conjugados CocE mutante-PEG, comparadas con la CocE libre se realizan como se describe anteriormente (véase Ejemplo 9).

[0173] Se lleva a cabo un análisis estadístico de los resultados obtenidos de los experimentos de pegilación. Se efectúa ANOVA aleatoria de doble vía en bloque aleatorio con pos-prueba de Dennett en conjuntos de datos con dos variables usando software GraphPad (San Diego, CA) Prism

para Windows y GraphPad. Se efectúa una prueba *t* pareada para experimentos con dos condiciones.

[0174] Puede realizarse asimismo la conjugación de CocE mutante a PEG ramificado de alto peso molecular (por ejemplo, hasta 60 KDa) (véase a este respecto, Reddy et al. (2002) *Advanced Drug Delivery Reviews* 54, 571-586).

[0175] Adicionalmente, PEGilación específica del sitio puede ser una alternativa viable para reducir la heterogenicidad funcional y estructural. La eliminación de residuos de cisteína cerca del sitio activo o la incorporación de residuos de cisteína en la superficie de la proteína puede servir como sustratos mejores de PEGilación (a través de acoplamiento de maleimida). Análogamente, puede emplearse acoplamiento con amina de PEG a CocE mediante, por ejemplo, la sustitución conservadora de residuos de arginina para cualesquiera CocE's mutantes (total de nueve lisinas en CocE tipo silvestre, siete de las cuales son lisinas superficiales).

[0176] Hallazgos preliminares han demostrado que la CocE tipo silvestre (WT) se enlazó exitosamente con polímeros de monometoxy-PEG (m-PEG) (peso molecular (MW): 5.5KDa) que contenían un grupo funcional succinimidil activado en un extremo, utilizando los procedimientos descritos anteriormente. Se recuperó un alto rendimiento de actividad CocE inicial (>70%) luego de la reacción de pegilación. Los espectros de masas MALDI-TOF revelaron cuatro picos primarios para los productos PEG-CocE, indicando la presencia de una mezcla heterogénea de los conjugados que contenían diferentes números de cadenas PEG (que variaban de 1 a 4, respectivamente) (véase a este respecto, Figura 19). Se ha sugerido en la literatura que la conjugación de simplemente 1-2 cadenas PEG por molécula de proteína podría producir efectos protectores inducidos por PEG (Veronese, FM and Harris, JM (2002b) *Advanced Drug Delivery Reviews* 54, 457-606; Avramis et al (2002) *Blood* 99, 1986-1994). A este

respecto, el método de pegilación empleado aquí satisface aparentemente tal requerimiento.

EJEMPLO 12: ENCAPSULACIÓN EN RBC

[0177] La encapsulación de CocE en RBC puede llevarse a cabo mediante un péptido PTD enlazado. LMWP se selecciona como el péptido PTD para transportar CocE mutante en RBC, debido a su potencia en transferir proteínas a través de la membrana de la célula (Park et al. (2005) FASEB Journal, in press) y su carencia de toxicidad (Chang et al. (2001) AAPS Journal 3, Article #17, #18 and #19). Para garantizar que la CocE mutante encapsulada se inserte permanentemente en RBC, la conexión entre CocE y LMWP puede degradarse automática y rápidamente una vez los conjugados CocE mutante-LMWP ingresen en RBC. Un conector, tal como un enlace disulfuro (S-S) que se degradará rápidamente en el interior de RBC debido a la presencia de glutatión citosólico elevado y actividad reductasa (Trouet et al. (1982) Proceeding of the National Academy of Science 79, 626-629), garantiza que la CocE permanecerá en el RBC.

[0178] Para producir conjugados CocE mutante-LMWP conectados con enlaces S-S, se activa primero con SPDP el grupo amina en el N-terminal de LMWP (éste es el único grupo -NH₂ en LMWP), y la LMWP activada se mezcla entonces con CocE mutante en la presencia de ditioneitol (DTT) para permitir la formación del enlace S-S con uno de los residuos de cisteína libres (cuatro en tipo silvestre) en la CocE mutante (Turner et al. (2002) Biochemistry 41, 12297-12307); de acuerdo con un procedimiento modificado, desarrollado previamente (Liang et al. (2000) AAPS Pharmaceutical Science 2, Article 7). CocE es estable cuando se almacena en DTT (Turner et al. (2002) Biochemistry 41, 12297-12307), sugiriendo que es improbable que el uso de estos grupos de cisteína libres para conjugación deteriore la actividad catalítica de esta enzima (la

mutación ya realizada de cada cisteína en CocE tipo silvestre en una serina resultó en no disminución de la actividad). El producto LWMP-CocE final se purifica entonces mediante una columna de heparina y se almacena por liofilización.

[0179] La encapsulación se obtiene incubando RBC con CocE mutante-LMWP durante 30-60 minutos. Debido a que la entrada en la célula, mediada por PTD, es independiente de la temperatura (Schwarze et al. (1999) Science 285, 1569-1572), la encapsulación se lleva a cabo a 4°C para preservar al máximo la funcionalidad del RBC. El proceso y la magnitud de atrapamiento de la CocE mutante en el RBC se monitorizan por microscopía confocal y análisis de citometría de flujo, usando CocE mutante etiquetada con FITC. La morfología de la CocE mutante atrapada en RBC se examina igualmente mediante SEM.

[0180] La caracterización básica — incluida la evaluación de la funcionalidad del RBC (por ejemplo, actividad de transferencia de oxígeno) y CocE mutante (por ejemplo, actividad hidrolizante de la cocaína, propiedades cinéticas tales como Km, Vm, etc.), fuga de CocE mutante del RBC (es decir, incubando el RBC cargado con CocE mutante en amortiguador y luego cuantificando la actividad enzimática en el sobrenadante), y la estabilidad de la CocE mutante atrapada contra degradación proteolítica — se lleva a cabo en amortiguador o en plasma. Los resultados obtenidos para la CocE mutante encapsulada en el RBC se comparan con los obtenidos para la enzima libre. Pruebas funcionales *in vivo* del RBC encapsulado con CocE mutante se efectúan como se describe anteriormente.

[0181] RBC humanos RBC (de American Red Cross, Detroit, MI) se utilizan para estudios *in vitro*. Para estudios animales *in vivo*, incluidas las pruebas funcionales y los estudios farmacocinéticos, sin embargo, se tienen que utilizar RBC autólogos de la misma especie animal

con el fin de evitar la incompatibilidad celular y posibles efectos tóxicos.

[0182] La vida media en la circulación ($t_{1/2}$) de la CocE mutante atrapada en RCB se determina inyectando CocE mutante marcada con ^{125}I (es decir, antes de su carga en los RBC) en ratones, de acuerdo con los mismos procedimientos descritos anteriormente. Cada conjunto de experimentos se componía de 24 ratones. Se sacrifican ratones (3) en intervalos de tiempo de 3, 6, 12, 24 horas y 3, 6, 10 y 15 días. Se recogen muestras de sangre y tejido, se pesan y se miden en cuanto al contenido de radioactividad. Se calculan los parámetros PK incluida la $t_{1/2}$ de eliminación, así como la distribución en el tejido utilizando un programa KINFT, como se describe anteriormente. Los resultados farmacocinéticos obtenidos para la CocE mutante encapsulada en los RBC se comparan con lo obtenidos para la CocE mutante libre.

[0183] Se lleva a cabo un análisis estadístico de los resultados obtenidos de los experimentos encapsulación en RBC. Se efectúa ANOVA aleatoria de doble vía en bloque aleatorio con pos-prueba de Dennett en conjuntos de datos con dos variables usando software GraphPad (San Diego, CA) Prism para Windows y GraphPad. Se efectúa una prueba t pareada para experimentos con dos condiciones.

[0184] Para examinar si lo ya sugerido podía cumplir los dos últimos requerimientos, se condujeron estudios preliminares de encapsulación en RBC mediada por PTD usando L-asparaginasa como una enzima modelo. LMWVP, un péptido PTD desarrollado previamente en el Laboratorio del Dr. Yang (Chang et al. (2001) AAPS Journal 3, Article #17, #1, and #19) con potente actividad demostrada de penetración de la membrana (Park et al. (2005) FASEB Journal, in press), se enlazó a asparaginasa empleando un procedimiento similar al descrito anteriormente. Los conjugados LMWP-ASNasa se incubaron entonces con RBC (recolectados de los ratones DBA/2) durante 2 horas a 4°C. Para

comparación, se prepararon también RBC-Ghosts que contenían ASNasa encapsulada de acuerdo con los procedimientos previamente establecidos (Updike et al. (1976) Science 193, 681-683). Los resultados preliminares demostraron que la eficiencia de carga del método mediado por LMWP fueron por lo menos comparable con, si no mejores que, la técnica convencional, de ruptura de la célula, basada en ósmosis. Una ventaja principal del método mediado por PTD, sin embargo, es que sólo requiere un solo paso para procesamiento; a diferencia de los otros métodos de ingreso a la célula que requieren pasos múltiples de los procedimientos de carga y lavado.

[0185] Figura 20 presenta imágenes de microscopio electrónico de barrido (SEM – scanning electron microscopy) tomadas de muestras de RBC normales, RBC-Ghost cargados con ASNasa y RBC cargados con LMWP-ASNasa. Como se observa, aunque los RBC cargados con ASNasa del método de ruptura osmótica/resellado (es decir, RBC-Ghost) exhibieron un cambio significativo en forma y morfología, los RBC cargados con LMWP-ASNasa mostraron una forma y morfología virtualmente indistinguibles (es decir, disco bicóncavo) de las de los RBC normales. Estos hallazgos están de acuerdo con los informados por muchos otros investigadores de que la encapsulación de células, mediada por PTD, no ocasiona ninguna perturbación o alteración significativas de la membrana de la célula (Dietz, GPH and Bahr, M (2004) Molecular Cell Neurosciences 27, 85-131; Schwarze et al. (1999) Science 285, 1569-1572; Suzuki et al. (2002) Journal of Biological Chemistry 275, 2437-2443).

[0186] Para evaluar aún más estos dos sistemas de encapsulación en RBC, se llevaron a cabo estudios preliminares de depuración. La vida media de la actividad de la ASNasa en plasma se evaluó luego de la inyección intravenosa de: (1) RBC-Ghost cargados con ASNs, y (2) RBC cargados con LMWP-ASNasa. Cada grupo animal se componía de 4 ratones DBA-2, y a cada ratón se le aplicaron 8 unidades de

actividad de ASNasa cargada. Se tomaron muestras de sangre en diferentes intervalos de tiempo de la vena de la cola, y se cuantificó la cantidad de actividad de ASNasa en la sangre entera mediante Nesslerización directa del amoníaco producido (Ho et al. (1970) Journal of Biological Chemistry 245, 3708-15). Los resultados demostraron que hubo un aumento casi doble en la vida media en circulación para los RBC encapsulados con LMWP-ASNasa ($t_{1/2}$: 9.2 días) comparada con aquella ($t_{1/2}$: 5.9 días) para el RBC-Ghost (véase a este respecto, Figura 21). Actualmente se desconoce que la diferencia entre las vidas medias de los RBC encapsulados y los RBC normales, no tratados. No obstante, estos resultados de un doble aumento de la $t_{1/2}$ sobre los RBC-Ghost demuestran el mérito de esta estrategia, como se informó en la literatura, de que incluso utilizando el método de encapsulación de RBC-Ghost, la actividad de la ASNasa *in vivo* ya se había prolongado de 26 horas para la ASNasa libre a 29 días para la ASNasa encapsulada en RBC-Ghost (Kravtsoff et al. (1996) European Journal of Clinical Pharmacology 49, 465-470): ya un aumento de 10 veces. Por lo tanto, otro aumento de 2 veces de esta $t_{1/2}$ por el método de encapsulación, descrito en esta especificación, es particularmente efectivo.

[0187] Para confirmar que la ASNasa encapsulada en RBC podía conservar aún sus funciones biológicas originales, se examinaron los efectos anti-neoplásicos tanto por RBC-Ghost-ASNasa como por RBC-ASNasa en ratones con tumores. Se cultivaron células de linfoma de ratón L5178Y, y a cada ratón DBA/2 se inyectaron intraperitonealmente 7×10^5 células cancerosas. Cinco días después de la implantación del tumor, se seleccionaron los ratones con pesos corporales similares y se repartieron en tres grupos: (1) grupo de control al cual se administró solución salina únicamente; (2) RBC-Ghost cargados con ASNasa; y (3) RBC cargados con LMWP-ASNasa. Cada grupo constaba de 5 ratones, y a cada ratón experimental se le administró 0.1 mL de los RBC encapsulados con droga (o Ghost). Los resultados mostraron que los tiempos medios de supervivencia para los grupos de control no tratados, tratados con ASNasa-

RBC-Ghost y tratados con LMWP-ASNasa-RBC fueron 10.0, 12.6 y 14.4 días, respectivamente (véase a este respecto, Figura 22). Debe observarse que aunque el tiempo de supervivencia entre los tres grupos difirió únicamente alrededor de 2 días, los efectos del tratamiento con la ASNasa encapsulada en RBC fue todavía bastante dramático – considerando el hecho de que tan sólo 0.1 mL de suspensión de RBC, que era equivalente a simplemente 5% del volumen total de sangre del ratón, se administró a cada ratón durante el tratamiento anti-neoplásico. En general, estos hallazgos con la enzima modelo ASNasa demostraron que la enzima encapsulada en RBC fue aún terapéuticamente activa. Se anota que la cocaína es más permeable a través de la membrana RBC que el sustrato asparaginasa.

[0188] Por lo tanto, la utilización de CocE encapsulada en RBC en tratar afecciones relacionadas con la cocaína es una estrategia efectiva porque la cocaína atraviesa fácilmente la membrana del RBC (y efectivamente alguna parte se concentra en los RBC) (Javaid et al. (1978) Journal of Chromatography 15, 105-113), la encapsulación mediada por PTD no altera las propiedades físicas y/o químicas del RBC, y la enzima encapsulada en RBC funciona como si fuera libre.

EJEMPLO 13: ELIMINACIÓN DE ENDOTOXINA

[0189] La descontaminación de endotoxina de los mutantes CocE puede efectuarse en varias formas. En el mejor de los casos, la descontaminación de endotoxina disminuye la concentración a niveles menores a 10 EU/mg de proteína. Métodos de descontaminación incluyen condiciones alternativas de cromatografía en columna de intercambio iónico, exclusión por tamaño, cromatografía de columna hidrófoba y en polietilenimina (PEI), ultrafiltración y extracción con tensioactivo. Se emplea un sistema de detección de endotoxina (PYROGENT 5000, Cambrex) para determinar el contenido de endotoxina de las preparaciones. El análisis se basa en el factor anti-LPS del lisado de amebocito de Limulus

(LAL - *Limulus ameobocyte lysate*). La sensibilidad del análisis está entre 0.01 y 100 EU/ml, bien dentro de los niveles requeridos. El análisis espectrofotométrico se diseña en un formato de placa de microvaloración de 96 pocillos. El fraccionamiento de la endotoxina y CocE y/o CocE mutante por los siguientes procedimientos puede examinarse tanto en cuanto al nivel de endotoxina como a la actividad de la cocaína esterasa. La actividad de la cocaína esterasa se mide utilizando un análisis espectrofotométrico que aprovecha la ventaja de la absorción intrínseca de la cocaína a 240 nm. Luego de la hidrólisis, los espectros de absorción revelan una dramática reducción en el pico 240 nm (Turner et al. (2002) Biochemistry 41, 12297-12307).

[0190] Cromatografía de intercambio aniónico: Condiciones actuales implican el uso de cromatografía líquida de rendimiento rápido (FPLC - *performance liquid chromatography*) en una columna Q-Sepharose a pH 8.0 para la descontaminación de endotoxina. Las condiciones de la solución amortiguadora (pH) pueden optimizarse para maximizar tanto la adsorción de CocE y/o CocE mutante como la separación de endotoxina. Se evalúan fracciones obtenidas por la técnica FLUTE ("*fluted fractions*") midiendo la actividad de la CocE o CocE mutante (análisis espectroscópico de la hidrólisis de cocaína, absorción a 240 nm). Los niveles de endotoxina se evalúan usando PYROGENT 5000 (véase más arriba).

[0191] Cromatografía de Exclusión de Tamaño y Ultrafiltración: La endotoxina puede existir como formas monoméricas ($MW \sim 1-2 \times 10^4$) o en forma micelar ($MW \sim 4 \times 10^5$ a 1×10^6), dependiendo de las condiciones de la solución amortiguadora. La presencia de detergentes tales como colato favorece la forma monomérica, mientras que los cationes divalentes (por ejemplo, Ca^{2+}) favorecen la forma micelar (Hirayama, C and Sakata, M (2002) Journal of Chromatography B Analytical Technology Biomedical Life Science 781, 419-432). Esta propiedad de la endotoxina se utiliza para separar la forma micelar (es

decir, en la presencia de cationes divalentes tales como Mg^{2+} o Ca^{2+}) de CocE y/o CocE mutante mediante cromatografía de filtración en gel empelando una resina de columna Sephadex 75 o Sephadex 200 (Pharmacia). La endotoxina micelar no debe retenerse en la columna y debe atravesar los espacios vacíos, mientras que CocE y/o CocE mutante deben eluirse como una proteína monodispersada correspondiendo a una proteína de 65 kDa. Análogamente, se evaluará la capacidad de unidades de ultrafiltración para separar las formas micelares de la endotoxina de CocE y/o CocE mutante. Actualmente se dispone de unidades de ultrafiltración con separaciones de peso molecular de 3×10^5 a 1×10^6 , bien dentro del rango requerido para retener endotoxina micelar pero no CocE en sí.

[0192] Cromatografía en Polietilenimina: Mitzner et al. (1993) y Morimoto et al. (1985) han usado PEI inmovilizada en microesferas de celulosa o en fibra de celulosa, respectivamente, para extraer endotoxina de preparaciones BSA. La columna de cromatografía en PEI es un intercambiador aniónico muy débil que, de hecho, puede sacar ventaja de algunas propiedades hidrófobas de la endotoxina y, por consiguiente, adsorberla preferencialmente. Varias preparaciones de microesferas de PEI-sílice se obtienen de Sigma, dependiendo del tamaño de las microesferas. Aunque las microesferas a base de sílice se asocian más clásicamente con aplicaciones de HPLC, empacaremos columnas para trabajo a menor presión y seleccionaremos los tamaños de ancho de malla de 200 μm .

[0193] Extracción con Triton X-114: La separación de fase con Triton X-114 se ha empleado exitosamente para separar endotoxina de albúmina y catalasa (Aida, Y and Pabst, MJ (1990) J Immunological Methods 132, 191-195). Aida y Pabst informan de una disminución de 1.000 veces en la concentración de endotoxina luego de un paso único de extracción con Triton X-114. Muestras de CocE y CocE mutante se incuban con volúmenes iguales de Triton 114 y se dejan incubar primero en hielo y luego a 37°C durante 15 minutos. La fase Triton X-114 que contiene la

endotoxina se retira mediante centrifugación. Como se anotó anteriormente, se ha demostrado que la CocE es considerablemente lábil al calor, pero puede protegerse en cierto modo por la presencia del sustrato, cocaína. Si la incubación de CocE con Triton X-114 a 37°C resulta en una inactivación significativa de CocE, la enzima se estabilizará incluyendo exceso de sustrato durante la extracción.

EJEMPLO 14: MUTAGÉNESIS DEL ORGANISMO HOSPEDERO COCE

[0194] Existen varias cepas bacterianas que exhiben crecimiento sensible a la temperatura en subproductos de cocaína esterasa, incluidas varias cepas de *Pseudomonas*, y estos organismos pueden adaptarse para crecimiento sensible a la temperatura en cocaína por la adición del gen CocE codificado en vectores de plásmidos apropiados. Por ejemplo, el gen CocE se secuenció originalmente de *Rhodococcus* MB1 subclonando fragmentos del gen en *Rhodococcus erythropolis* CW25, una bacteria incapaz de metabolizar cocaína pero capaz de crecer en los subproductos de cocaína esterasa ecgonina metil éster y benzoato (Bresler et al. (2000) Applied & Environmental Microbiology 66, 904-908). El gen CocE se ha subclonado en vectores lanzadera pJAK-14 y pMMB67EH (véase a este respecto, Ejemplo 1). Estos plásmidos son capaces de expresión en cualquier bacteria gram-negativa incluidas *Pseudomonas* y, adicionalmente, el plásmido pMMB67EH posibilita niveles altos de expresión y facilidad de transformación mediante conjugación bacteriana con el uso del plásmido cooperador pRK2013. La transformación de cualquiera de los dos plásmidos en bacterias que exhiben crecimiento sensible a la temperatura en subproductos de cocaína esterasa (por ejemplo, cepas de *Pseudomonas*), permite el crecimiento sensible a la temperatura en placas que contienen cocaína como fuente exclusiva de carbono. La mutagénesis de las dos cepas de *Pseudomonas*, que contienen el *Rhodococcus* MB1 original y el plásmido CocE, se lleva a cabo usando

radiación con luz UV a 260nm. Se determina la concentración de la exposición de modo que se elimine el 90-95% de las células. Las células restantes se recuperan en medio nutriente durante 1 hora a 26°C, se recogen y se enriquecen en la presencia de cocaína como se describe previamente (Britt, et al. (1992) Journal of Bacteriology 174, 2087-2094). El enriquecimiento se lleva a cabo a 37°C para seleccionar las variantes termoestables de la CocE. Finalmente, las células se esparcen sobre placas con medio agar mínimo que contienen 10mM de cocaína y se incuban a 37°C. Se desarrollan colonias únicas y se examinan con respecto a actividad CocE a 37°C. La amplificación por PCR del gen CocE se realiza en mutantes que se descubrió que producen CocE activa y soluble a 37°C, y los productos de amplificación se subclonan en el plásmido pET-22B(+) para posterior caracterización. Como preludio a la mutagénesis de CocE, puede ser necesario mutagenizar las cepas *Pseudomonas* nativas con el fin de preseleccionar en cuanto a no sensibilidad a la temperatura a 37°C cuando crecen en productos de hidrólisis de cocaína, y luego demuestran sensibilidad a la temperatura en cocaína a 37°C luego de clonarse en CocE.

EJEMPLO 15: MÉTODO DE DETECCIÓN SELECTIVA DE GRAN PRODUCTIVIDAD PARA IDENTIFICACIÓN DE MUTANTES CocE TERMOESTABLES

[0195] Se implementaron varios métodos de detección selectiva de gran productividad para la identificación de variantes CocE termoestables. Debido a que la enzima tipo silvestre es conocida por tener inestabilidad térmica a temperaturas por encima de 30°C, luego de la transformación en células BL21 de *E. coli*, se subcultivan colonias y se induce la expresión de proteína a 16°C. A continuación las proteínas expresadas se examinan con respecto a actividad esterasa a temperaturas de 30°C y más. Después de varias rondas de mutagénesis y experimentación a temperaturas crecientes se logran los mutantes termoestables. Se prepara entonces cada CocE mutante individual, se purifica y examina con respecto

a actividad y termoestabilidad a 37°C, como se describe más arriba (véase los Ejemplos 1 y 4)

[0196] Colonias bacterianas, que contienen polipéptidos mutantes, se detectan selectivamente directamente de placas de agar mediante huella en filtro de nitrocelulosa de placas de replicación, seguida por lisis de las bacterias y fijación de proteína. La determinación de la actividad enzimática a diferentes temperaturas se logra monitorizando la acumulación de ácido benzoico, el subproducto ácido de la (-)cocaína. Una huella húmeda de nitrocelulosa se pone sobre un papel de filtro seco, saturado previamente con una mezcla de cocaína con un pH de 7.4 sin solución amortiguadora y un indicador de pH que pasa de incoloro a color luego de la acidificación, por ejemplo, rojo metilo. Se identifica la enzima activa por el cambio de color y se recolectan las colonias en la forma apropiada. El método de detección, basado en la acidificación a través de la formación de ácido benzoico, se emplea para la detección de la expresión celular de anticuerpos catalíticos que hidrolizan la cocaína en el grupo éster de benzoilo, el mismo sitio escindido por CocE. Alternativamente, la detección por huella de nitrocelulosa se obtiene por exposición a un derivado tiol de cocaína y detección subsiguiente de grupos sulfhidrilo mediante un sistema indicador de metal pesado precipitante (por ejemplo, a base de mercurio).

[0197] Las colonias bacterianas que contienen los polipéptidos mutantes se detectan también selectivamente subcultivando en medio líquido y examinando directamente en cuanto a la presencia de actividad cocaína esterasa empleando un derivado tiol de cocaína y detección utilizando el indicador tiol colorimétrico, reactivo de Ellman (véase a este respecto, Figura 6). El reactivo de Ellman forma rápidamente un enlace disulfuro con los grupos tiol libres y libera un ión tiolato coloreado que absorbe a 412 nm. Los cultivos incubados de un día para otro a 16°C en la presencia de IPTG para inducir la expresión de proteína (20µl) se mezclan

con 1mM de derivado de cocaína benzoiltioéster, y 500uM de reactivo de Ellman en 100mM de fosfato sódico, pH 7.4, a un volumen final de 200µl. Los resultados (véase a este respecto, Figura 6) indican que las células que contienen la enzima CocE tipo silvestre son capaces de escindir el benzoiltioéster en niveles mucho más elevados que las células solas.

[0198] Finalmente, las colonias bacterianas que contienen polipéptidos mutantes se detectan selectivamente subcultivando en medio líquido, seguido por lisis y aislamiento de polipéptidos mutantes usando un medio de afinidad. Por ejemplo, células lisadas se lavan a través de placas con filtro de agarosa-níquel que permiten la recolección y elución subsiguiente de proteínas marcadas 6xHIS (por ejemplo, Ni-NTA Superflow 96-Bio-robot kit (Qiagen)). Alternativamente, las células se desintegran por lisis en placas de microvaloración recubiertas con níquel, que permiten la unión de proteínas de fusión 6xHIS y la extracción subsiguiente de contaminantes (por ejemplo, placas inmovilizantes con quelato de níquel (Nunc) o placas flash con quelato de níquel NEN (Perkin Elmer)). Análogamente, células lisadas se incuban con microesferas recubiertas con níquel (tales como las microesferas de agarosa magnéticas Ni-NTA (Qiagen)), seguidas por extracción subsiguiente de las proteínas contaminantes. La actividad de la proteína esterasa aislada se examina entonces usando cualquiera de los análisis mencionados previamente (tales como el análisis de actividad espectrofotométrico (Ejemplos 1 y 4), el análisis de actividad de la cocaína tritiada (Ejemplo 1), el análisis de actividad con indicación del pH del ácido benzoico, los sistemas de detección de cocaína con el derivado tiol, el empleo de aptámeros de cocaína (Stojanovic, M. N., de Prada, P. & Landry, D. W. (2001) J Am Chem Soc 123, 4928-31; Stojanovic, M. N. & Landry, D. W. (2002) J Am Chem Soc 124, 9678-9) monitorizando los cambios en fluorescencia luego de la degradación de la cocaína, o mediante el uso de un sustrato genérico de esterasa tal como 4-nitrofenil acetato y monitorización de los cambios colorimétricos a 420nm como se describe previamente (Halgasova, N. et al.

(1994) Biochem J 298 Pt 3, 751-5; O'Conner, C.J. & Manuel, R.D. (1993) J Dairy Sci. 76:3674-3682).

**EJEMPLO 16: ANÁLISIS PRELIMINAR DEL POLIPÉPTIDO
MUTANTE N197K**

[0199] Análisis preliminares del polipéptido mutante N197K (SEQ ID NO: 42) mostraron excelente estabilidad luego de 1 hora a 37°C el día 0. Los valores Vmax y Km se muestran en la Tabla 5. Una segunda verificación el día 3 mostró una estabilidad similar. Los valores Vmax y Km se muestran en la Tabla 6. Los valores Km más elevados el día 3 se debieron a la reutilización de la cocaína vieja. La filtración en gel de las muestras incubadas a 37°C mostró la formación de agregados.

TABLA 5: Prueba inicial de N197K (Día 0)

	N197K (0)	N197K(60)
Ecuación 1		
Valores de ajuste óptimo		
VMAX	2928	2187
KM	34.77	24.24

TABLA 6: Prueba inicial de N197K (Día 3)

	N197K (0)	N197K(60)
Ecuación 1		
Valores de ajuste óptimo		
VMAX	3357	3085
KM	102.2	113.5

**EJEMPLO 17: IDENTIFICACIÓN DE POLIPÉPTIDOS CocE
MUTANTES TÉRMICAMENTE ESTABLES**

[0200] Polipéptidos CocE mutantes térmicamente estables se identificaron determinando el $t_{1/2}$ de los polipéptidos CocE mutantes. En pocas palabras, las enzimas se preincubaron a 37°C durante tiempos diferentes. Las mediciones de la actividad se determinaron a temperatura de la sala (RT – *room temperature*) (25°C). Las enzimas mutantes con $t_{1/2}$ de más de 12 minutos (el $t_{1/2}$ de la CocE tipo silvestre) se consideraron térmicamente estables (véase, por ejemplo, Figura 27 y Tabla 7). Como se demostró por el polipéptido CocE mutante de combinación T172R/G173Q, en varias incorporaciones, la combinación de dos mutaciones únicas con ninguna estabilidad o estabilidad más baja puede resultar en una combinación térmicamente estable.

TABLA 7: Polipéptidos CocE mutantes térmicamente estables

Mutante	Estabilidad @37°C ($t_{1/2}$)
T122A	No
Q123E	No
S159A	No
S140A	No
S167A/W52L	No
T172R	~46 minutos
V121D	No
L163V	No
F189A	No
F189A/T172R	~40 minutos (similar a T172R)
C107S	No
W220A	No
F189L	No

A193D	No
T172R/A193D	~40 minutos (similar a T172R)
G173Q	~25 minutos
T254R	No
N42V	No
T172R/G173Q	~326 minutos
G171Q/T172R/G173Q	No
G171A	No
G173A	No
wt-I175-G-D185	No
wt-T176-G-G-D185	No
T172R/G173Q-I175-G-D185	
T172R/G173Q-I175-G-G-A186	-75 minutos
T172R/G173Q-T176-G-G-D185	-75 minutos
S177Q	No
D45R	No
F47R	No
L169K	~274 minutos
L174R	No
A181K	No
S179R	No
F189K	25 minutos
V190K	No
A194K	No
R182K	No

**EJEMPLO 18: PROTECCIÓN CONTRA AGREGACIÓN DE LOS
MUTANTES DE COCAÍNA ESTERASA TERMOESTABLES**

[0201] La protección contra agregación de los mutantes CocE

termoestables se evaluó usando cromatografía de exclusión molecular. Concretamente, se preincubaron las enzimas a 37°C durante 0 minutos o 60 minutos y se resolvieron mediante cromatografía de exclusión molecular. Los resultados para la CocE tipo silvestre, T172R y T172R/G173Q se muestran en Figura 28.

EJEMPLO 19: ESPECTROS DE BAJA ABSORCIÓN UV (LOW-UV SPECTRA)

[0202] Datos de espectros CD de baja absorción UV se obtuvieron usando un espectropolarímetro Aviv Mode 400, con la ayuda de Norma Greenfield, UMDNJ, que empleaba un soporte de 5 celdas y 0.2mg/ml de concentraciones de proteína. Los valores de los datos brutos se calibraron en PBS de control, se emparejaron (la curva se alisó estadísticamente) y se restauraron ("*deconvoluted*") utilizando el algoritmo CCA, como se describe por A. Perczel, K. Park, and G.D. Fasman, [Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm: a practical guide. Analytical Biochemistry 203, 83-93 (1992).] Este algoritmo encuentra el número mínimo de curvas necesarias para reconstruir un conjunto de datos, y expresa el porcentaje en que cada curva contribuye al conjunto de datos como una función de la temperatura.

[0203] Más adelante se presenta un análisis de restauración CCA con baja absorción UV ("*a low UV CCA denconvolution analysis*") de CocE tipo silvestre (WT) y 4 mutantes, obtenido en una fusión de espectros CD únicos usando un soporte de 5 celdas. El experimento se llevó a cabo durante un período de 8 horas, de 0-80°C.

[0204] Se observó fusión dependiente de la temperatura entre las longitudes de onda examinadas (200-250nm), y los espectros emparejados (alisados estadísticamente) para cada mutante se muestran en

Figura 29. La restauración mediante el algoritmo CCA indicó que cada uno de los espectros se describió mejor por un conjunto de tres curvas, como se muestra en Figura 30. Esto sugiere que la fusión de CocE es por lo menos un proceso de dos pasos, que se mueve de una curva original (curva 1) a un paso de desenvolvimiento intermedio (curva 2) y, finalmente, una proteína completamente desnaturalizada (curva 3). La contribución porcentual (%) que cada temperatura ejerció en describir estas tres curvas se ilustra en Figura 31. Se empleó análisis de respuesta a la dosis para aproximar la temperatura a la cual el espectro inicial se fusiona (1), la formación y fusión del estado intermedio (2), y la acumulación de la proteína completamente fusionada (3). Estos números de recolectaron y graficaron en Figura 32 y se muestran en Tabla 8.

TABLA 8: Puntos de fusión de cada paso

	Fusión #1	Formación #2	Fusión #2	Formación #3
—◆— WT	37.04	34.57	50.98	59.86
—■— T172R	39.22	38.11	54.56	56.85
—●— T172R-A193D	38.11	46.47	47.68	58.72
—×— T172R-F189K	38.8	46.81	48.42	57.23
—*— T172R-G173Q	40.62	45.61	46.17	56.61

[0205] El mutante más termoestable T172R-G173Q (según se determinó en otros análisis) mostró la temperatura de fusión más alta de la curva original 1 (40°C vs 37°C para el tipo silvestre), y la temperatura más baja para la desaparición del intermedio de la curva 2 (46°C vs 50°C para el tipo silvestre) y la aparición de la curva de fusión completa 3 (56°C vs 59°C para el tipo silvestre).

[0206] En resumen, es evidente que todos los mutantes experimentan un proceso de fusión de 2 pasos.

EJEMPLO 20: ESTABILIZACIÓN UTILIZANDO PRODUCTOS E INHIBIDORES

[0207] La cocaína esterasa (CocE) escinde la cocaína para producir ácido benzoico y ecgonina metil éster. Concretamente, se investigaron sustratos alternativos e inhibidores de cocaína, así como compuestos capaces de termoestabilizar la enzima, generalmente sustituyendo amida y análogos de tiol en el enlace éster reactivo, o eliminando el enlace (para inhibidores), sustituyendo análogos de benzolilo en lugar del grupo saliente ácido benzoico, y/o eliminando o alterando el grupo metil éster en la porción ecgonina de la molécula. Tal como se discute a continuación, se determinó que algunos sustratos, productos e inhibidores estabilizaron la desnaturalización térmica de la CocE tipo silvestre, así como evitaron la agregación inducida térmicamente en electroforesis en gel.

[0208] La cocaína es el sustrato natural de la cocaína esterasa (CocE). La escisión de la cocaína se monitorizó por una caída en absorbencia a 240nm. La cocaína (rango mM) impidió la formación inducida a 37°C de agregados de CocE de alto peso molecular (concentraciones de enzima de 0.1 mg/ml) (véase, por ejemplo, Figura 33). La cocaína (cantidades uM) estabilizó la pérdida de actividad inducida a 37°C (véase, por ejemplo, Figura 34), si bien el mecanismo de esta estabilización es complicado debido a la inhibición del sustrato en concentraciones más altas.

[0209] El ácido benzoico es el producto natural de la CocE y un débil inhibidor de la escisión por CocE de 4-nitrofenil acetato (K_i 310uM). El ácido benzoico impidió la formación inducida a 37°C de agregados de CocE de alto peso molecular (concentraciones de enzima de 0.1 mg/ml) (véase, por ejemplo, Figura 35). El ácido benzoico estabilizó (cantidades uM) la pérdida de actividad inducida a 37°C (véase, por ejemplo, Figura

36), si bien el mecanismo de esta estabilización es complicado debido a la inhibición del sustrato en concentraciones más altas.

[0210] CocE cataliza la escisión de 4-nitrofenil acetato (4NPA) en 4-nitrofenol (4NP) y acetato. Se monitorizó la reacción de escisión mediante observación de la formación de producto a 400 nm. Tanto el 4NP como el 4NPA (rango mM) impidieron la formación inducida a 37°C de agregados de CocE de alto peso molecular (concentraciones de enzima de 0.1 mg/ml).

[0211] El ácido fenilbórico es un potente inhibidor de CocE (K_i 250nM). El ácido fenilbórico estabilizó la agregación inducida a 37°C de CocE con una EC_{50} de 0.2UM mediante análisis de densitometría.

[0212] Usando lo anterior, se desarrolla un análisis de detección selectiva para identificar selectivamente pequeñas moléculas que podrían estabilizar análogamente la enzima la enzima pero que no necesariamente ocupan el sitio activo. Las moléculas identificadas como moléculas estabilizadoras se emplean para estabilizar las proteínas divulgadas en esta especificación hasta que estén listas para uso.

REIVINDICACIONES

Lo que se reivindica es:

1. Un polipéptido de cocaína esterasa (CocE) mutante aislado que comprende una secuencia de aminoácidos de SEQ ID NO: 1, en donde por lo menos un residuo de aminoácido se sustituye de tal modo que el polipéptido CocE mutante tenga actividad esterasa con termoestabilidad incrementada a 37°C comparado con la CocE tipo silvestre.
2. El polipéptido CocE aislado de la reivindicación 1, en donde se sustituyen por lo menos dos residuos de aminoácido.
3. El polipéptido CocE aislado de la reivindicación 2, en donde se sustituyen por lo menos tres residuos de aminoácido.
4. El polipéptido CocE aislado de la reivindicación 3, en donde se sustituyen por lo menos cuatro residuos de aminoácido.
5. El polipéptido CocE aislado de la reivindicación 4, en donde se sustituyen por lo menos cinco residuos de aminoácido.
6. Un polipéptido de cocaína esterasa mutante aislado que comprende una secuencia de aminoácidos seleccionada del grupo conformado por SEQ ID NO: 3 (L163V); SEQ ID NO: 7 (V225I); SEQ ID NO: 8 (I218L); SEQ ID NO: 9 (A310D); SEQ ID NO: 10 (A149S); SEQ ID NO: 11 (S159A); SEQ ID NO: 12 (S265A); SEQ ID NO: 13 (S56G); SEQ ID NO: 14 (W220A); SEQ ID NO: 16 (S140A); SEQ ID NO: 17 (F189L); SEQ ID NO: 18 (A193D); SEQ ID NO: 19 (T254R); SEQ ID NO: 20 (N42V); SEQ ID NO: 21 (V262L); SEQ ID NO: 22 (L508G); SEQ ID NO: 23 (Y152H); SEQ ID NO: 24 (V160A); SEQ ID NO: 25 (T172R); SEQ ID NO: 26 (Y532F); SEQ ID NO: 27 (T74S); SEQ ID NO: 28 (W285T); SEQ ID NO: 29 (L146P); SEQ ID NO: 30 (D533S); SEQ ID NO: 31 (A194R); SEQ ID NO: 32 (G173Q); SEQ ID NO: 33 (C477T); SEQ ID NO: 34

(K531A); SEQ ID NO: 35 (R41I); SEQ ID NO: 36 (L119A); SEQ ID NO: 37 (K46A); SEQ ID NO: 38 (F84Y), SEQ ID NO: 39 (T172R-G173Q); SEQ ID NO: 40 (L169K); SEQ ID NO: 41 (F189A), SEQ ID NO: 42 (N197K), SEQ ID NO: 43 (R182K), SEQ ID NO: 44 (F189K), SEQ ID NO: 45 (V190K), SEQ ID NO: 46 (Q191K), y SEQ ID NO: 47 (A194K), o u fragmento funcional de las mismas.

7. Un ácido nucleico aislado que codifica el polipéptido CocE mutante de una cualquiera de las reivindicaciones 1-6.

8. Un ácido nucleico aislado que comprende una secuencia nucleotídica, que se hibrida a SEQ ID NO: 2 o al complemento de SEQ ID NO: 2 bajo condiciones de alta restricción, en donde el ácido nucleico aislado codifica un polipéptido CocE mutante que tiene actividad esterasa con termoestabilidad incrementada a 37°C, comparado con CocE tipo silvestre.

9. Un ácido nucleico aislado que codifica un polipéptido CocE mutante que comprende una secuencia de aminoácidos que tiene por lo menos 85% de identidad de secuencia con la secuencia de ácido nucleico de SEQ ID NO: 2, en donde el polipéptido CocE mutante codificado tiene actividad esterasa con termoestabilidad incrementada a 37°C, comparado con CocE tipo silvestre.

10. El ácido nucleico aislado de la reivindicación 7 en donde la identidad de secuencia se selecciona del grupo conformado por 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, y 99% por lo menos.

11. El polipéptido o ácido nucleico de una cualquiera de las reivindicaciones 1-10 en donde el aumento en termoestabilidad del polipéptido CocE mutante sobre la CocE tipo silvestre es por lo menos

alrededor de 2.1 kcal/mol, por lo menos alrededor de 2.2 kcal/mol, por lo menos alrededor de 2.3 kcal/mol, por lo menos alrededor de 2.4 kcal/mol, por lo menos alrededor de 2.5 kcal/mol, por lo menos alrededor de 2.6 kcal/mol, por lo menos alrededor de 2.7 kcal/mol, por lo menos alrededor de 2.8 kcal/mol, por lo menos alrededor de 2.9 kcal/mol, por lo menos alrededor de 3.0 kcal/mol, por lo menos alrededor de 3.1 kcal/mol, por lo menos alrededor de 3.2 kcal/mol, por lo menos alrededor de 3.3 kcal/mol, por lo menos alrededor de 3.4 kcal/mol, por lo menos alrededor de 3.5 kcal/mol, por lo menos alrededor de 3.6 kcal/mol, por lo menos alrededor de 3.7 kcal/mol, por lo menos alrededor de 3.8 kcal/mol, por lo menos alrededor de 3.9 kcal/mol, por lo menos alrededor de 4.0 kcal/mol, por lo menos alrededor de 4.1 kcal/mol, por lo menos alrededor de 4.2 kcal/mol, por lo menos alrededor de 4.3 kcal/mol, por lo menos alrededor de 4.4 kcal/mol, o por lo menos alrededor de 4.5 kcal/mol.

12. El polipéptido o ácido nucleico de una cualquiera de las reivindicaciones 1-11 en donde el polipéptido CocE mutante tiene inmunogenicidad reducida, comparado con la CocE tipo silvestre.

13. El polipéptido o ácido nucleico de una cualquiera de las reivindicaciones 1-12 en donde el polipéptido CocE mutante tiene por lo menos alrededor de 60%, por lo menos alrededor de 65%, por lo menos alrededor de 70%, por lo menos alrededor de 75%, por lo menos alrededor de 80%, por lo menos alrededor de 85%, por lo menos alrededor de 90%, por lo menos alrededor de 95%, por lo menos alrededor de 100%, por lo menos alrededor de 105%, por lo menos alrededor de 110%, por lo menos alrededor de 115%, por lo menos alrededor de 120%, por lo menos alrededor de 125%, por lo menos alrededor de 130%, por lo menos alrededor de 135%, o por lo menos alrededor de 140% de la actividad esterasa del polipéptido CocE tipo silvestre.

14. El polipéptido o ácido nucleico de una cualquiera de las

reivindicaciones 1-12 en donde el polipéptido CocE mutante conserva por lo menos una eficiencia catalítica sustancialmente la misma o mayor del polipéptido CocE tipo silvestre.

15. Una composición farmacéutica que contiene el polipéptido CocE mutante o el polipéptido CocE mutante codificado por el ácido nucleico de una cualquiera de las reivindicaciones 1-14 y una sustancia portadora o excipiente farmacéuticamente aceptable.

16. El polipéptido, polipéptido codificado por el ácido nucleico, o la composición farmacéutica de una cualquiera de las reivindicaciones 1-15, en donde el polipéptido CocE mutante se pegila.

17. El polipéptido, polipéptido codificado por el ácido nucleico, o la composición farmacéutica de una cualquiera de las reivindicaciones 1-16, en donde el polipéptido CocE mutante se encapsula en un glóbulo rojo.

18. El polipéptido, polipéptido codificado por el ácido nucleico, o la composición farmacéutica de una cualquiera de las reivindicaciones 1-17, en donde el polipéptido CocE mutante se estabiliza por un sustrato o inhibidor.

19. El polipéptido, polipéptido codificado por el ácido nucleico, o la composición farmacéutica de una cualquiera de las reivindicaciones 1-5 y 7-18, en donde el polipéptido comprende una pluralidad de sustituciones de residuo de aminoácido seleccionadas del grupo conformado por: T122A; 123E; S159A; S140A; S167A/W52L; T172R; V121D; L163V; F189A; F189A/T172R; C107S; W220A; F189L; A193D; T172R/A193D; G173Q; T254R; N42V; T172R/G173Q; G171Q/T172R/G173Q; G171A; G173A; wt-M75-G-D185; wt-T176-G-G-D185; T172R/G173Q-I175-G-D185; T172R/G173Q-I175-G-G-A186; T172R/G173Q-T176-G-G-D185; S177Q; D45R; F47R; L169K; L174R; A181K; S179R; F189K; V190K; A194K; y

R182K.

20. Un método de tratar una afección inducida por la cocaína, que comprende administrar a un sujeto necesitado de ello una cantidad efectiva del polipéptido CocE mutante, el polipéptido CocE mutante codificado por el ácido nucleico, o la composición farmacéutica de una cualquiera de las reivindicaciones 1-19.

21. El método de la reivindicación 20, en donde la afección inducida por la cocaína se selecciona del grupo conformado por sobredosis de cocaína, toxicidad de la cocaína, adicción a la cocaína y dependencia de la cocaína.

22. Un método de detección selectiva de gran productividad para la identificación de polipéptidos CocE mutantes termoestables, que comprende los pasos de:

- transformar establemente una célula con un ácido nucleico que codifica un polipéptido CocE mutante;
- expresar el polipéptido CocE mutante en la célula;
- aislar el polipéptido CocE expresado;
- medir la actividad esterasa del polipéptido CocE mutante aislado a una o más temperaturas predeterminadas de por lo menos alrededor de 30°C hasta alrededor de 50°C, para determinar la termoestabilidad de los polipéptidos CocE mutantes aislados;
- seleccionar el polipéptido CocE mutante aislado con actividad esterasa a la temperatura(s) predeterminada.

23. El método de detección selectiva de gran productividad de la reivindicación 22 en donde medir la actividad esterasa de los polipéptidos mutantes aislados comprende:

- contactar el polipéptido CocE mutante aislado con (i) cocaína y un indicador de pH o (ii) un tio-derivado de cocaína y un indicador de tiol;

detectar un cambio en el indicador de pH o el indicador de tiol; en donde un cambio del indicador de pH o el indicador de tiol se correlaciona con la formación de ácido benzoico de la hidrólisis de cocaína o derivado de cocaína por el polipéptido CocE mutante.

24. El método de detección selectiva de gran productividad de una cualquiera de las reivindicaciones 22-23 que comprende además repetir uno o más pasos del método de detección selectiva por lo menos una vez con una temperatura incrementada para medir la actividad esterasa.

25. El método de detección selectiva de gran productividad de la reivindicación 24 en donde un primer ciclo de detección selectiva tiene una primera temperatura para medir la actividad esterasa de alrededor de 30°C, y un ciclo subsiguiente de detección selectiva tiene una temperatura para medir la actividad esterasa de alrededor de 45°C.

26. El método de detección selectiva de gran productividad de una cualquiera de las reivindicaciones 22-25 en donde la expresión del polipéptido CocE mutante ocurre a una temperatura a la cual la CocE tipo silvestre conserva sustancialmente actividad catalítica.

27. El método de detección selectiva de gran productividad de una cualquiera de las reivindicaciones 22-25 en donde la expresión del polipéptido CocE mutante ocurre a una temperatura a la cual el polipéptido CocE tipo silvestre se divide sustancialmente en cuerpos de inclusión.

FIG. 1

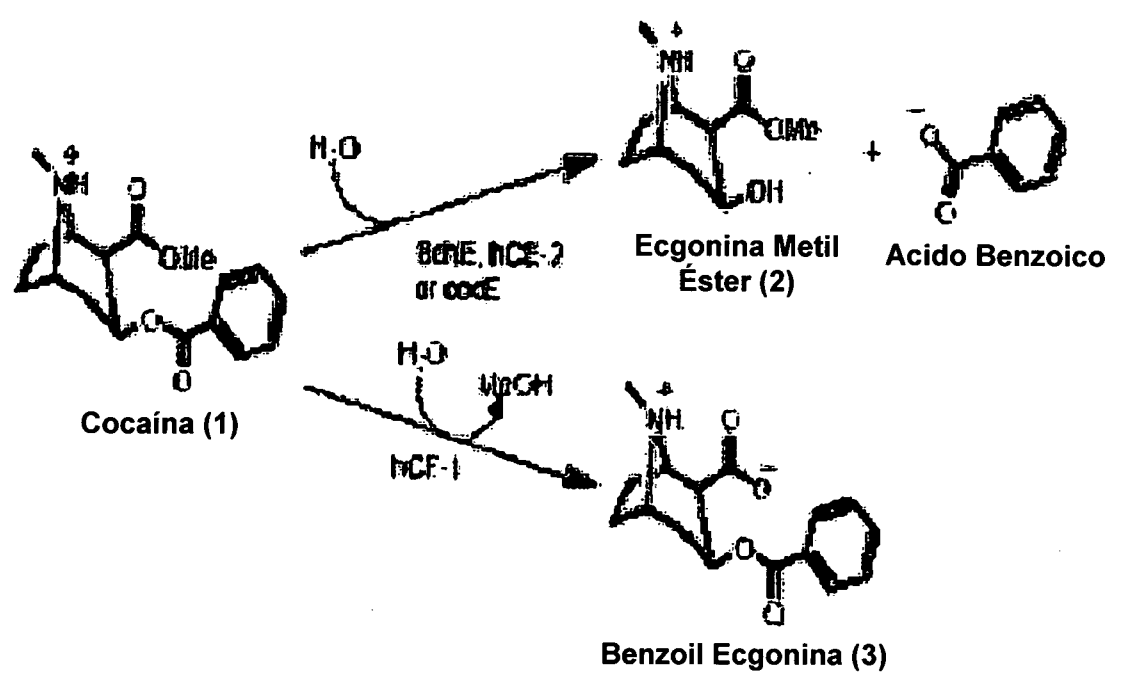


FIG. 2

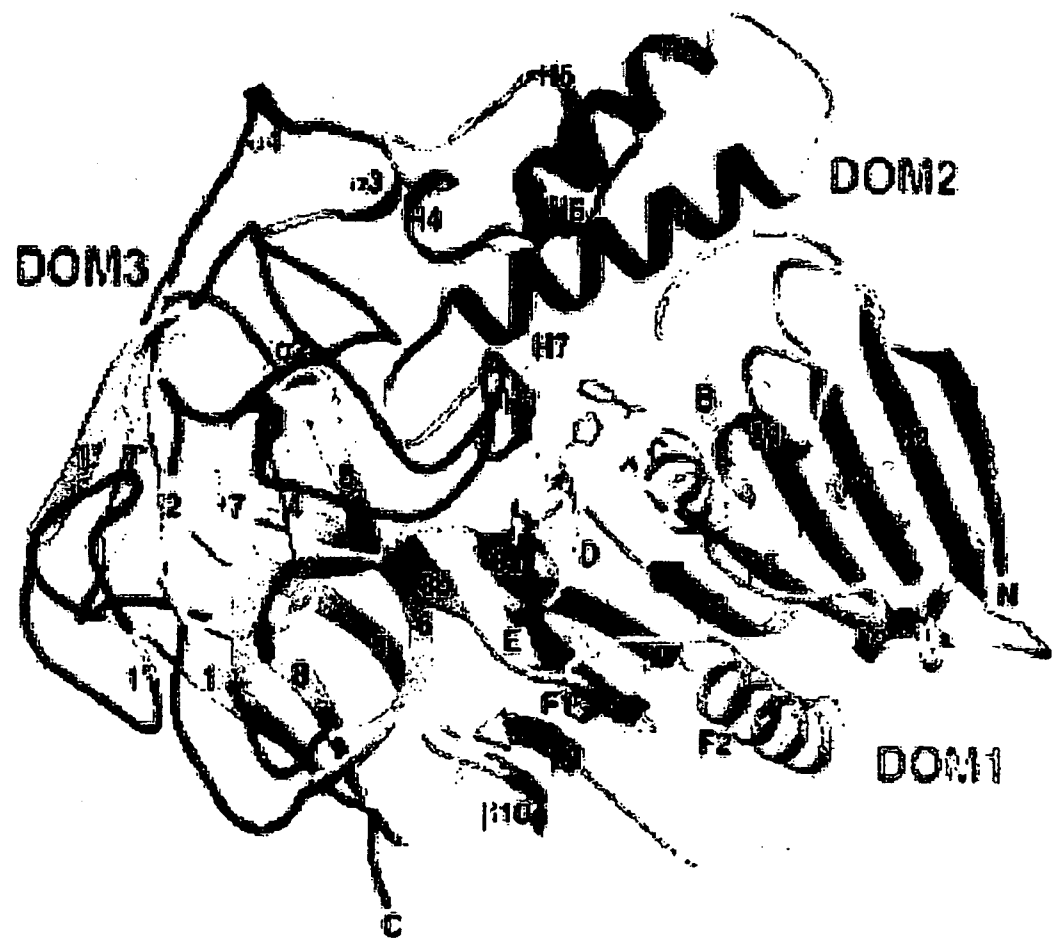


FIG. 3

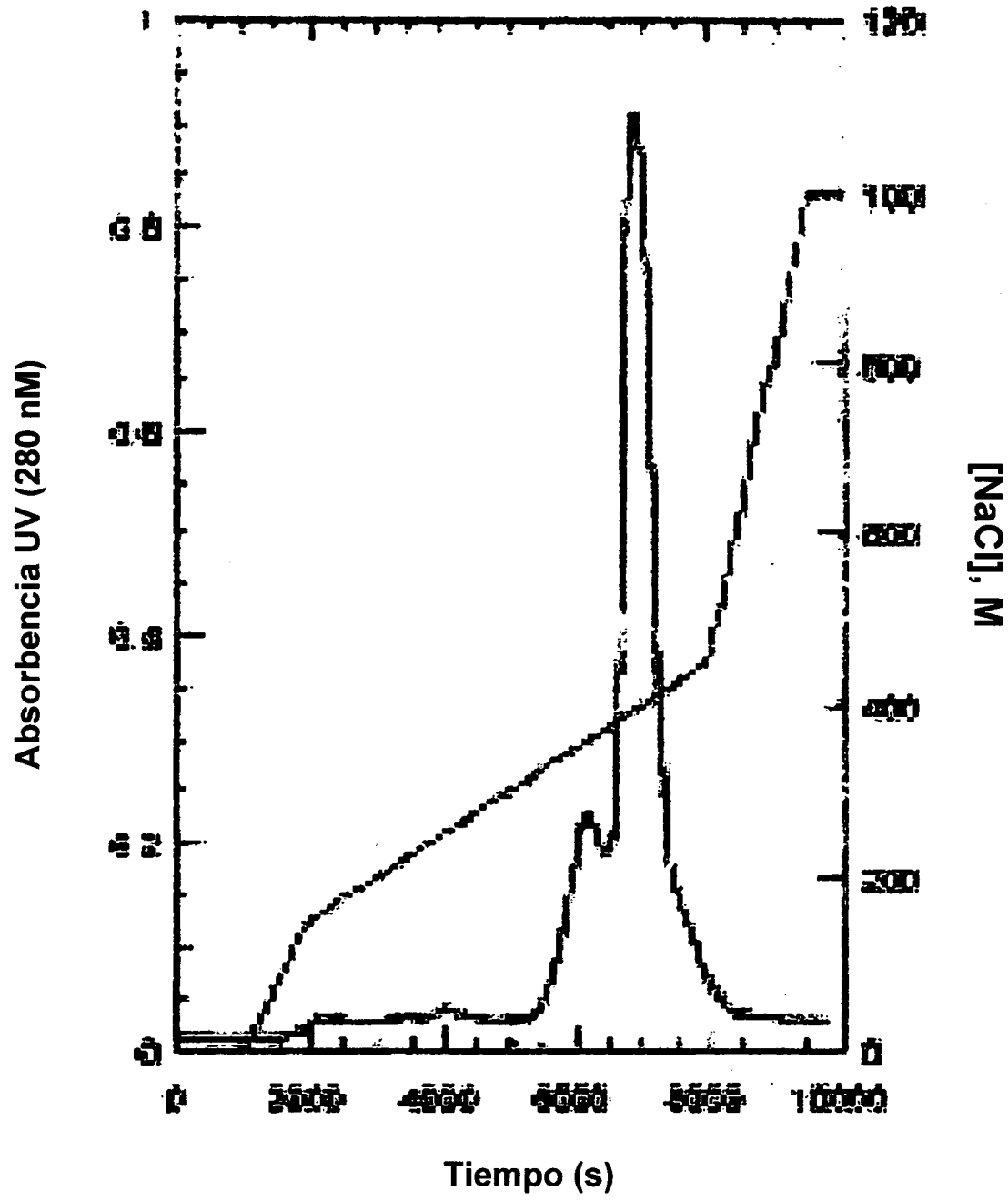


FIG. 4

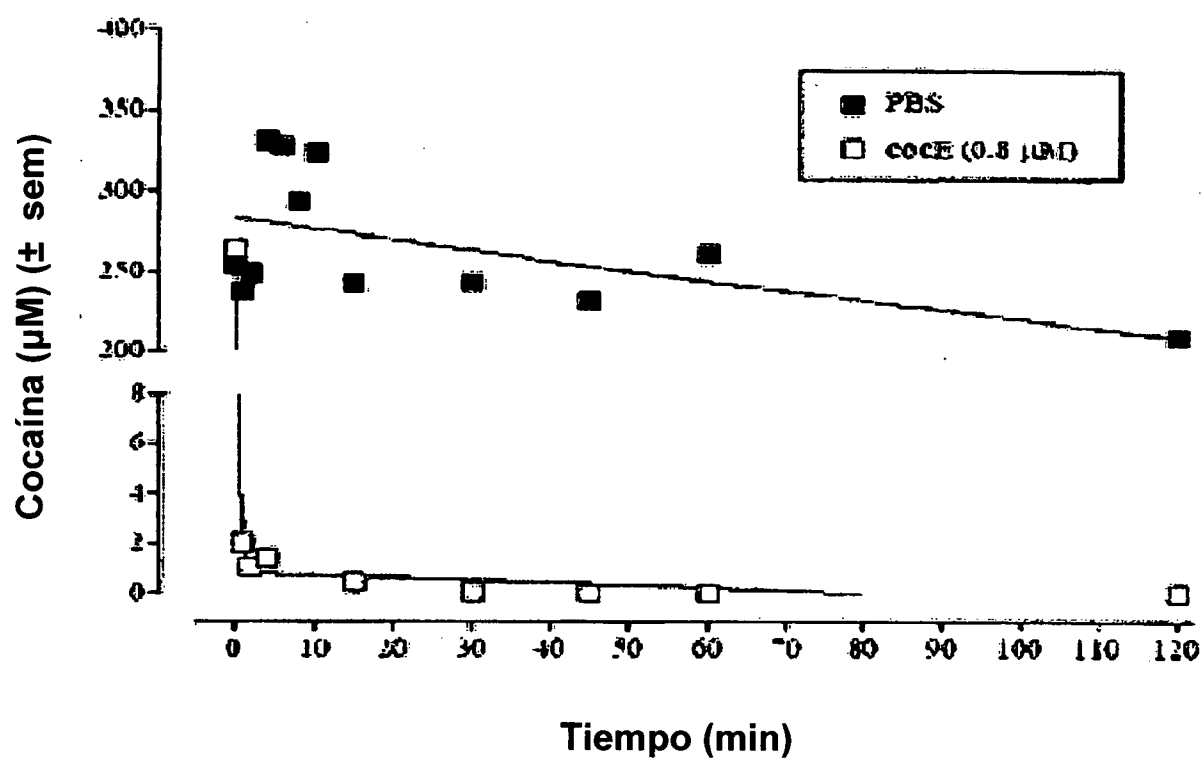


FIG. 5

Repetición de mutantes S159A & T172R (3-6-06)

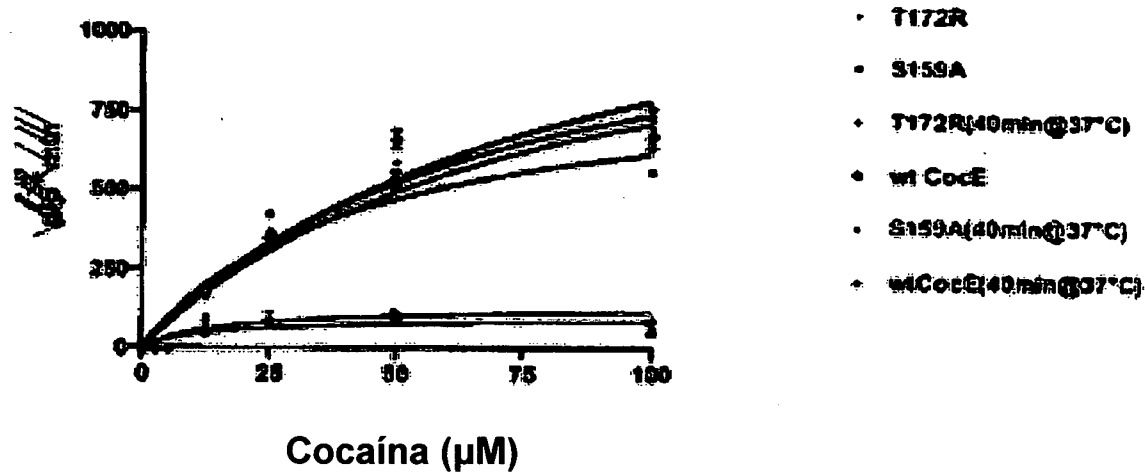


FIG. 6

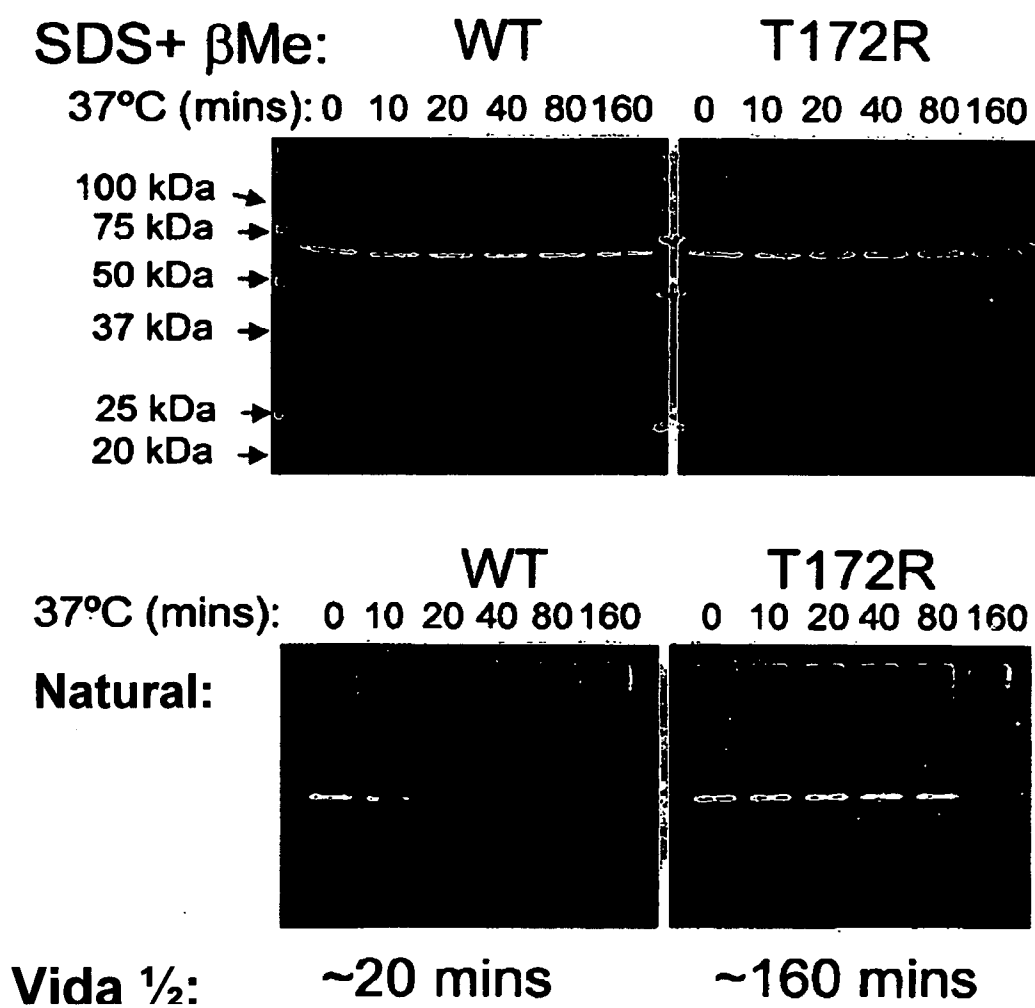


FIG. 7

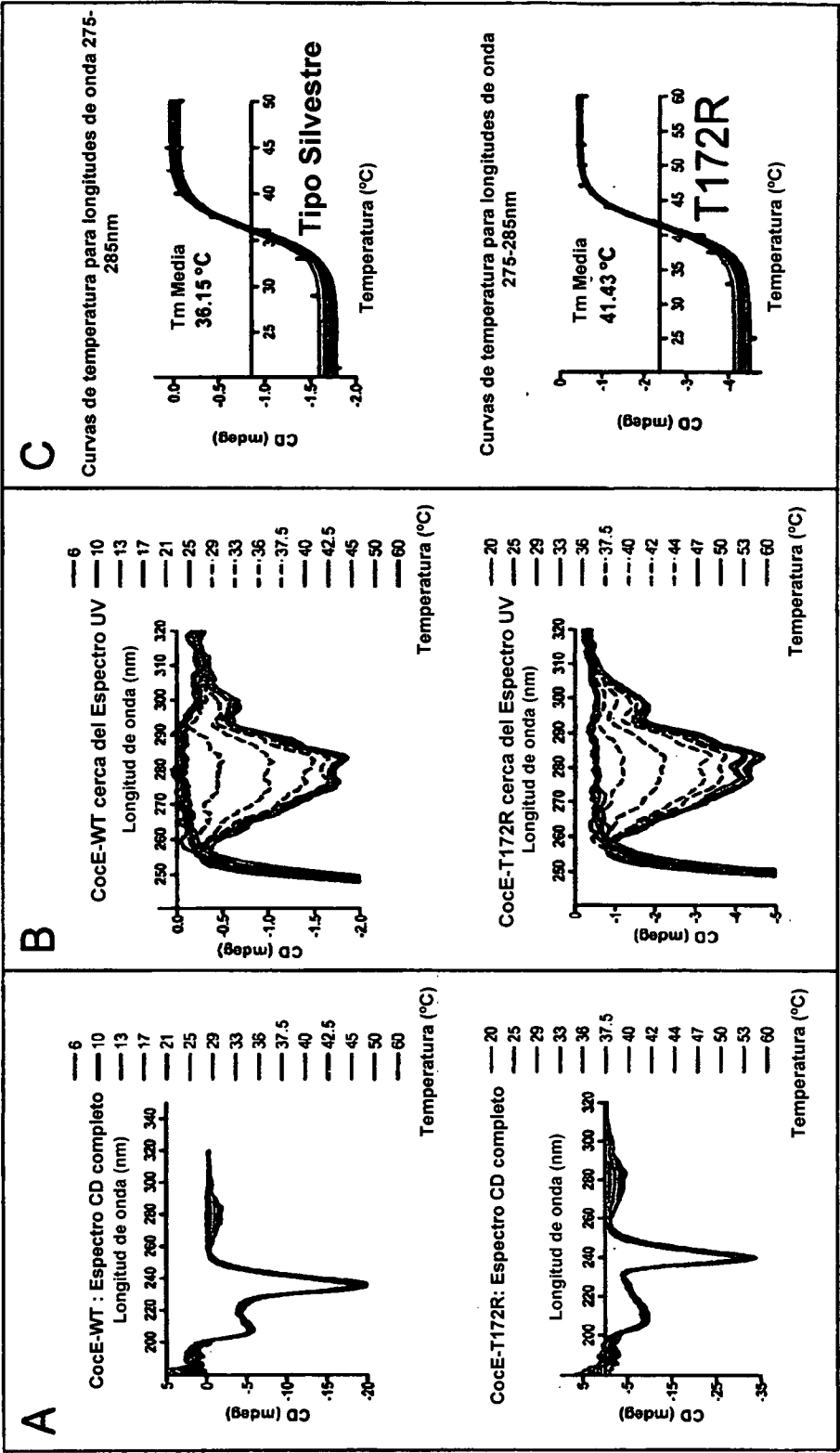
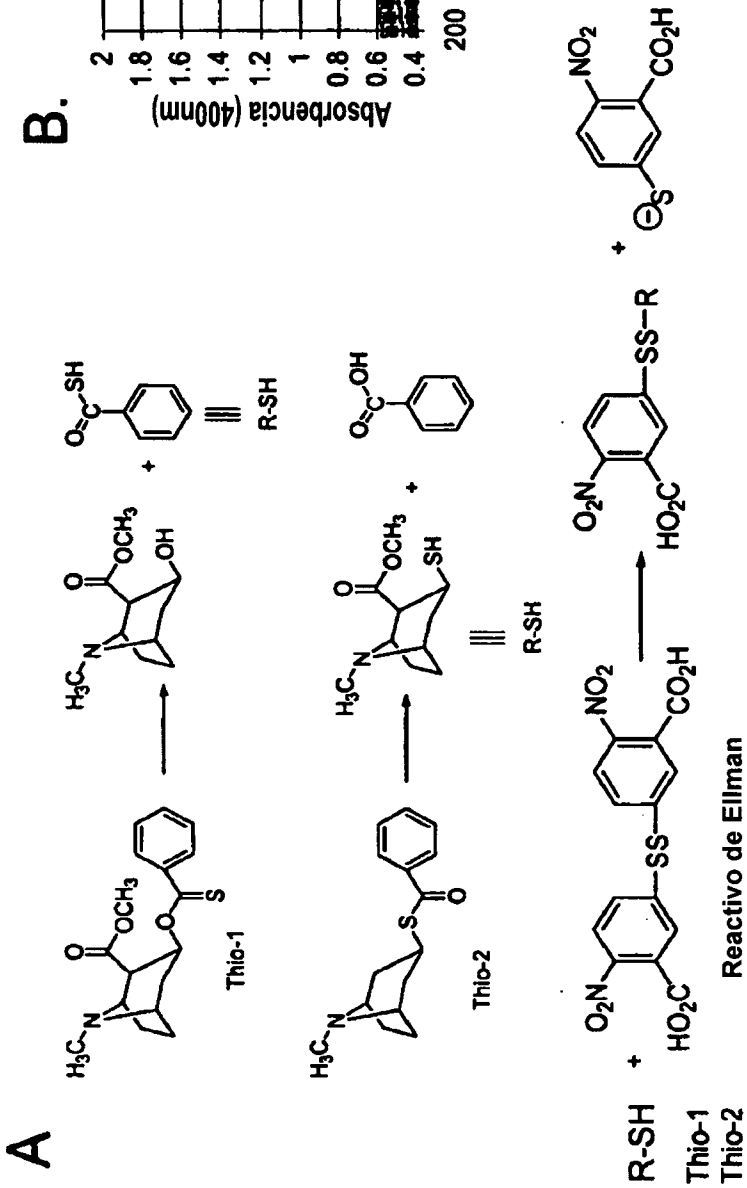
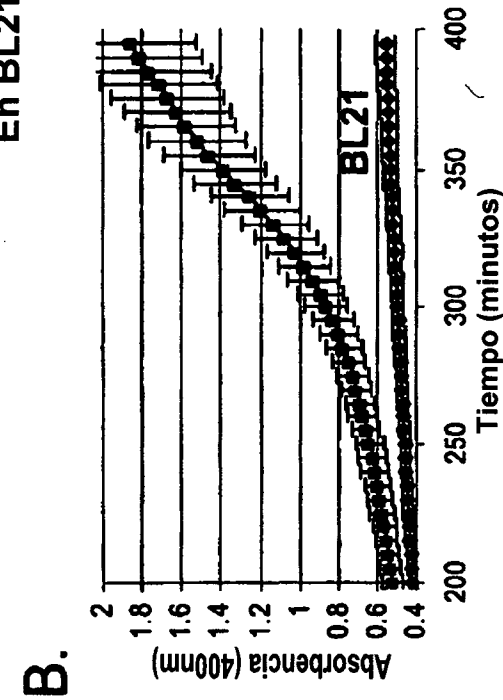


FIG. 8

CocE en células pET22
En BL21



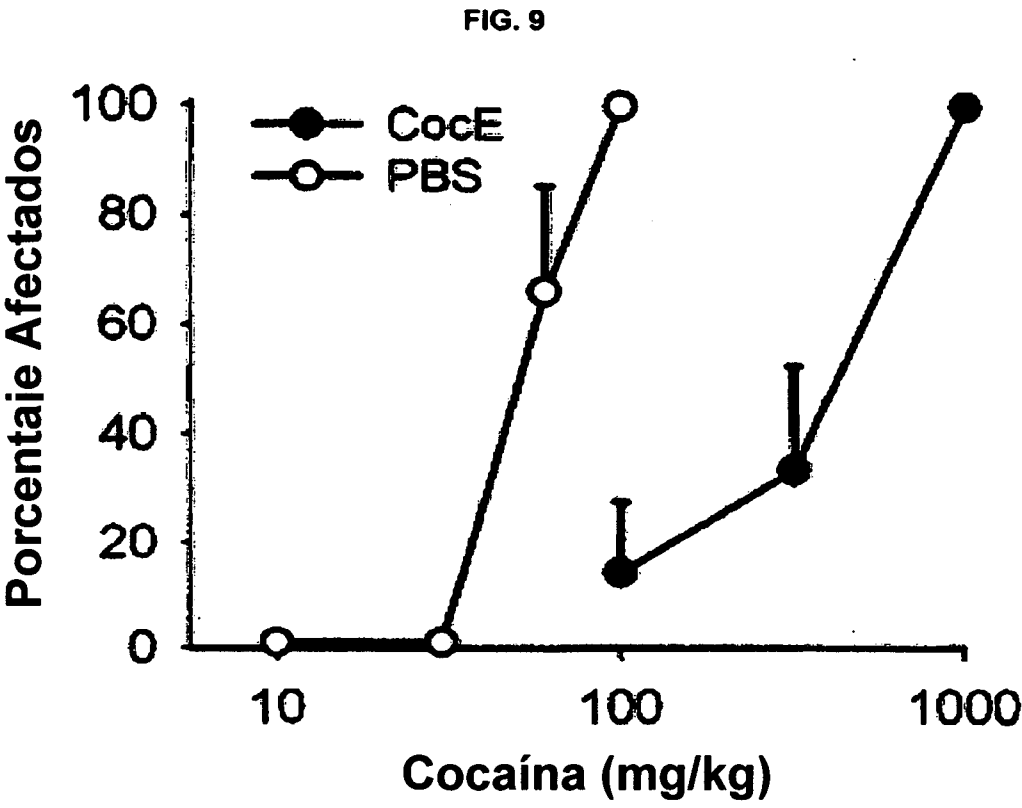


FIG. 10

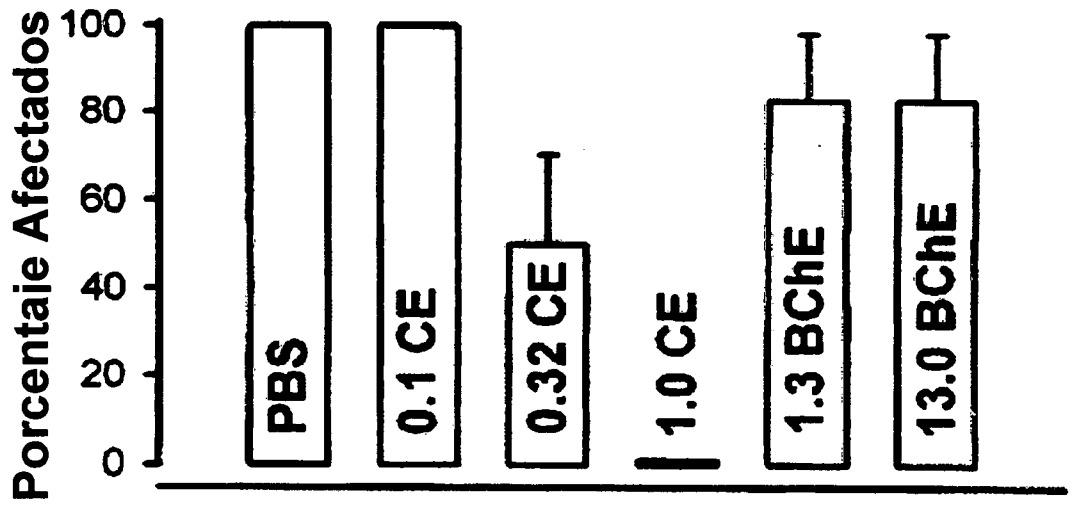


FIG. 11

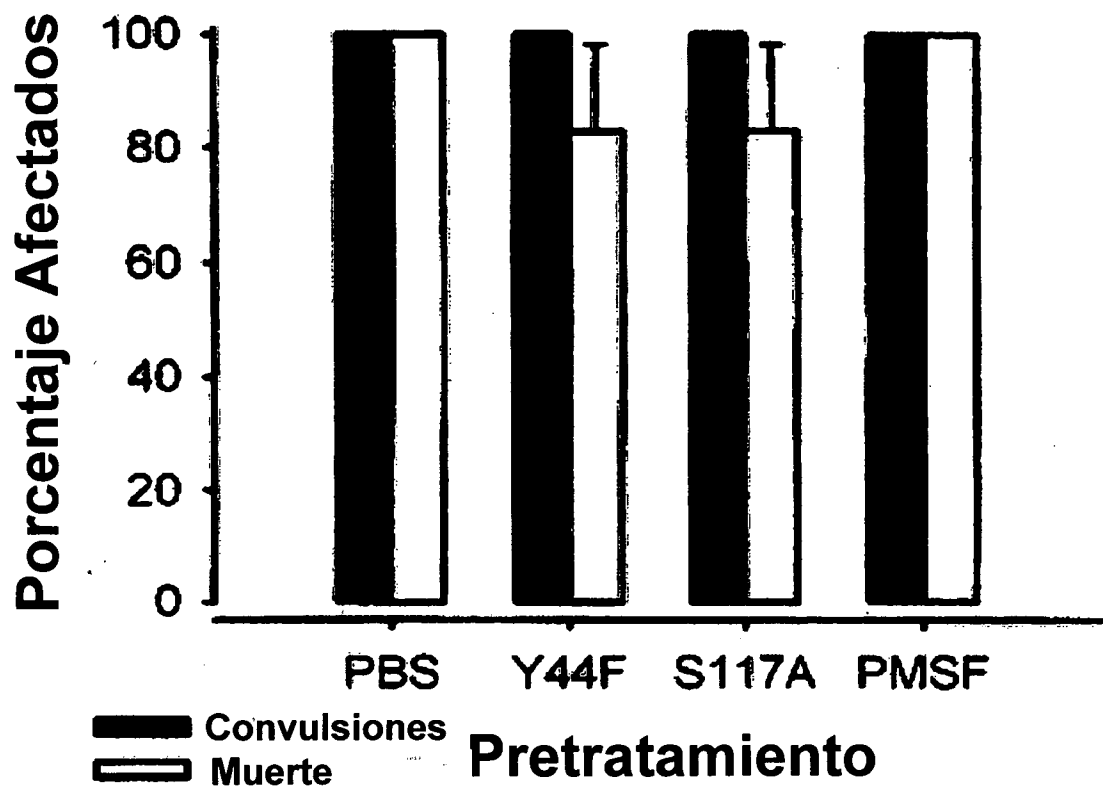


FIG. 12

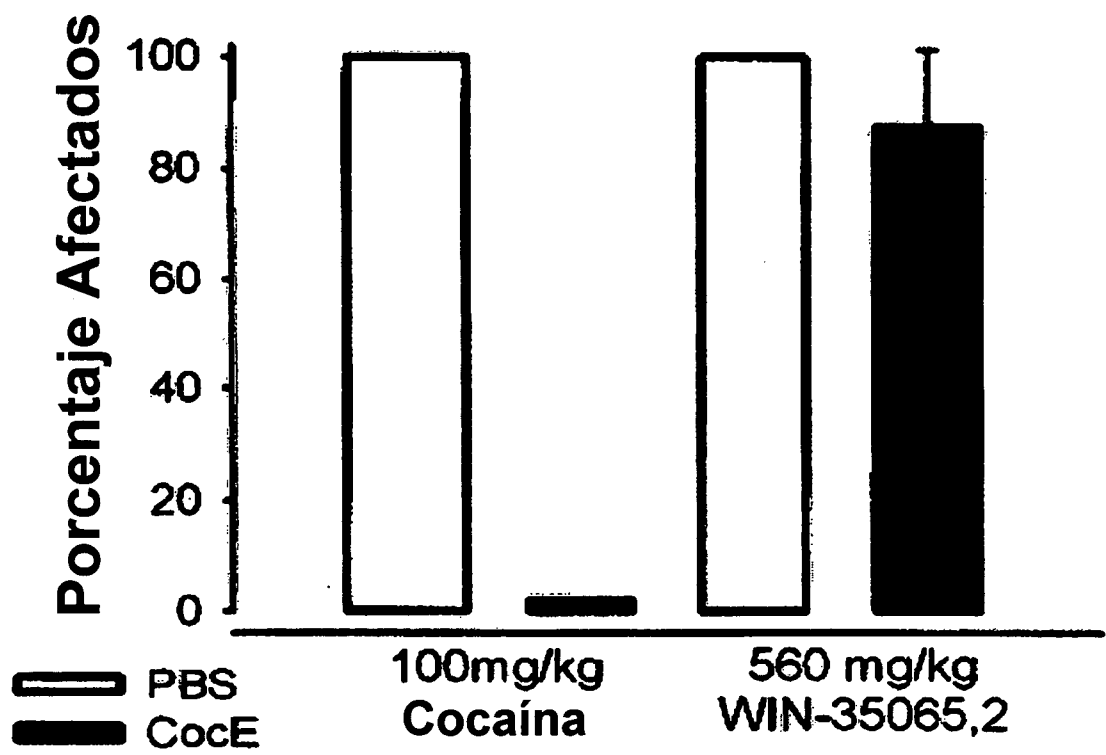


FIG. 13

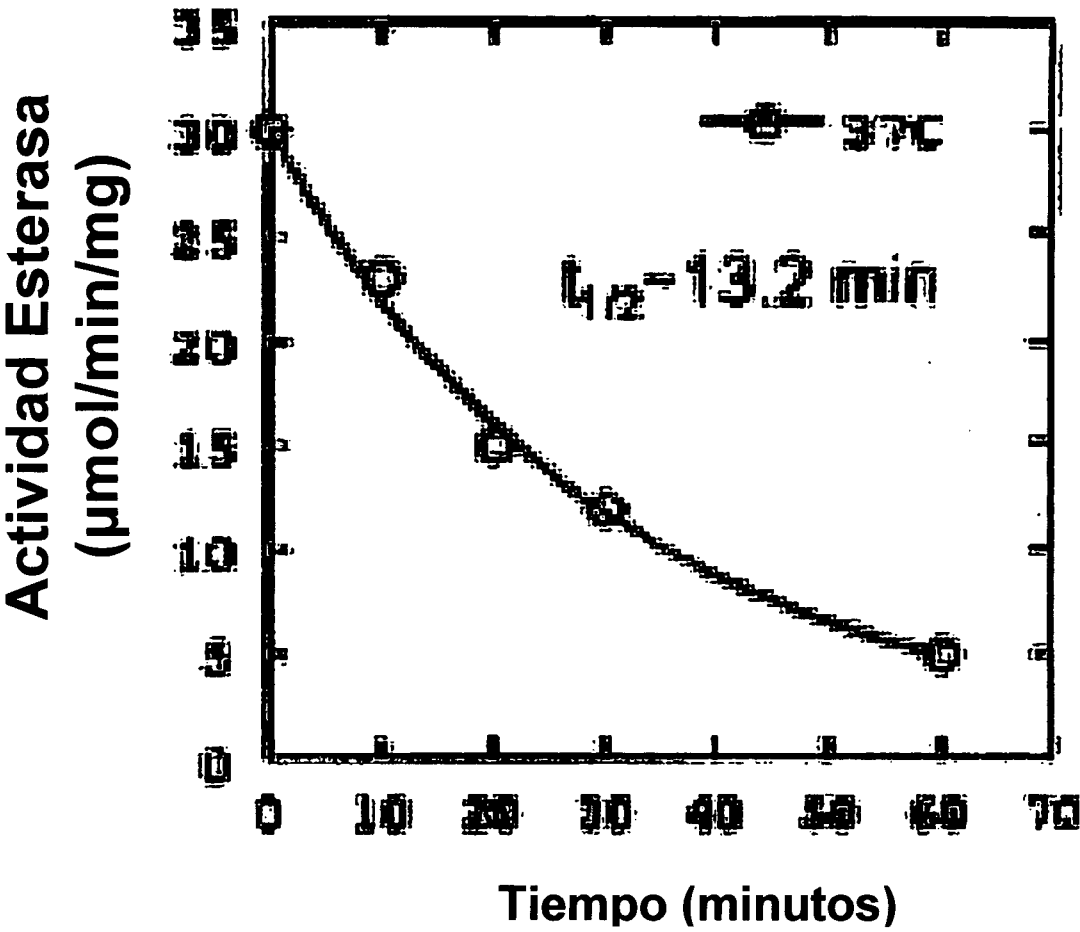


FIG. 14

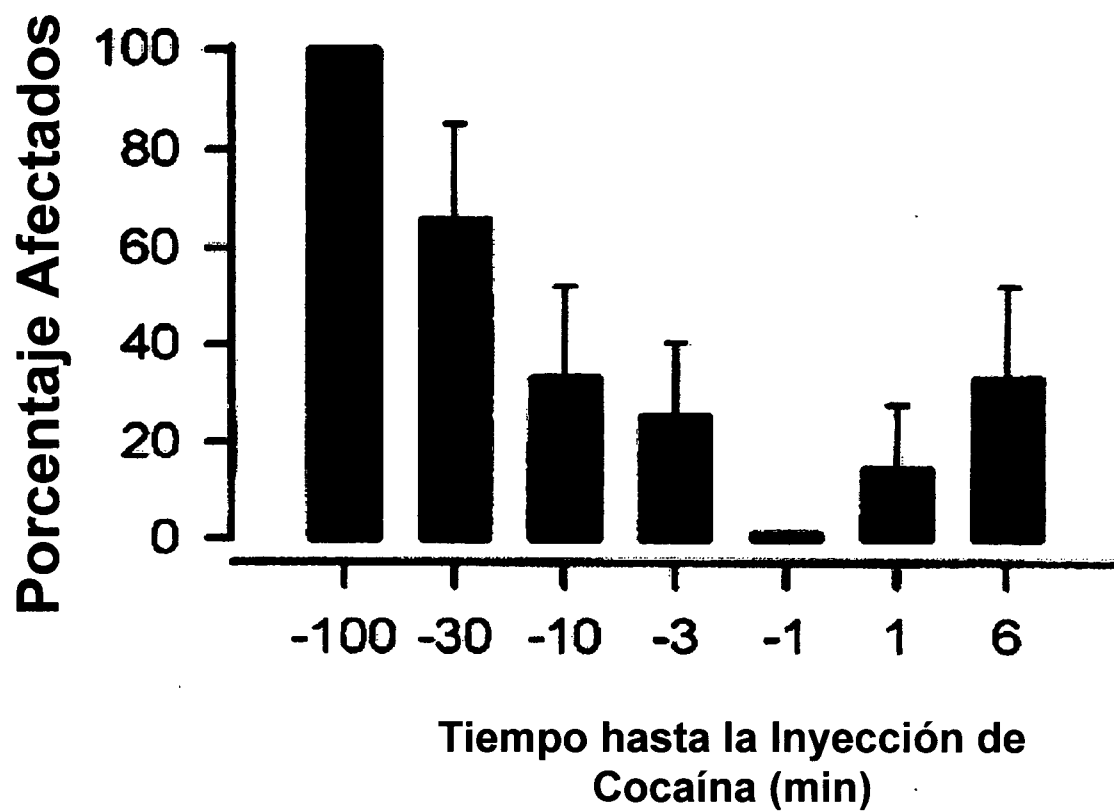


FIG. 15

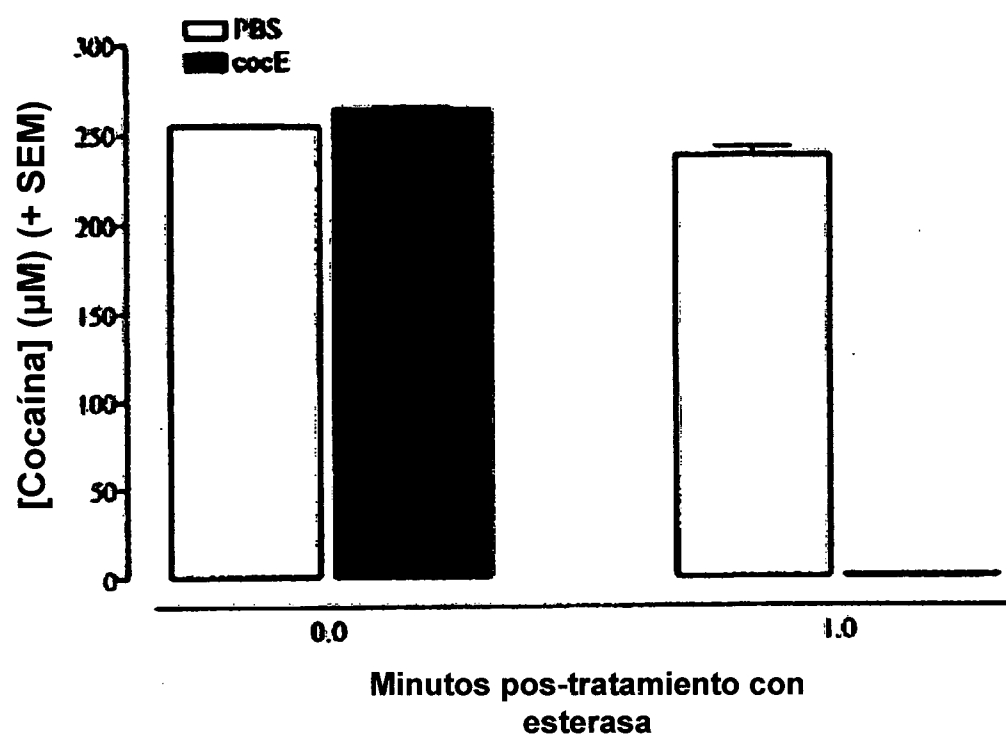


FIG. 16

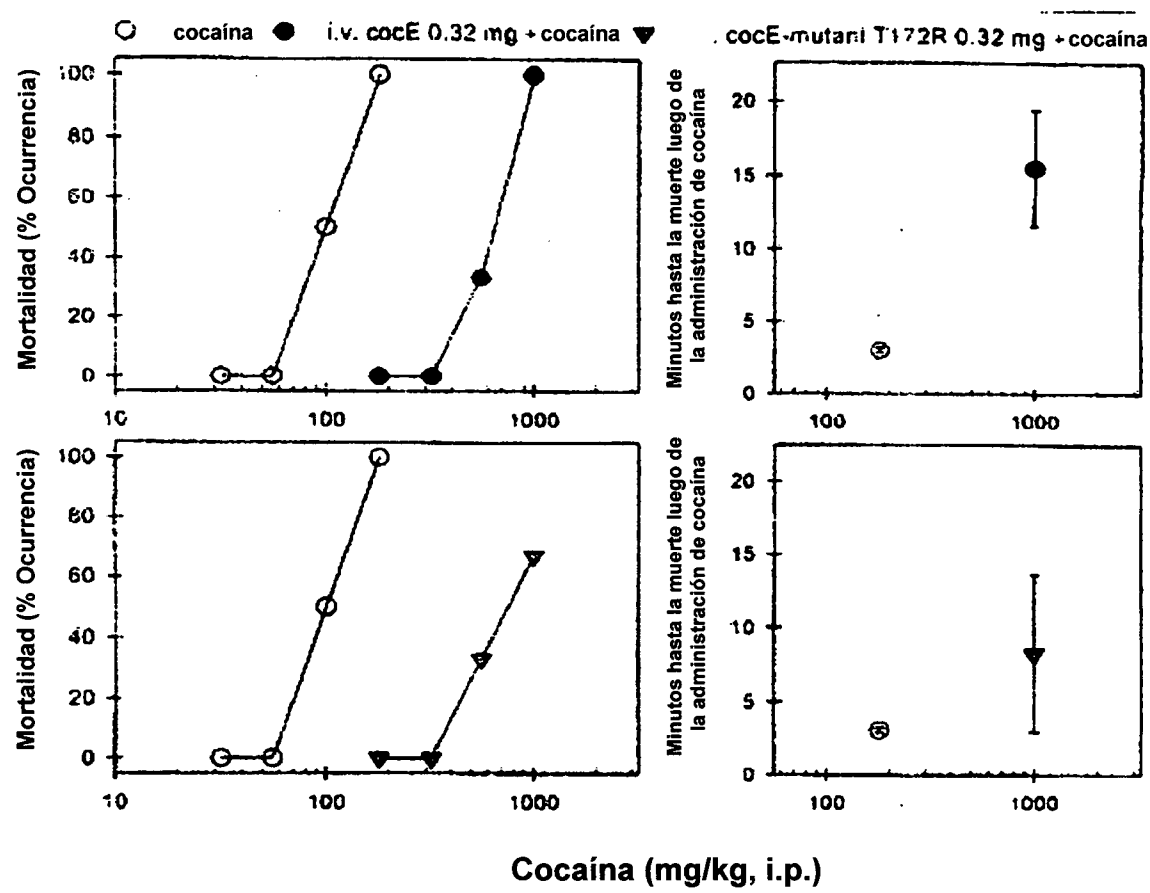


FIG. 17

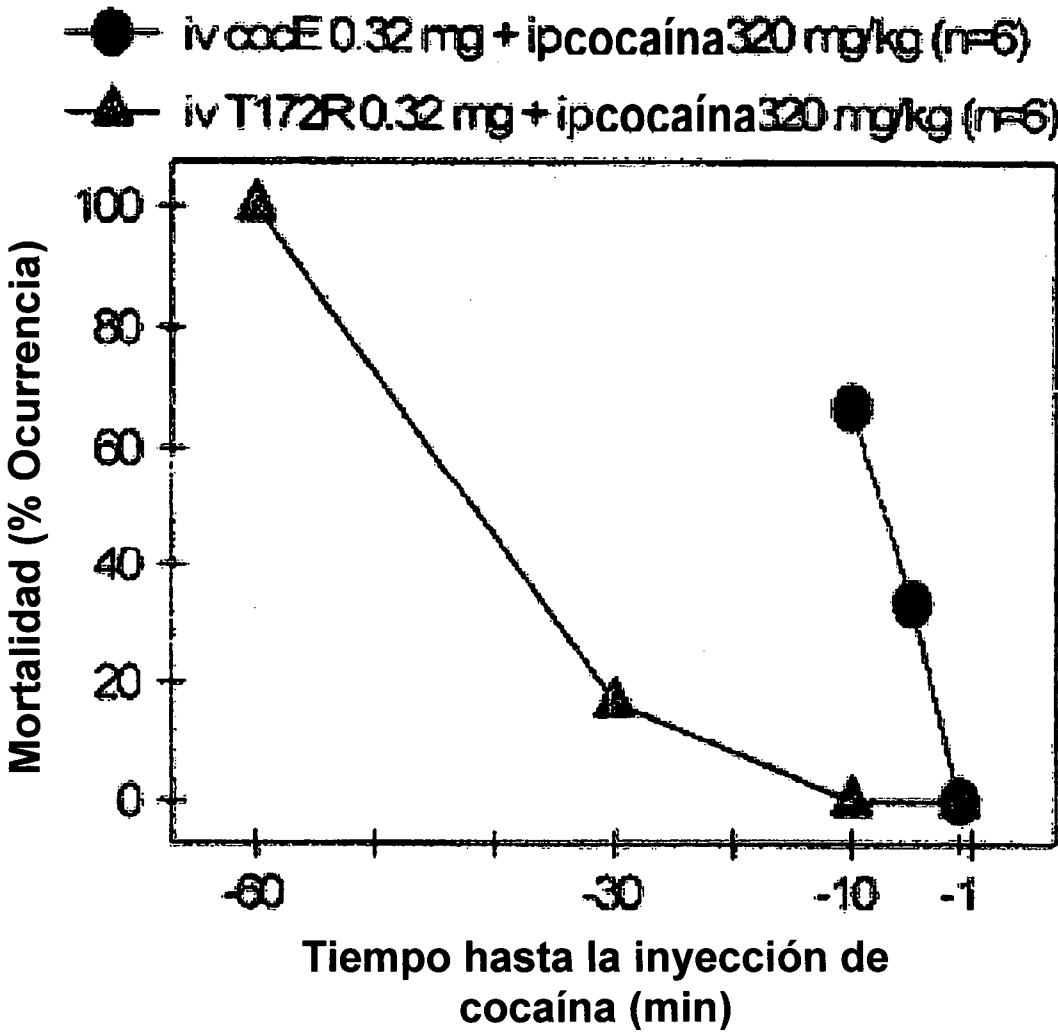


FIG. 18

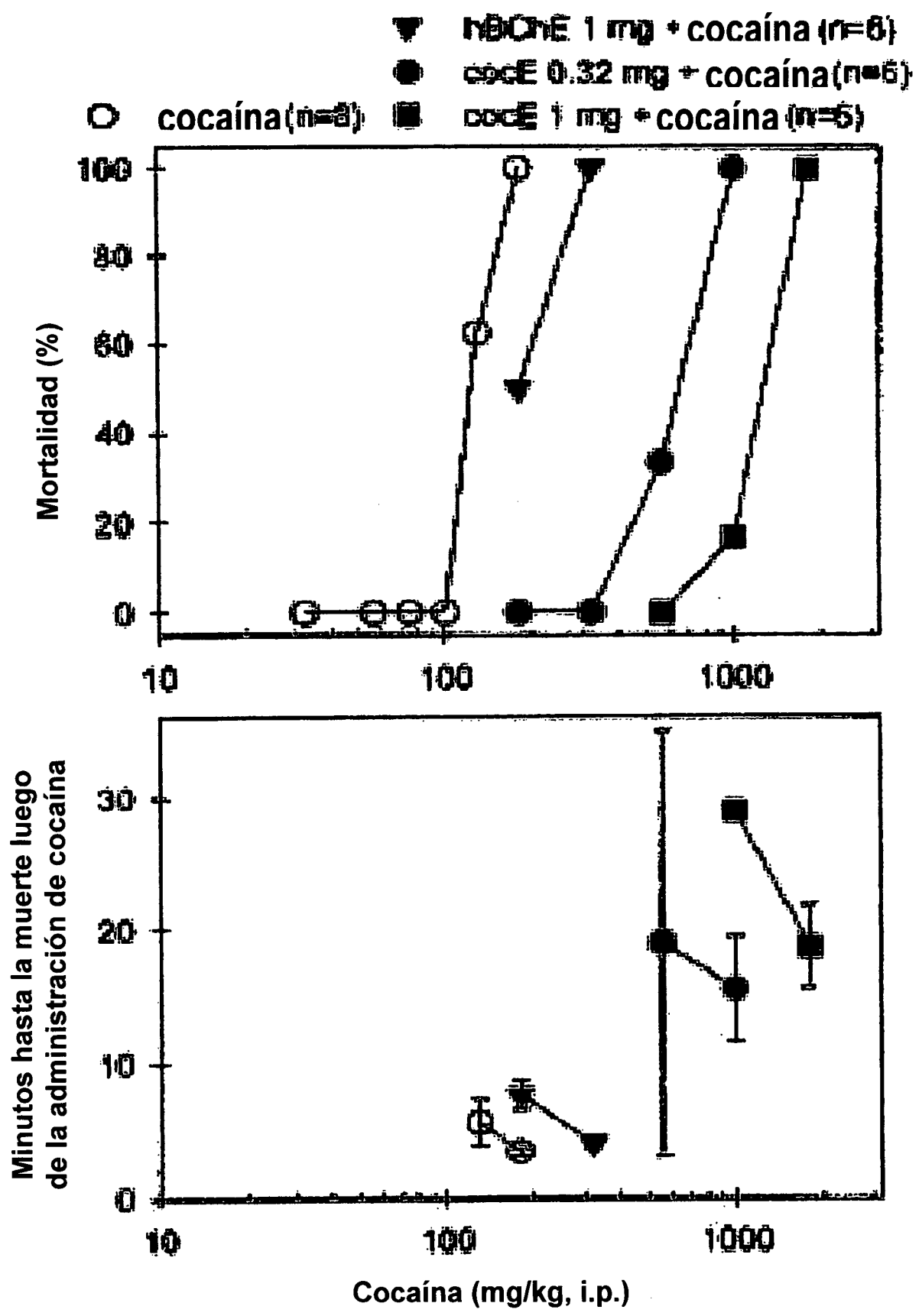


FIG. 19

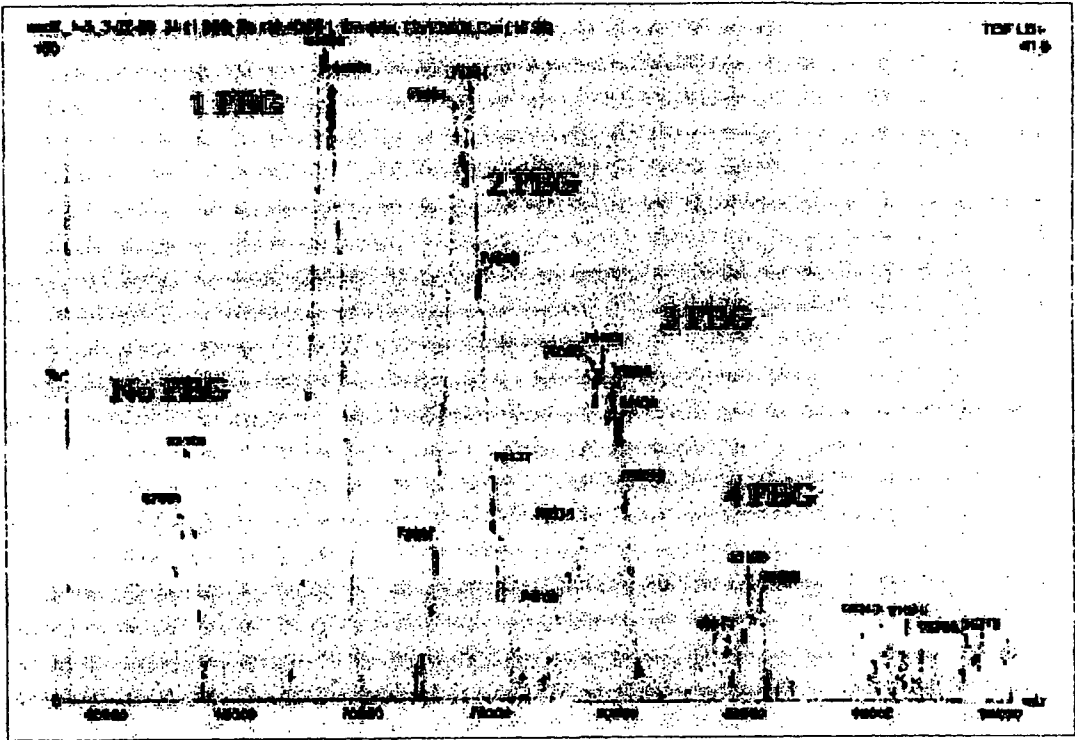
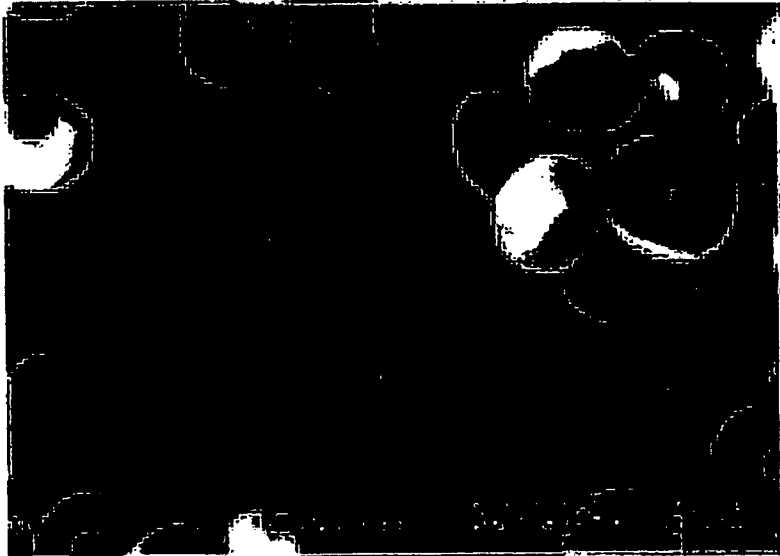
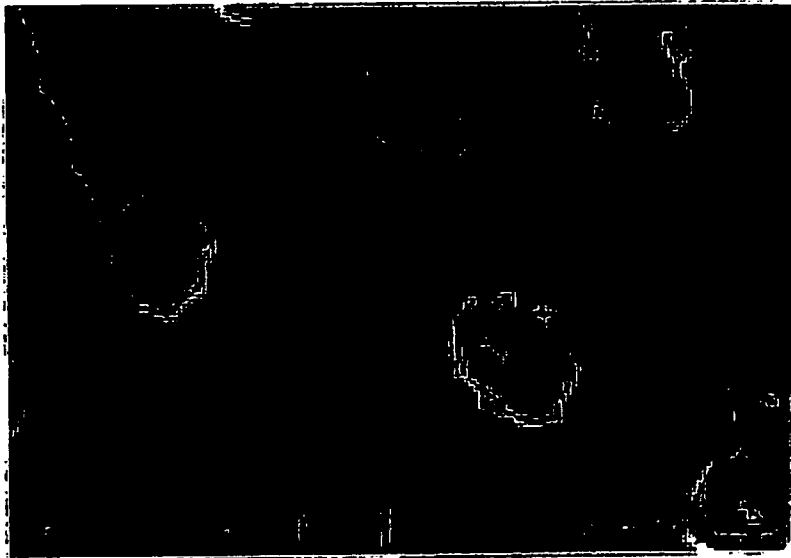


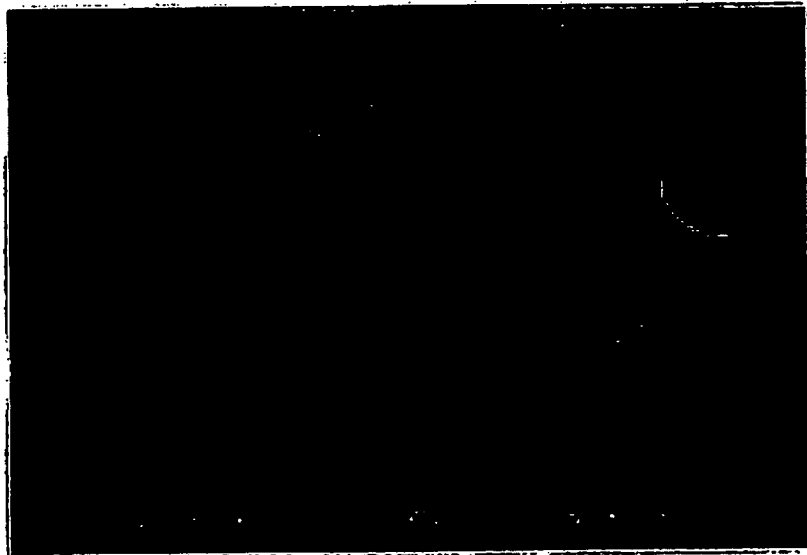
FIG. 20



A



B



C

FIG. 21

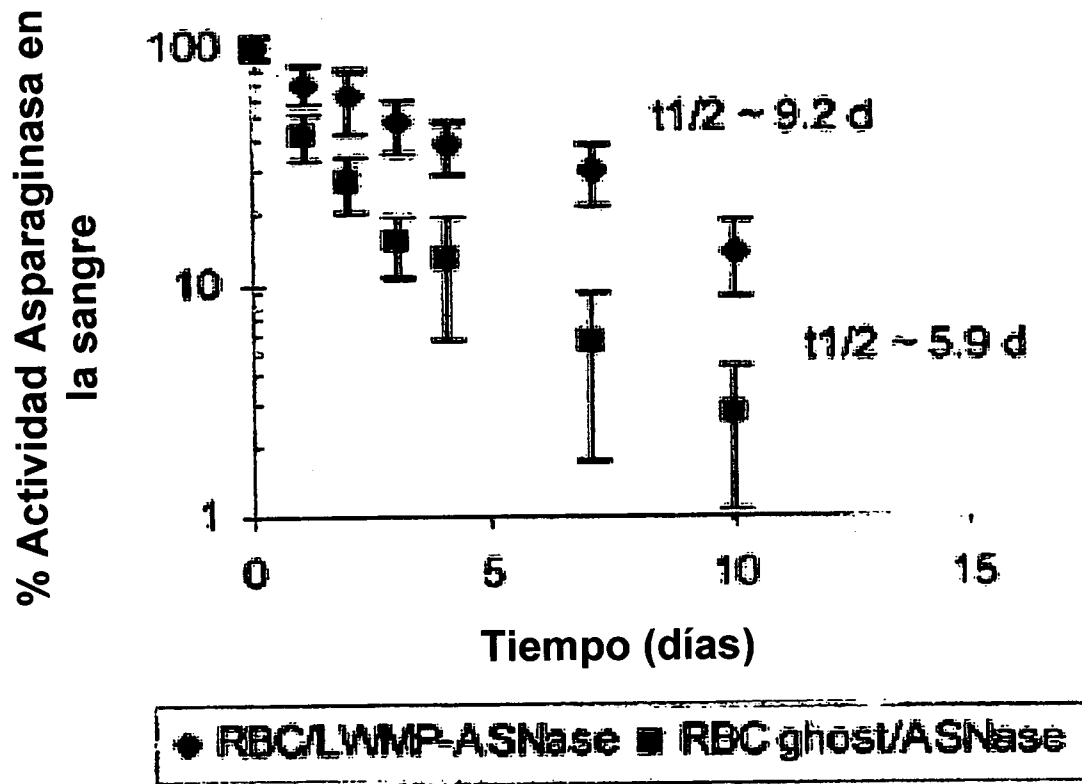


FIG. 22

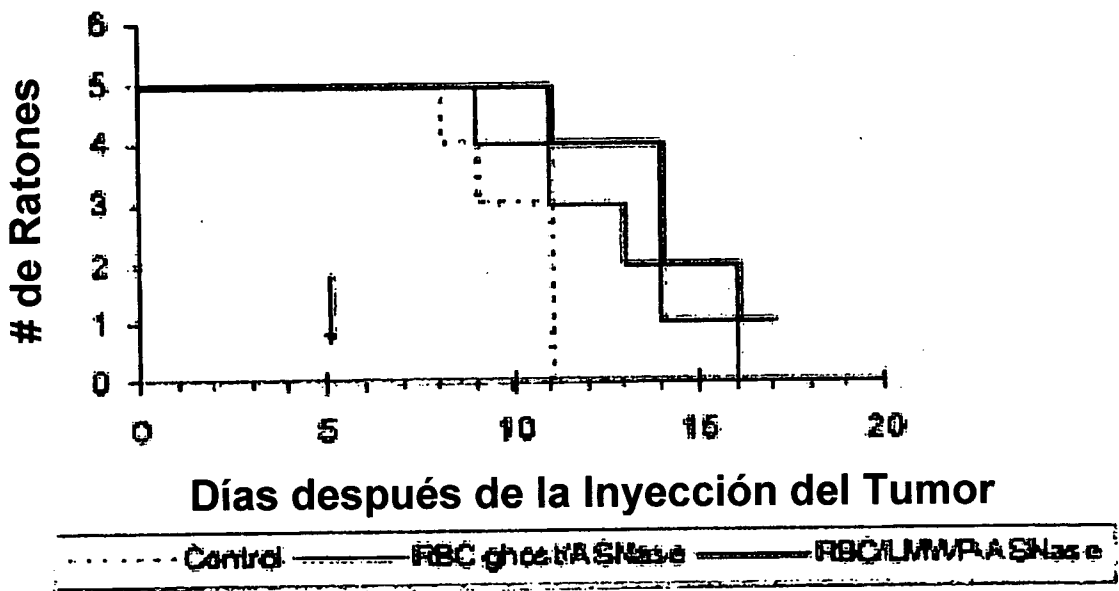


FIG. 23

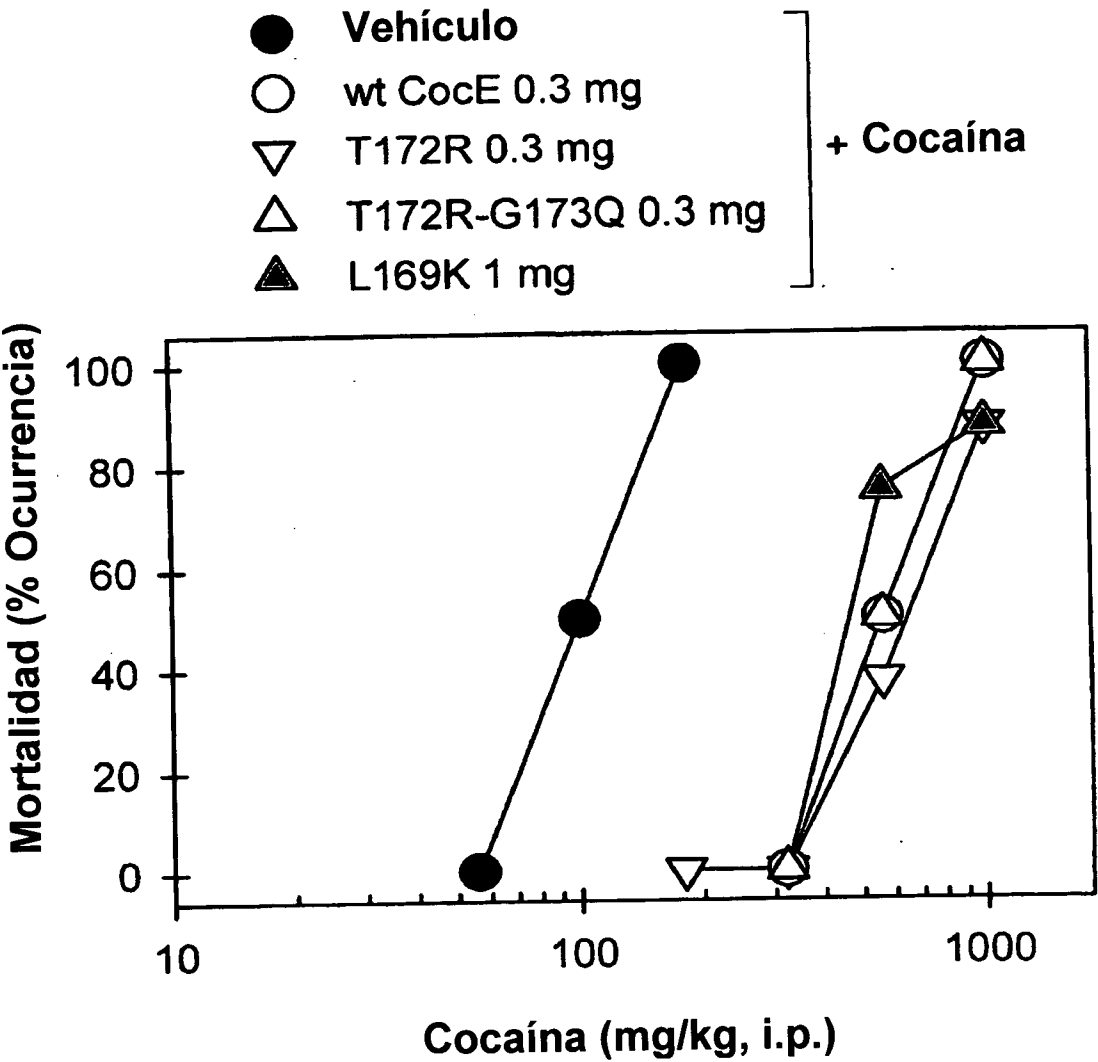


FIG. 24

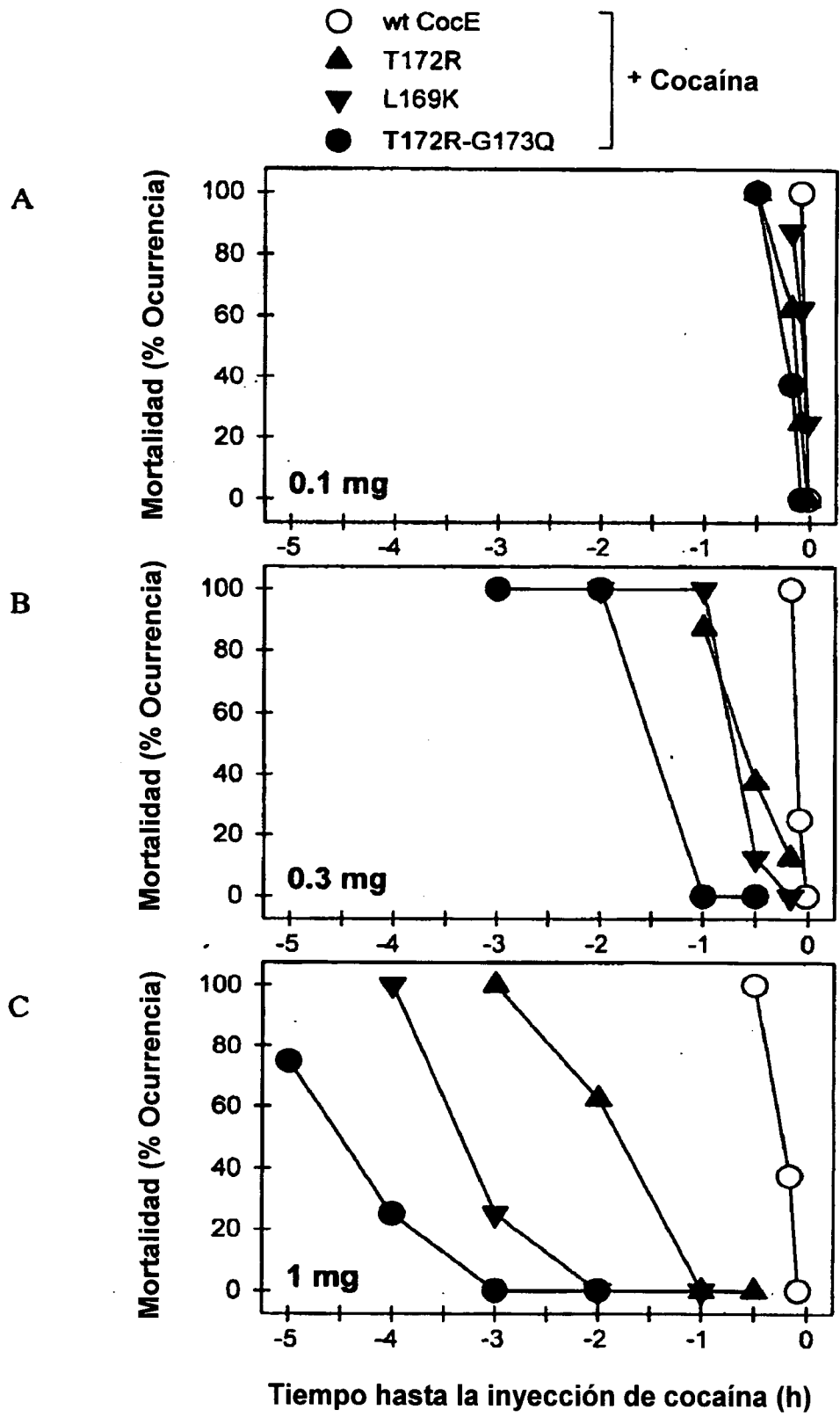


FIG. 25

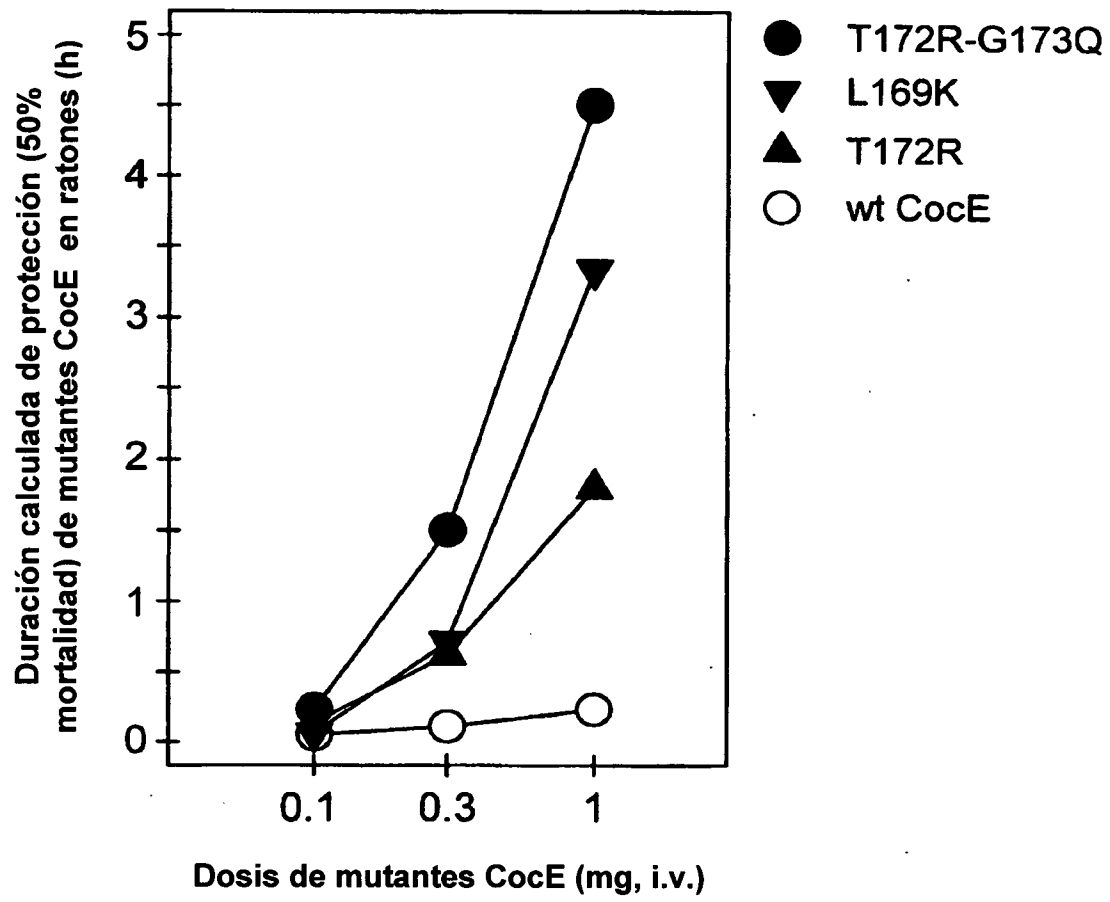


FIG. 26

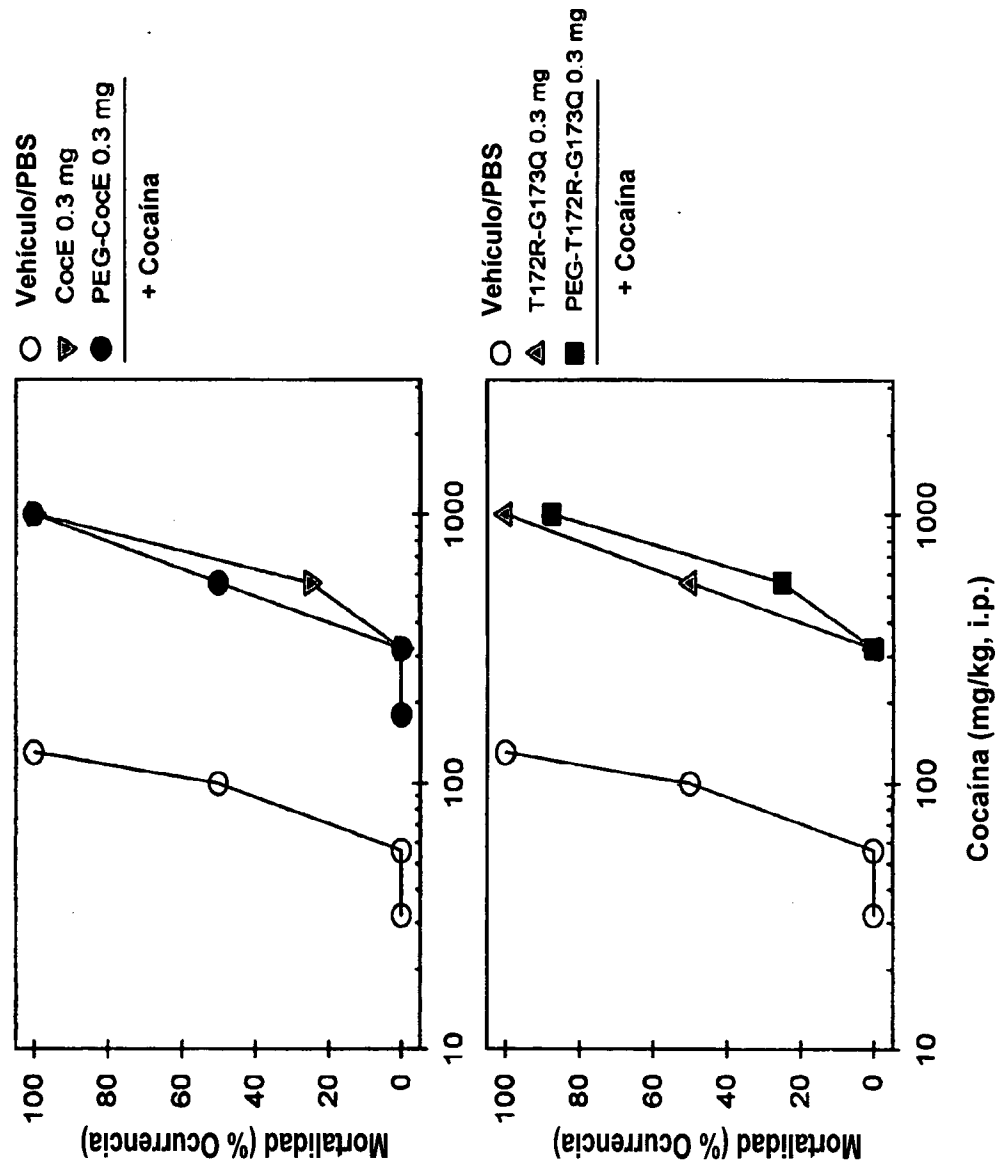
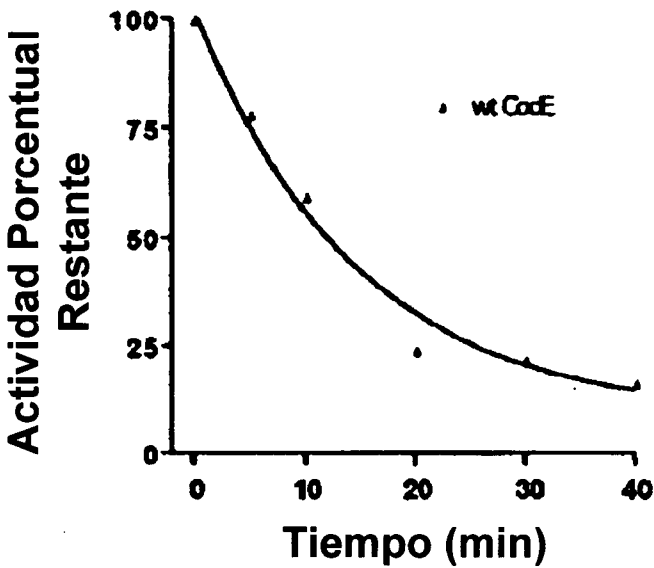
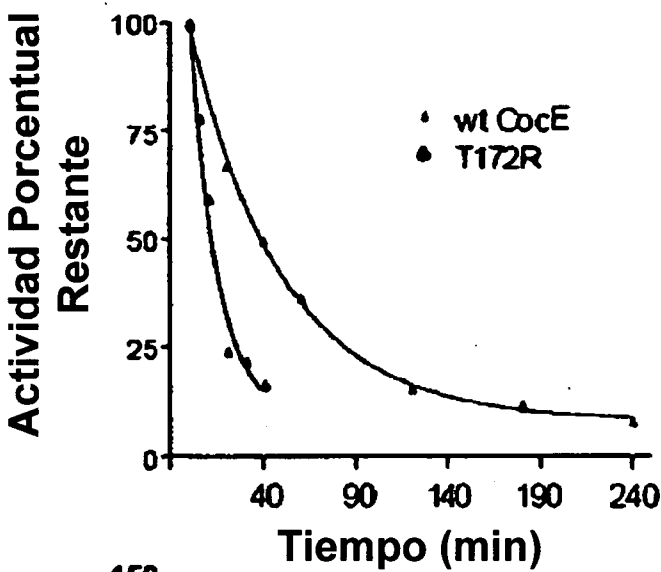


FIG. 27

A



B



C

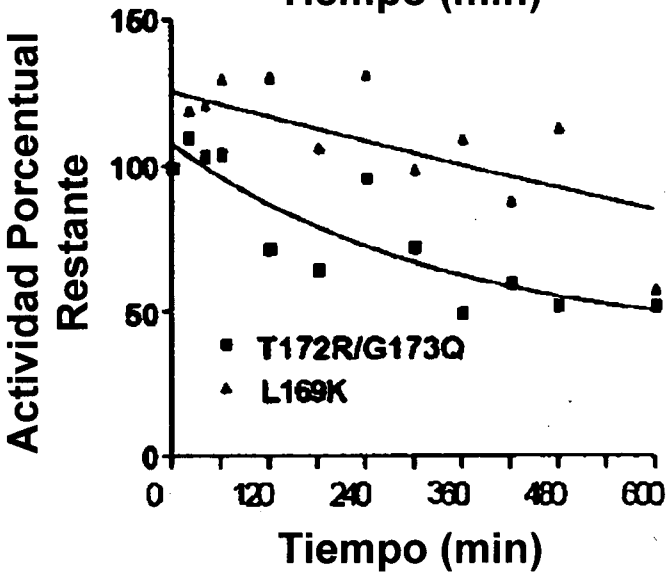


FIG. 28

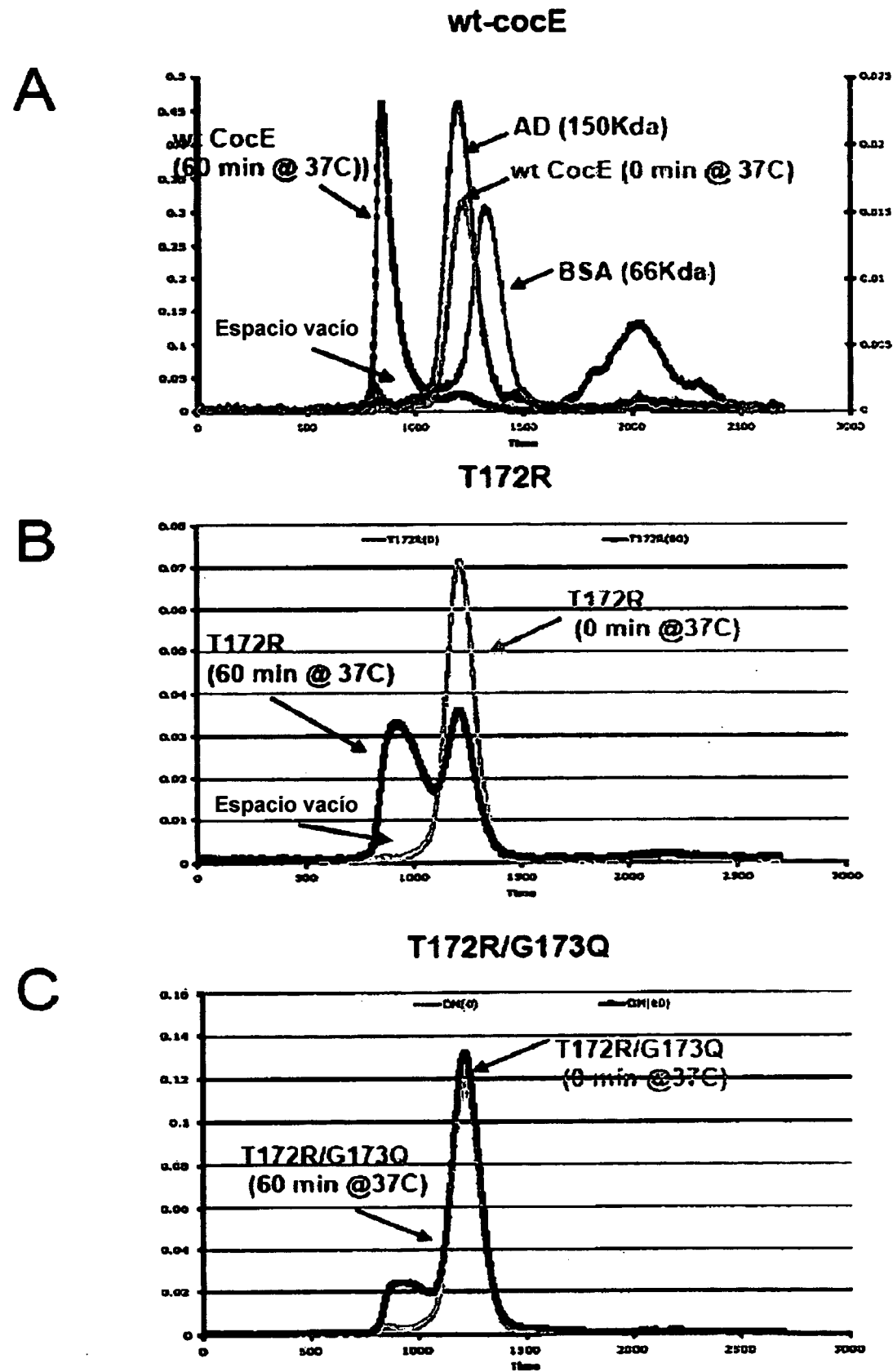


FIG. 29

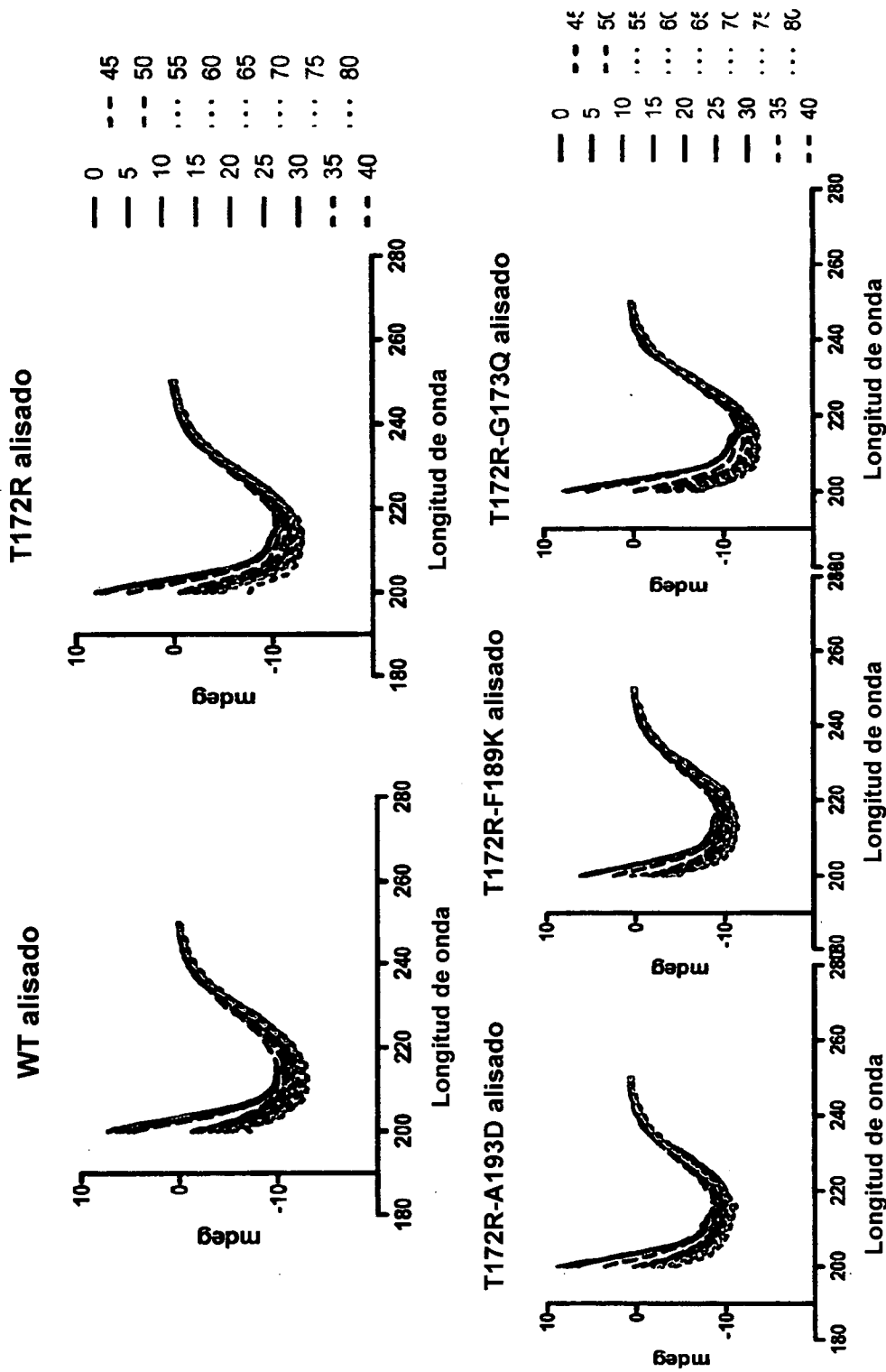


FIG. 30

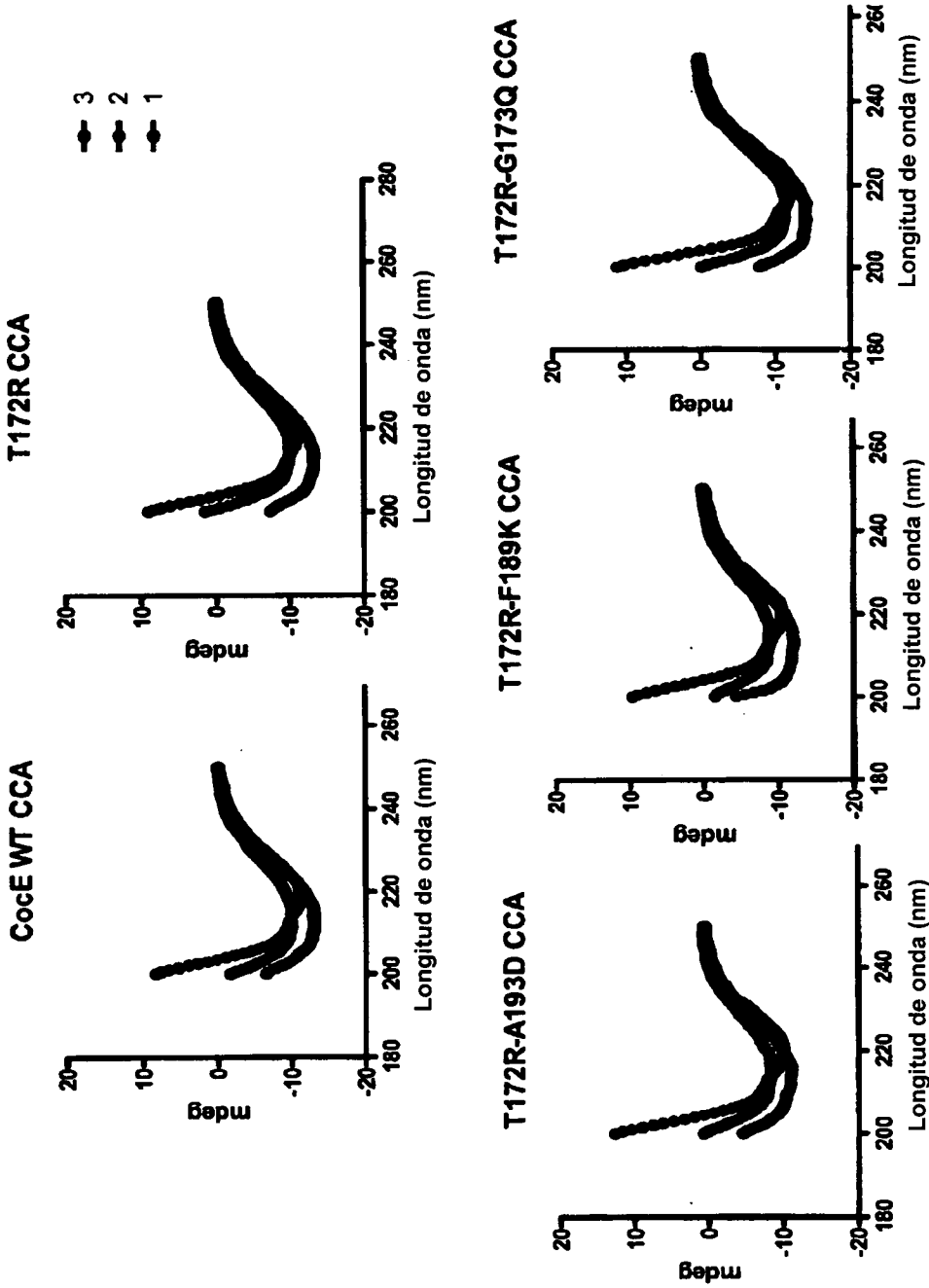


FIG. 31

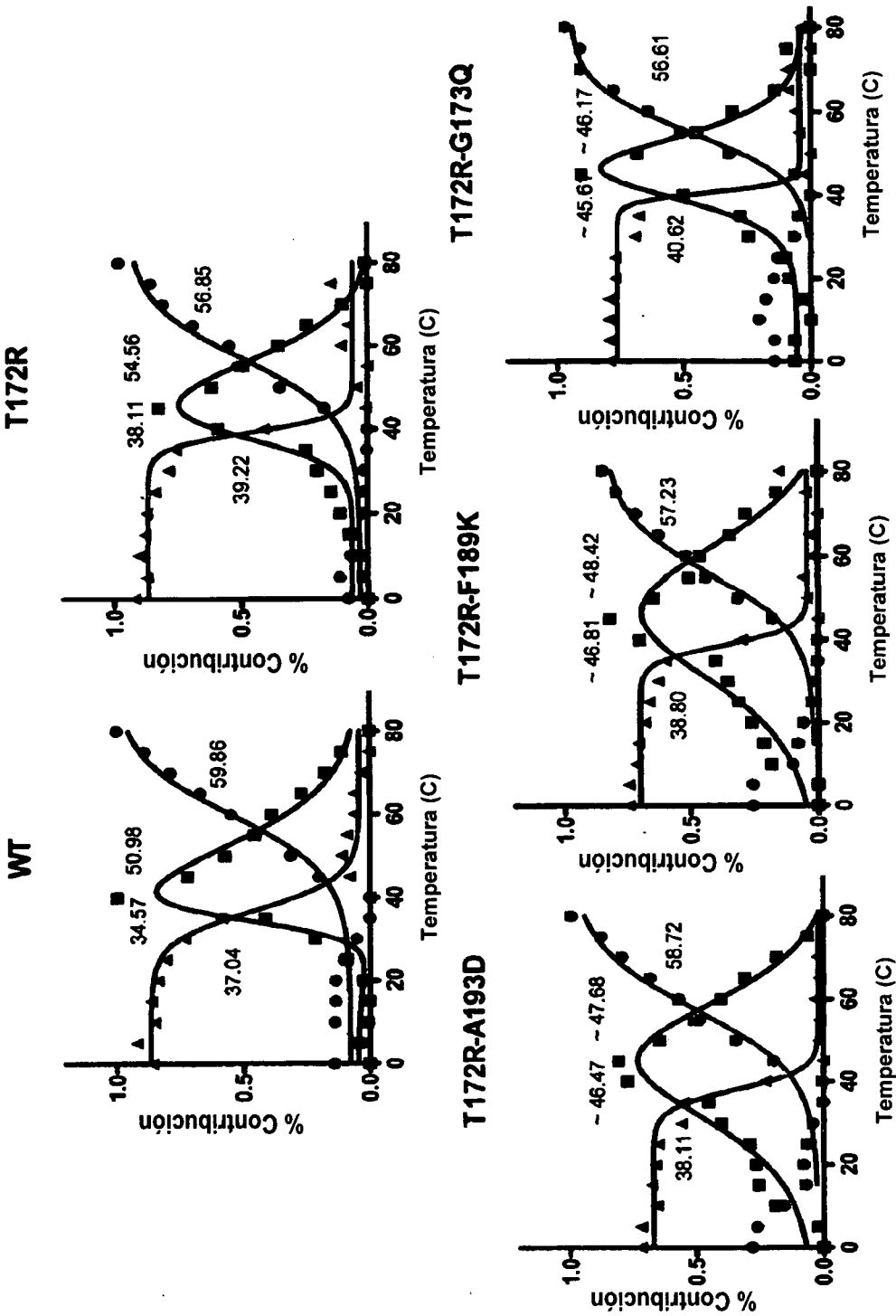
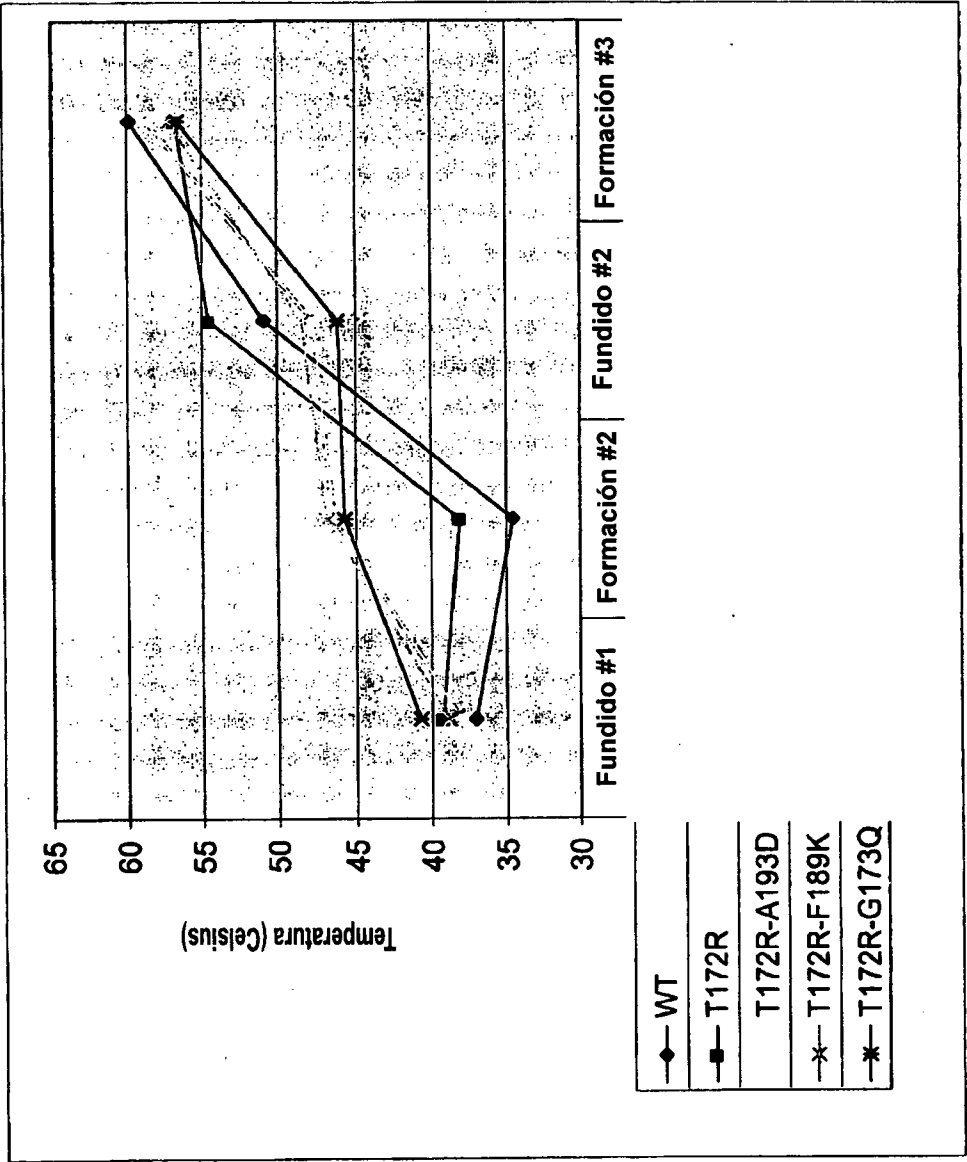


FIG. 32



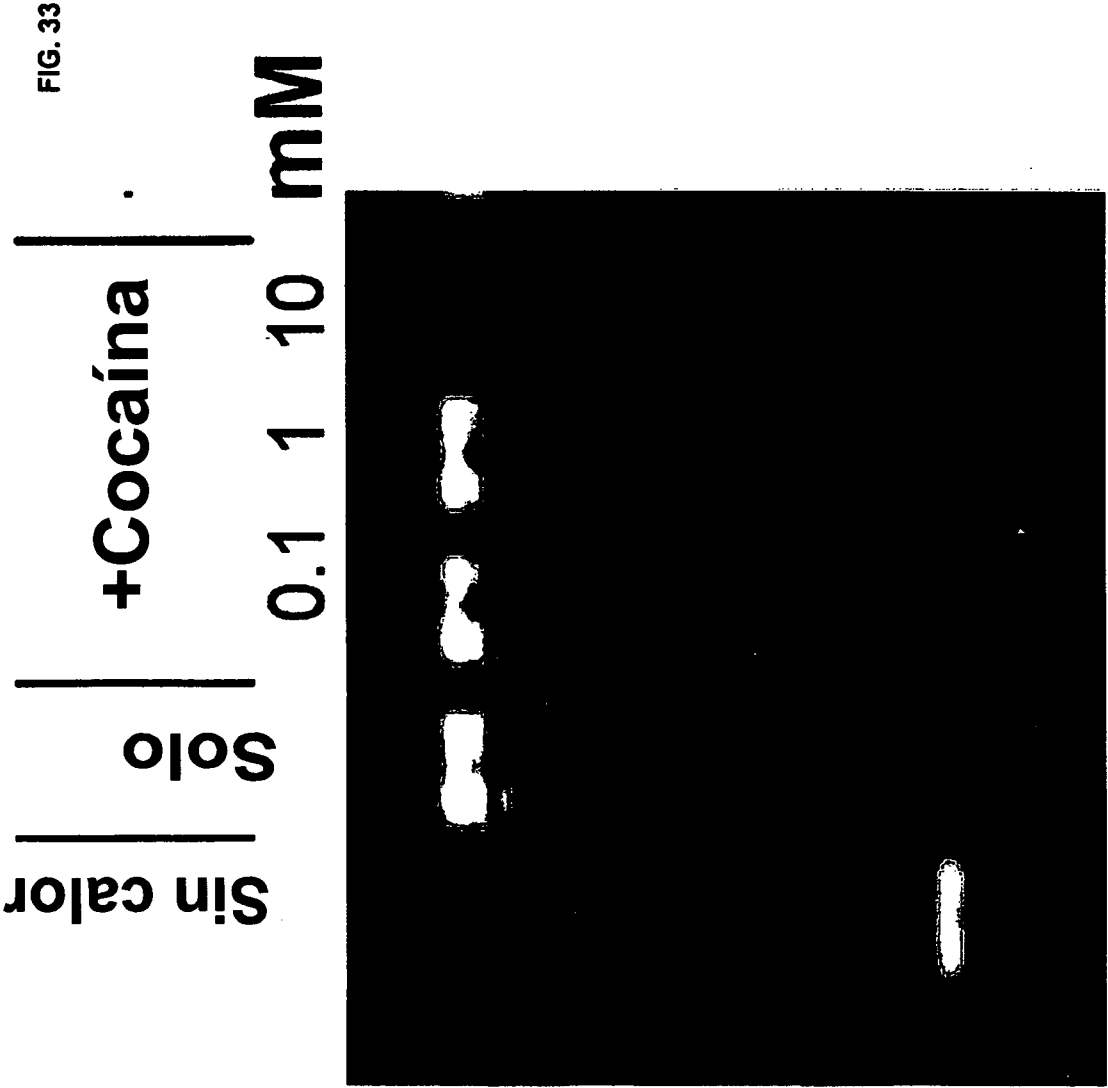
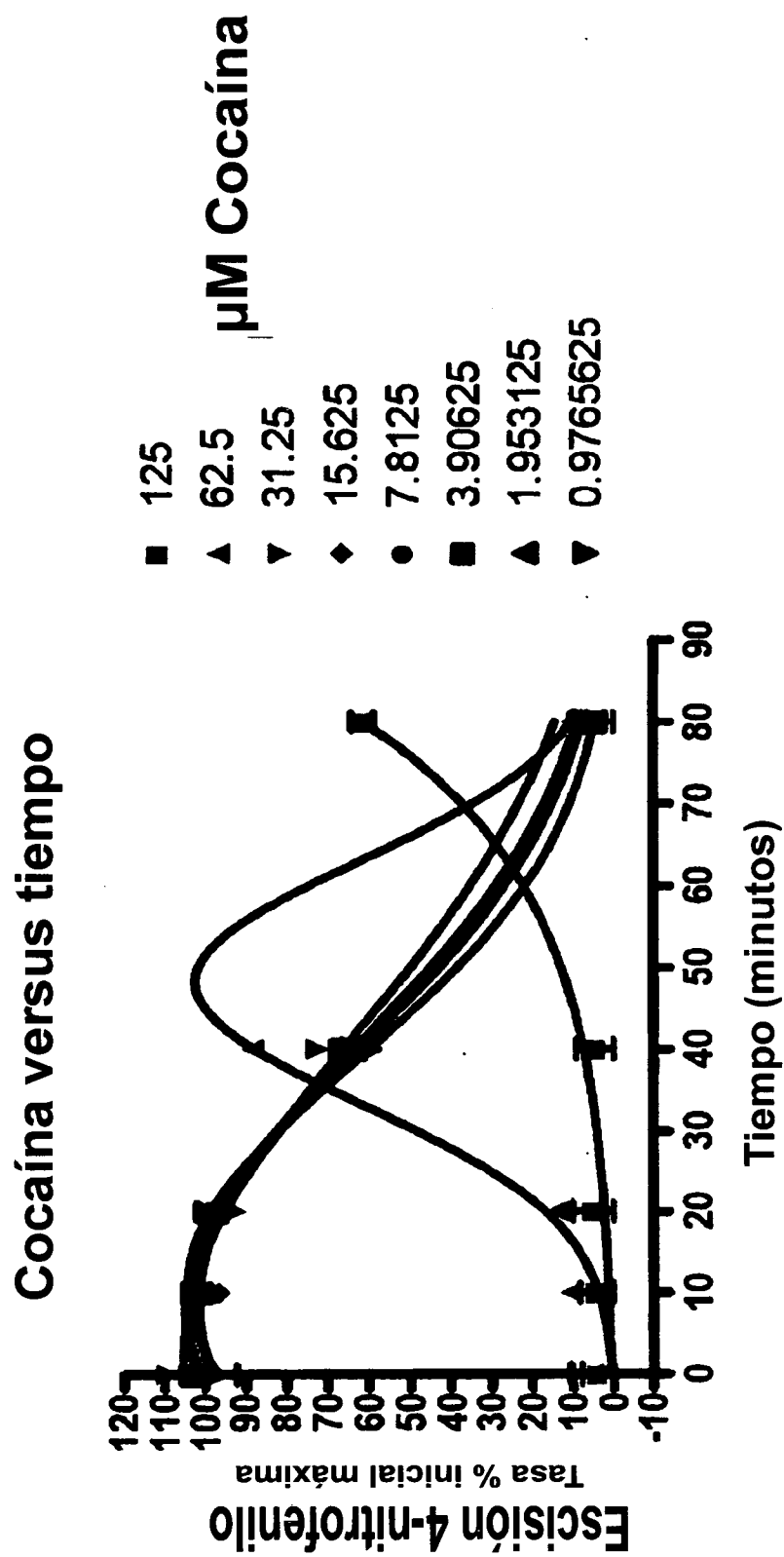


FIG. 34





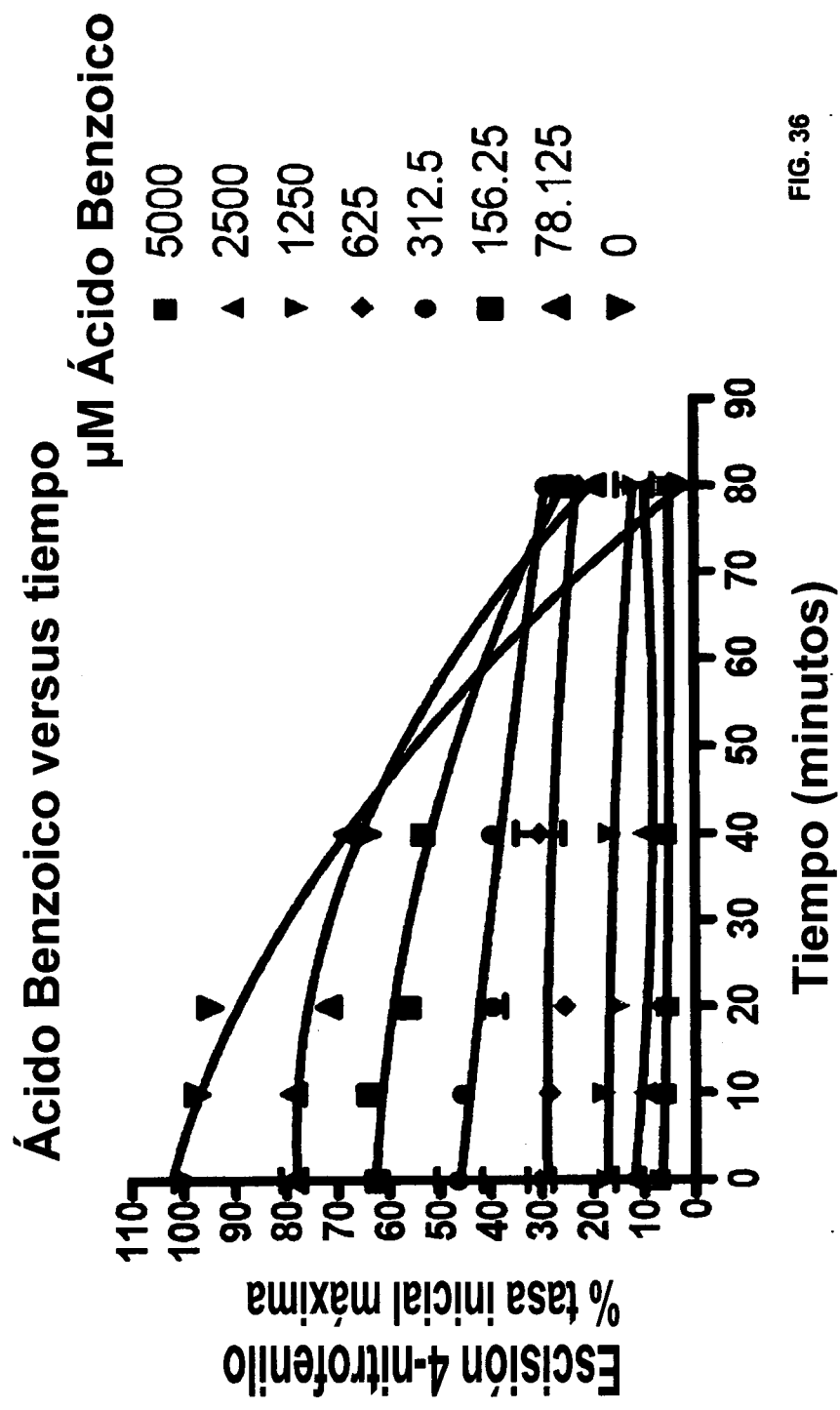


FIG. 36

Tiempo incubado 37°C
(la actividad de escisión 4NP
debe disminuir con el tiempo, a
menos que se estabilice)

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SEQUENCE LISTING

<110> Columbia University
Zhan, Chang-Guo
Landry, Donald
Sunahara, Roger
MacDonald, Joanne
Narasimhan, Diwa
Woods, James
Holden Ko, Mei_Chuan
Deng, Shi-Xian

<120> ANTI-COCAINE COMPOSITIONS AND TREATMENT

<130> 88800730-0003

<140> PCTUS0715762

<141> 2007-08-03

<150> 60/819,569

<151> 2006-07-10

<160> 47

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<210> 1

<211> 574

<212> PRT

<213> Rhodococcus sp. MB1

<400> 1

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20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
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 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
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 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly

370

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Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
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Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
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Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
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accgcaattc gaataccggc ggagtaatcg cacgggaaca gtcgaagag atgtgcaccg	1680
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Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
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Glu Ala Val Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
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Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
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Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
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405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
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Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
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 Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45
 Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Asp Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
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 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
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<223> Mutant CocE polypeptide

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 1 5 10 15

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 Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
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 Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Asp Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
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 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
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 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
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 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
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 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
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 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
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 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
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 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
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 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
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 500 505 510
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515

SEQ_LIST_2nd_Revised_88800730_0078

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Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 5
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 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
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Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
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 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
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SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 6
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 6

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Glu Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
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290

SEQ_LIST_2nd_Revised_88800730_0078
295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 7
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CoCE polypeptide

<400> 7

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
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SEQ_LIST_2nd_Revised_88800730_0078
185 190

180

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Ile Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 8
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 8

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
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SEQ_LIST_2nd_Revise_88800730_0078

65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Leu Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

SEQ_LIST_2nd_Revised_88800730_0078

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

SEQ_LIST_2nd_Revised_88800730_0078

<210> 9

<211> 574

<212> PRT

<213> Artificial

<220>

<223> Mutant CoCE polypeptide

<400> 9

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Asp Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 10
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 10

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ser Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

SEQ_LIST_2nd_Revised_88800730_0078

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 11
 <211> 574
 <212> PRT
 <213> Artificial
 <220>

SEQ_LIST_2nd_Revised_88800730_0078

<223> Mutant CocE polypeptide

<400> 11

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ala Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 12
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 12

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ala Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 13
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 13

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Gly Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

SEQ_LIST_2nd_Revised_88800730_0078

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515

SEQ_LIST_2nd_Revised_88800730_0078

515

520

525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

- <210> 14
- <211> 574
- <212> PRT
- <213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 14

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Ala Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala

SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 15
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide
<400> 15

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Ala Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
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290

SEQ_LIST_2nd_Revised_88800730_0078
295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525
 Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

- <210> 16
- <211> 574
- <212> PRT
- <213> Artificial

- <220>
- <223> Mutant CocE polypeptide
- <400> 16

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ala Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu

SEQ_LIST_2nd_Revised_88800730_0078

180

185

190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 17
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 17

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
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SEQ_LIST_2nd_Revised_88800730_0078

65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Leu Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

SEQ_LIST_2nd_Revised_88800730_0078

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

SEQ_LIST_2nd_Revised_88800730_0078

<210> 18
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CoCE polypeptide

<400> 18
Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15
Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30
Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45
Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60
Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80
Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95
Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110
Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125
Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140
Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160
Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175
Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190
Asp Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 19
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 19

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Arg Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

SEQ_LIST_2nd_Revised_88800730_0078

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 20
 <211> 574
 <212> PRT
 <213> Artificial

<220>

SEQ_LIST_2nd_Revised_88800730_0078

<223> Mutant CocE polypeptide

<400> 20

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Val Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 21
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 21

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Leu Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

SEQ_LIST_2nd_Revised_88800730_0078

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30
 Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45
 Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

SEQ_LIST_2nd_Revised_88800730_0078

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Gly Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 Página 53

SEQ_LIST_2nd_Revised_88800730_0078
520 525

515

520

525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210>	23
<211>	574
<212>	PRT
<213>	Artificial

<220>
<223> Mutant CocE polypeptide

<400> 23

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp His Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 Página 55

SEQ_LIST_2nd_Revised_88800730_0078
410 415

405
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525
 Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540
 Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560
 Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210>	24
<211>	574
<212>	PRT
<213>	Artificial

<220>
<223> Mutant CocE polypeptide

<400> 24

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val₃₅ Pro Val Leu Leu Val₄₀ Arg Asn Pro Tyr Asp₄₅ Lys Phe Asp

SEQ_LIST_2nd_Revised_88800730_0078

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Ala
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
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290

SEQ_LIST_2nd_Revised_88800730_0078
295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 25
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 25

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Arg Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
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SEQ_LIST_2nd_Revised_88800730_0078

180

185

190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 26
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 26

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
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SEQ_LIST_2nd_Revised_88800730_0078

65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

SEQ_LIST_2nd_Revised_88800730_0078

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Phe Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

SEQ_LIST_2nd_Revised_88800730_0078

<210> 27
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 27

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Ser Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 28

<211> 574

<212> PRT

<213> Artificial

<220>

<223> Mutant CoCE polypeptide

<400> 28

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Thr Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

SEQ_LIST_2nd_Revised_88800730_0078

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525
 Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540
 Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560
 Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 29
 <211> 574
 <212> PRT
 <213> Artificial
 <220>

SEQ_LIST_2nd_Revised_88800730_0078

<223> Mutant CocE polypeptide

<400> 29

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Pro Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 30

<211> 574

<212> PRT

<213> Artificial

<220>

<223> Mutant CocE polypeptide

<400> 30

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Ser Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 31
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CoCE polypeptide

<400> 31

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30
 Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45
 Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Arg Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

SEQ_LIST_2nd_Revised_88800730_0078

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525 530 535 540 545 550

4/4

347

10531

515 SEQ_LIST_2nd_Revised_88800730_0078
520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 32
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CoCE polypeptide

<400> 32

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gln Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
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SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 33
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 33

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr

290

SEQ_LIST_2nd_Revised_88800730_0078
295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Thr Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 34
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 34

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
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SEQ_LIST_2nd_Revised_88800730_0078

180
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380
 Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Ala Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 35
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> R41I

<400> 35

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Ile Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
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SEQ_LIST_2nd_Revised_88800730_0078

65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
305 310 315 320

SEQ_LIST_2nd_Revised_88800730_0078

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

SEQ_LIST_2nd_Revised_88800730_0078

<210> 36
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CoCE polypeptide

<400> 36

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Ala Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 37

<211> 574

<212> PRT

<213> Artificial

<220>

<223> Mutant CocE polypeptide

<400> 37

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Ala Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

SEQ_LIST_2nd_Revised_88800730_0078

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 38
 <211> 574
 <212> PRT
 <213> Artificial

<220>

SEQ_LIST_2nd_Revised_88800730_0078

<223> Mutant CocE polypeptide

<400> 38

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Tyr Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 39

<211> 587

<212> PRT

<213> Artificial

<220>

<223> Mutant CoCE polypeptide

<400> 39

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Arg Gln Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255
 Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270
 Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285
 Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300
 Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320
 His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335
 Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350
 Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365
 Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
565 570 575

Ala Ala Ala Leu Glu His His His His His His
580 585

- <210> 40
- <211> 587
- <212> PRT
- <213> Artificial
- <220>
- <223> Mutant CocE polypeptide
- <400> 40

SEQ_LIST_2nd_Revised_88800730_0078

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15
 Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30
 Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45
 Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60
 Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80
 Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95
 Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110
 Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125
 Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140
 Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160
 Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Arg Gly Leu Ile Thr
 165 170 175
 Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190
 Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205
 Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220
 Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240
 Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

SEQ_LIST_2nd_Revised_88800730_0078

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 Página 97

500 SEQ_LIST_2nd_Revised_88800730_0078
505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
565 570 575

Ala Ala Ala Leu Glu His His His His His His
580 585

<210> 41
<211> 587
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 41

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Arg Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Ala Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 Página 99

370

SEQ_LIST_2nd_Revised_88800730_0078
375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
565 570 575

Ala Ala Ala Leu Glu His His His His His His
580 585

<210> 42
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Mutant CoCE polypeptide

SEQ_LIST_2nd_Revised_88800730_0078

<400> 42

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Lys Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
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SEQ_LIST_2nd_Revised_88800730_0078

245

250

255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
 565 570 575

His His His His His His
 580

<210> 43
 <211> 582
 <212> PRT
 <213> Artificial

<220>
 <223> Mutant CocE polypeptide

<400> 43

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125 130 135 140 145 150

115

SEQ_LIST_2nd_Revised_88800730_0078

120

125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Lys Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

SEQ_LIST_2nd_Revised_88800730_0078

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
 565 570 575

His His His His His His
 580

<210> 44

<211> 582

<212> PRT

<213> Artificial

<220>

<223> Mutant CocE polypeptide

SEQ_LIST_2nd_Revised_88800730_0078

<400> 44

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Lys Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510
Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525
Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540
Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560
Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575
His His His His His His
580

<210> 45
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide
<400> 45

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15
Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30
Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45
Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60
Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80
Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95
Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

SEQ_LIST_2nd_Revised_88800730_0078

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Lys Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

SEQ_LIST_2nd_Revised_88800730_0078

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

- <210> 46
- <211> 582
- <212> PRT
- <213> Artificial
- <220>

SEQ_LIST_2nd_Revised_88800730_0078

<223> Mutant CocE polypeptide

<400> 46

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Lys Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe Val Gly Glu Ser Leu Arg Thr Phe Val Ala Val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu Val Val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Gln Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly Val Pro Lys Val Arg
 325 330 335

Leu Phe Val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

<210> 47
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Mutant CocE polypeptide

<400> 47

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

SEQ_LIST_2nd_Revised_88800730_0078

Met Phe Gly val Ser Tyr Leu Gly val Thr Gln Trp Gln Ala Ala val
 115 120 125

Ser Gly val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe val Gln Leu
 180 185 190

Ala Lys Ile Leu Asn Asp val Ala Gly Ala Ala Ser val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp val Ile Asp Gln
 210 215 220

val val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

Phe Glu Arg Leu Gly Gly Leu Ala Thr Pro Ala Leu Ile Thr Ala Gly
 245 250 255

Trp Tyr Asp Gly Phe val Gly Glu Ser Leu Arg Thr Phe val Ala val
 260 265 270

Lys Asp Asn Ala Asp Ala Arg Leu val val Gly Pro Trp Ser His Ser
 275 280 285

Asn Leu Thr Gly Arg Asn Ala Asp Arg Lys Phe Gly Ile Ala Ala Thr
 290 295 300

Tyr Pro Ile Gln Glu Ala Thr Thr Met His Lys Ala Phe Phe Asp Arg
 305 310 315 320

His Leu Arg Gly Glu Thr Asp Ala Leu Ala Gly val Pro Lys val Arg
 325 330 335

Leu Phe val Met Gly Ile Asp Glu Trp Arg Asp Glu Thr Asp Trp Pro
 340 345 350

Leu Pro Asp Thr Ala Tyr Thr Pro Phe Tyr Leu Gly Gly Ser Gly Ala
 355 360 365

3/4

10531

306

SEQ_LIST_2nd_Revised_88800730_0078

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly-Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

SEQ_LIST_2nd_revised_88800730_0078
LISTADO DE SECUENCIAS

<110> Columbia University
Zhan, Chang-Guo
Landry, Donald
Sunahara, Roger
MacDonald, Joanne
Narasimhan, Diwa
Woods, James
Holden Ko, Mei-Chuan
Deng, Shi-Xian

<120> COMPOSICIÓN ANTI-COCAÍNA Y TRATAMIENTO

<130> 88800730-0003

<140> PCTUS0715762

<141> 2007-08-03

<150> 60/819,569

<151> 2006-07-10

<160> 47

<170> Versión PatentIn 3.3

<210> 1

<211> 574

<212> PRT

<213> Rhodococcus sp. MB1

<400> 1

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

accgatctga cgatgaccga tctgatggag ttcttccggc actggcatgc cgggcgttcg 2160
caggttcaga tctcaccggc gtttgggatc gaccgcaaaa cctgacttgc acaagttgac 2220
ggcggtttcc ggactcggct gacatttgcg caagtagaag gccccgcggg ttcgggattt 2280
ggcgcactaa cgggtcggta aaatctggcg agtggcggcg tacattcgca aggtcaggac 2340
cgcatcgggg gcgaccgcgg tgcagatcgc cgtcaagcag ggacgtcgcg acaaggtgcg 2400
tcgagcacct cggttccgca cacaccgaca gcgagcttgc tgcgctactg caggccgccg 2460
aggagaagct gcaggtcgga cagtaggaac tcgacctcga cctcggcggg agcggggatc 2520
gagggtcggg gatcgcgggc aagcgggtccc gctggttgat cgaggcgatc gagaccgat 2580
gacggcggct cggcttcgac gtgatcgacg acgaggtgtt catctaactg gtgatcggca 2640
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<210> 3
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 3

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15
Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30
Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45
Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60
Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80
Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95
Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110
Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 4
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 4

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078

515

520

525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 5
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 5

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 6
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 6

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 7
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 7

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 Página 15

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 8
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 8

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 Página 17

SEQ_LIST_2nd_Revised_88800730_0078

<210> 9
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 9

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 10
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 10

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEO_LIST_2nd_Revised_88800730_0078

<223> Polipéptido CocE mutante

<400> 11

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ala Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
 210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
 225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 12
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<212> PRT
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<220>
<223> Polipéptido CocE mutante

<400> 12

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525
 Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540
 Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560
 Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 13
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 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 13

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078
520 525

515

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 14
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 14

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078
410 415

405

410

415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu phe val ser ser ser Ala val Asp Thr Asp phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510 .

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

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<210> 15
<211> 574
<212> PRT
<213> Artificial
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<220> Polipéptido CocE mutante

<400> 15

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revise_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 16
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 16

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ala Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 Página 37

SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 17
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 17

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
Página 39

SEQ_LIST_2nd_Revised_88800730_0078

<210> 18
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 18

Met val Asp Gly Asn Tyr Ser val Ala Ser Asn val Met val Pro Met
 1 5 10 15

Arg Asp Gly val Arg Leu Ala val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro val Pro val Leu Leu val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe val Arg
 50 55 60

Asp Gly Tyr Ala val val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe val Pro His val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn val Gly
 100 105 110

Met Phe Gly val Ser Tyr Leu Gly val Thr Gln Trp Gln Ala Ala val
 115 120 125

Ser Gly val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe val Gln Leu
 180 185 190

Asp Ala Ile Leu Asn Asp val Ala Gly Ala Ala Ser val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 19
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 19

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

<223> Polipéptido CocE mutante

<400> 20

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Val Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 21
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 21

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 22
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 22

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078

515

520

525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 23
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 23

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp His Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

- <210> 24
- <211> 574
- <212> PRT
- <213> Artificial
- <220>
- <223> Polipéptido CocE mutante
- <400> 24

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

- <210> 25
- <211> 574
- <212> PRT
- <213> Artificial
- <220>
- <223> Polipéptido CocE mutante
- <400> 25

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Arg Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
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SEQ_LIST_2nd_Revised_88800730_0078

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 26
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 26

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
Página 61

SEQ_LIST_2nd_Revised_88800730_0078

<210> 27
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 27

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Ser Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 28
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 28

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

<223> Polipéptido CocE mutante

<400> 29

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Pro Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 30
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 30

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Ser Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

- <210> 31
- <211> 574
- <212> PRT
- <213> Artificial
- <220>
- <223> Polipéptido CocE mutante
- <400> 31

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

SEQ_LIST_2nd_Revised_88800730_0078

515

520

525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 32
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 32

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

SEQ_LIST_2nd_Revised_88800730_0078
405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 33
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 33

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

SEQ_LIST_2nd_Revised_88800730_0078

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
 565 570

<210> 34
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 34

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
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SEQ_LIST_2nd_Revised_88800730_0078

<210> 36
 <211> 574
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 36

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Ala Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
 130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
 145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
 165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
 180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
 195 200 205

SEQ_LIST_2nd_Revised_88800730_0078

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 37
<211> 574
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 37

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Ala Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

SEQ_LIST_2nd_Revised_88800730_0078

<223> Polipéptido CocE mutante

<400> 38

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Tyr Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Gln Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg
565 570

<210> 39
<211> 587
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 39

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400
 Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415
 Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430
 Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445
 Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460
 Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480
 Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495
 Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510
 Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525
 Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540
 Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560
 Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
 565 570 575
 Ala Ala Ala Leu Glu His His His His His His
 580 585

<210> 40
 <211> 587
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 40

SEQ_LIST_2nd_Revised_88800730_0078

500

505

510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
 565 570 575

Ala Ala Ala Leu Glu His His His His His His
 580 585

<210> 41
 <211> 587
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

<400> 41

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
 1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
 20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
 35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
 50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
 65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
 85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
 100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
 115 120 125

SEQ_LIST_2nd_Revised_88800730_0078

370375380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
385390395400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
405410415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
420425430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
435440445

Leu phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
450455460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
465470475480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
485490495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500505510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515520525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530535540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545550555560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Lys Leu
565570575

Ala Ala Ala Leu Glu His His His His His His
580585

<210> 42
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

- <210> 43
- <211> 582
- <212> PRT
- <213> Artificial
- <220>
- <223> Polipéptido CocE mutante
- <400> 43

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val

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SEQ_LIST_2nd_Revised_88800730_0078

Ala Asn Thr Ser Thr Gly Gly Gly Thr Leu Ser Thr Ser Ile Ser Gly
 370 375 380

Thr Glu Ser Ala Asp Thr Tyr Leu Tyr Asp Pro Ala Asp Pro Val Pro
 385 390 395 400

Ser Leu Gly Gly Thr Leu Leu Phe His Asn Gly Asp Asn Gly Pro Ala
 405 410 415

Asp Gln Arg Pro Ile His Asp Arg Asp Asp Val Leu Cys Tyr Ser Thr
 420 425 430

Glu Val Leu Thr Asp Pro Val Glu Val Thr Gly Thr Val Ser Ala Arg
 435 440 445

Leu Phe Val Ser Ser Ser Ala Val Asp Thr Asp Phe Thr Ala Lys Leu
 450 455 460

Val Asp Val Phe Pro Asp Gly Arg Ala Ile Ala Leu Cys Asp Gly Ile
 465 470 475 480

Val Arg Met Arg Tyr Arg Glu Thr Leu Val Asn Pro Thr Leu Ile Glu
 485 490 495

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
 500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
 515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
 530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
 545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
 565 570 575

His His His His His His
 580

<210> 44
 <211> 582
 <212> PRT
 <213> Artificial

<220>
 <223> Polipéptido CocE mutante

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510 ,

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

<210> 45
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 45

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

SEQ_LIST_2nd_Revised_88800730_0078

<223> Polipéptido CocE mutante

<400> 46

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

Met Phe Gly Val Ser Tyr Leu Gly Val Thr Gln Trp Gln Ala Ala Val
115 120 125

Ser Gly Val Gly Gly Leu Lys Ala Ile Ala Pro Ser Met Ala Ser Ala
130 135 140

Asp Leu Tyr Arg Ala Pro Trp Tyr Gly Pro Gly Gly Ala Leu Ser Val
145 150 155 160

Glu Ala Leu Leu Gly Trp Ser Ala Leu Ile Gly Thr Gly Leu Ile Thr
165 170 175

Ser Arg Ser Asp Ala Arg Pro Glu Asp Ala Ala Asp Phe Val Lys Leu
180 185 190

Ala Ala Ile Leu Asn Asp Val Ala Gly Ala Ala Ser Val Thr Pro Leu
195 200 205

Ala Glu Gln Pro Leu Leu Gly Arg Leu Ile Pro Trp Val Ile Asp Gln
210 215 220

Val Val Asp His Pro Asp Asn Asp Glu Ser Trp Gln Ser Ile Ser Leu
225 230 235 240

SEQ_LIST_2nd_Revised_88800730_0078

Ala Gly Glu Ile Tyr Glu Val Ala Ile Asp Met Leu Ala Thr Ser Asn
500 505 510

Val Phe Leu Pro Gly His Arg Ile Met Val Gln Val Ser Ser Ser Asn
515 520 525

Phe Pro Lys Tyr Asp Arg Asn Ser Asn Thr Gly Gly Val Ile Ala Arg
530 535 540

Glu Gln Leu Glu Glu Met Cys Thr Ala Val Asn Arg Ile His Arg Gly
545 550 555 560

Pro Glu His Pro Ser His Ile Val Leu Pro Ile Ile Lys Arg Leu Glu
565 570 575

His His His His His His
580

<210> 47
<211> 582
<212> PRT
<213> Artificial

<220>
<223> Polipéptido CocE mutante

<400> 47.

Met Val Asp Gly Asn Tyr Ser Val Ala Ser Asn Val Met Val Pro Met
1 5 10 15

Arg Asp Gly Val Arg Leu Ala Val Asp Leu Tyr Arg Pro Asp Ala Asp
20 25 30

Gly Pro Val Pro Val Leu Leu Val Arg Asn Pro Tyr Asp Lys Phe Asp
35 40 45

Val Phe Ala Trp Ser Thr Gln Ser Thr Asn Trp Leu Glu Phe Val Arg
50 55 60

Asp Gly Tyr Ala Val Val Ile Gln Asp Thr Arg Gly Leu Phe Ala Ser
65 70 75 80

Glu Gly Glu Phe Val Pro His Val Asp Asp Glu Ala Asp Ala Glu Asp
85 90 95

Thr Leu Ser Trp Ile Leu Glu Gln Ala Trp Cys Asp Gly Asn Val Gly
100 105 110

107391

From the INTERNATIONAL BUREAU

PCTNOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 03 October 2007 (03.10.2007)	To: BLOSSER, G., Harley Sonnenschein Nath & Rosenthal LLP P.O. Box 061080 Wacker Drive Station, Sears Tower Chicago, IL 60606-1080 ETATS-UNIS D'AMERIQUE	
Applicant's or agent's file reference 88800730-0003PCT		IMPORTANT NOTIFICATION
International application No. PCT/US2007/015762		International filing date (day/month/year) 10 July 2007 (10.07.2007)
International publication date (day/month/year) Not yet published		Priority date (day/month/year) 10 July 2006 (10.07.2006)
Applicant COLUMBIA UNIVERSITY et al		

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. (If applicable) The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

(If applicable) An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
10 July 2006 (10.07.2006)	60/819,569	US	10 September 2007 (10.09.2007)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Dorothee Mülhausen

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Telephone No. +41 22 338 74 01

Form PCT/IB/304 (October 2005)

1/DEPCMUBO

10929/

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 88800730-0078	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below	
International application No. PCT/US07/15762	International filing date (day/month/year) 10 July 2007 (10.07.2007)	(Earliest) Priority Date (day/month/year) 10 July 2006 (10.07.2006)
Applicant COLUMBIA UNIVERSITY		

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 3 sheets.

☐ 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:
- ☒ 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**,
- ☒ 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**,
- ☒ 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. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/15762

A. CLASSIFICATION OF SUBJECT MATTER

IPC: A61K 38/46(2006.01)

USPC: 514/183

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. : 514/183

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2,257,972 A (BRITT et al) 27 January 1993. (27.01.1993), abstract.	1-27
A	US 5,730,985 A (BARBER et al) 24 May 1998 (24.05.1998), abstract	1-27
A	US 5,977,314 A (LANDRY et al) 2 November 1999 (02.11.1999), abstract	1-27

☐ 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	"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
"E" earlier application or patent published on or after the international filing date	"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
"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)	"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
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 August 2008 (28.08.2008)

Date of mailing of the international search report

29 SEP 2008

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (571) 273-3201

Authorized officer

ANNA PAGONAKIS

Telephone No. 571-270-3505

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/15762

Continuation of B. FIELDS SEARCHED Item 3:
EAST (USPat, USPGPub, Derwent, EPO)
STN

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
SHEILA R. GIBSON
SONNENSCHN NATH & ROSENTHAL LLP
P.O. BOX 061080
WACKER DRIVE STATION, SEARS TOWER
CHICAGO, IL 60606-1080

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Applicant's or agent's file reference 88800730-0078		Date of mailing (day/month/year) 29 SEP 2008
International application No. PCT/US07/15762		International filing date (day/month/year) 10 July 2007 (10.07.2007)
International Patent Classification (IPC) or both national classification and IPC IPC: A61K 38/46 (2006.01) USPC: 514/183		Priority date (day/month/year) 10 July 2006 (10.07.2006)
Applicant COLUMBIA UNIVERSITY		

1. This opinion contains indications relating to the following items:

- | | | |
|-------------------------------------|--------------|--|
| ☒ | Box No. I | Basis of the opinion |
| ☐ | Box No. II | Priority |
| ☐ | Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ | Box No. IV | Lack of unity of invention |
| ☒ | Box No. V | Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ | Box No. VI | Certain documents cited |
| ☐ | Box No. VII | Certain defects in the international application |
| ☐ | Box No. VIII | Certain observations on the international application |

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Date of completion of this opinion 28 August 2008 (28.08.2008)	Authorized officer ANNA PAGONAKIS Telephone No. 571-270-3505
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Form PCT/ISA/237 (cover sheet) (April 2007)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US07/15762

Box No. I Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:

☒ 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)).2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:

a. type of material

☐ a sequence listing☐ table(s) related to the sequence listing

b. format of material

☐ on paper☐ in electronic form

c. time of filing/furnishing

☐ contained in the international application as filed.☐ filed together with the international application in electronic form.☐ furnished subsequently to this Authority for the purposes of search.4. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITYInternational application No.
PCT/US07/15762**Box No. V Reasoned statement under Rule 43 bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)

Claims 1-27 YESClaims NONE NO

Inventive step (IS)

Claims 1-27 YESClaims NONE NO

Industrial applicability (IA)

Claims 1-27 YESClaims NONE NO

2. Citations and explanations:

Claims 1-27 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the claimed polypeptide.

Claims 1-27 meet the criteria set out in PCT Article 33(4) since the claimed invention is related to therapeutic utility of the claimed polypeptide and thus meet industrial applicability in the medical industry.

RECORD COPY

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

PCT/US2007/015762

17391-439

For receiving Office use only	
PCT/US 07/15762	
International Application No.	
10-07-2007	
International Filing Date	
RO/US	
Name of receiving Office and "PCT International Application"	
Applicant's or agent's file reference (if desired) (12 characters maximum) 88800730-0003PCT	

Box No. I TITLE OF INVENTION	
ANTI-COCAINE COMPOSITIONS AND TREATMENT	
Box No. II APPLICANT ☐ This person is also inventor	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
Columbia University 412 Low Memorial Library 535 West 116th Street, MC4308 New York, New York 10027 US	
Telephone No.	
Facsimile No.	
Applicant's registration No. with the Office	
State (that is, country) of nationality: US	
State (that is, country) of residence: US	
This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
GIBSON, Sheila R. Sonnenschein Nath & Rosenthal LLP PO Box 061080 Wacker Drive Station, Sears Tower Chicago, Illinois 60606-1080 US	
Telephone No. 312-876-8000	
Facsimile No. 312-876-7934	
Agent's registration No. with the Office 54,120	
☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
<i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) LANDRY, Donald 29 Claremont Avenue Apartment 2-South New York, New York 10027 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) ZHAN, Chang-Guo 2193 Allegheny Way Lexington, Kentucky 40523 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: CN	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) WOODS, James H. Department of Pharmacology, University of Michigan Medical School, 1301 MSRB III Ann Arbor, Michigan 48109 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) SUNAHARA, Roger 1130 Olivia Avenue Ann Arbor, Michigan 48104 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: CA	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
<i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) NARASIMHAN, Diwahar L. 1507 Pine Valley Boulevard #7 Ann Arbor, Michigan 48104 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: IN	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) MACDONALD, Joanne 605 W. 156th Street, Apt. 40 New York, New York 10032 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: AU	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) YANG, Victor 3629 Tanglewood Drive Ann Arbor, Michigan 48105 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) KO, Mei-Chuan Holden Department of Pharmacology, University of Michigan Medical School, 1301 MSRB III Ann Arbor, Michigan 48109-0632 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: TW	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S) <i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> DENG, Shi-Xian 34 Odgen Avenue White Plains, New York 10605 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality: US	State <i>(that is, country)</i> of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> TESMER, John J. 2450 Adare Road Ann Arbor, Michigan 48104 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality: US	State <i>(that is, country)</i> of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> LEE, Tien-Yi Ann Arbor, Michigan US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality: TW	State <i>(that is, country)</i> of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> KWON, Young Min Ann Arbor, Michigan US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality: KR	State <i>(that is, country)</i> of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S) <i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) GAO, Daquan 3929 Stonyrun Drive Louisville, Kentucky 40220 US	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality: CN	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality:	State (that is, country) of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality:	State (that is, country) of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality:	State (that is, country) of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.) Applicant's registration No. with the Office
State (that is, country) of nationality:	State (that is, country) of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Supplemental Box*If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*
 - (i) *If more than one person is to be indicated as applicant and/or inventor and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*
 - (ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States Indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*
 - (iii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*
 - (iv) *if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*
 - (v) *if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*
2. *If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such a case, write the name or two-letter code of each designated State concerned and the indication "patent of addition," "certificate of addition," "inventor's certificate of addition" or "utility certificate of addition," the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other parent grant or the date of filing of the parent application (Rules 4.11(a)(iii) and 49bis.1(a) or (b)).*
3. *If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such a case, write "United States of America" or "US" and the indication "continuation" or "continuation-in-part" and the number and the filing date of the parent application (Rules 4.11(a)(iv) and 49bis.1(d)).*

BOX IV (CONTINUED)

BLOSSER, G. Harley
 BOCK, Joel N.
 BURTON, Thomas J.
 CARLEY, Donald
 CRAWFORD, David E.
 FAHMI, Tarek N.
 GIBSON, Sheila R.
 HANLEY, Matthew E.
 MAHER, David W.
 MCGINLEY, Brian R.
 METZGER, David R.
 MICHAEL, Martin P.
 RAUCH, Christopher P.
 SABETT, Randy V.
 STANLEY, Bryan

SONNENSCHN NATH & ROSENTHAL LLP
 PO Box 061080
 Wacker Drive Station, Sears Tower
 Chicago, Illinois 60606-1080
 US

Sheet No. ... 7 ...

Box No. V DESIGNATIONS

The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents. However,

- ☐ DE Germany is not designated for any kind of national protection
☐ JP Japan is not designated for any kind of national protection
☐ KR Republic of Korea is not designated for any kind of national protection
☐ RU Russian Federation is not designated for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 10 July 2006	60/819569	US	US	
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

Transmit certified copy: the receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office) identified above as:

- ☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (). (See also the Notes to Box No. VI: further information must be provided to support a request to restore the right of priority.)

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA / US

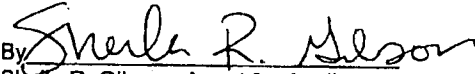
Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year) Number Country (or regional Office)

Box No. VIII DECLARATIONS

The following declarations are contained in Boxes Nos. VIII (i) to (v) (mark the applicable check-boxes below and indicate in the right column the number of each type of declaration):

		Number of declarations
☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING			
This international application contains:		This international application is accompanied by the following item(s) (mark the applicable check-boxes below and indicate in right column the number of each item):	Number of items
(a) on paper, the following number of sheets:		1. ☒ fee calculation sheet	1
request (including declaration and supplemental sheets)	8	2. ☐ original separate power of attorney	
description (excluding sequence listing and/or tables related thereto)	80	3. ☐ original general power of attorney	
claims	6	4. ☐ copy of general power of attorney; reference number, if any:	
abstract	1	5. ☐ statement explaining lack of signature	
drawings	36	6. ☐ priority document(s) identified in Box No. VI as item(s):	
Sub-total number of sheets	131	7. ☐ translation of international application into (language):	
sequence listing	113	8. ☐ separate indications concerning deposited microorganism or other biological material	
tables related thereto		9. ☐ sequence listing in electronic form (indicate type and number of carriers)	
(for both, actual number of sheets if filed on paper, whether or not also filed in electronic form; see (c) below)		(i) ☐ copy submitted for the purposes of international search under Rule 13ter only (and not as part of the international application)	
Total number of sheets	244	(ii) ☐ (only where check-box (b)(i) or (c)(i) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter	
(b) ☐ only in electronic form (Section 801(a)(i))		(iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing mentioned in left column	
(i) ☐ sequence listing		10. ☐ tables in electronic form related to sequence listing (indicate type and number of carriers)	
(ii) ☐ tables related thereto		(i) ☐ copy submitted for the purposes of international search under Section 802(b-quater) only (and not as part of the international application)	
(c) ☒ also in electronic form (Section 801(a)(ii))		(ii) ☐ (only where check-box (b)(ii) or (c)(ii) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Section 802(b-quater)	
(i) ☒ sequence listing		(iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column	
(ii) ☐ tables related thereto		11. ☒ other (specify): Return postcard; credit card payment form	2
Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the			
☒ sequence listing:			
☐ tables related thereto:			
(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)			
Figure of the drawings which should accompany the abstract:	2	Language of filing of the international application:	English
Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE			
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).			
Columbia University			
By 			
Sheila R. Gibson, Agent for Applicants			
All designated states except US			
For receiving Office use only			
1. Date of actual receipt of the purported international application:		10-07-2007	
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		IAP6 Rec'd PCT/PTO 10 JUL 2007	
4. Date of timely receipt of the required corrections under PCT Article 11(2):		2. Drawings: ☐ received: ☐ not received:	
5. International Searching Authority (if two or more are competent): ISA / US		6. ☐ Transmittal of search copy delayed until search fee is paid	
For International Bureau use only			
Date of receipt of the record copy by the International Bureau:			

REQUISITOS MINIMOS PARA ADMISION A TRAMITE
PCT

PATENTE DE INVENCION



MODELO DE UTILIDAD []

- ◆ Indicación de que se solicita la concesión de una patente []
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud []
- ◆ Descripción de la invención []
- ◆ Dibujos de ser estos pertinentes []
- ◆ Comprobante de pago de las tasas establecidas []

COMPLETA []

INCOMPLETA []

DISEÑO INDUSTRIAL []

- ◆ Indicación de que se solicita el registro de Diseño Industrial []
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud []
- ◆ Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño []
- ◆ Comprobante de pago de las tasas establecidas []

COMPLETA []

INCOMPLETA []

ESQUEMA DE TRAZADO []

- ◆ Indicación de que se solicita el registro de un Esquema de Trazado []
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud []
- ◆ Representación gráfica del esquema trazado []
- ◆ Comprobante de pago de las tasas establecidas []

COMPLETA []

INCOMPLETA []

Firma Responsable

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Fecha



No. 09-010531- -00000-0000

Fecha: 2009-02-04 16:27:29 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eve: 378 FASENACIONAL
Act. 411 PRESENTACION Folios: 446

Carrera 13 No. 27-00 Piso 6, 7 y 10

Corinujador: 3 82 08 40

Fax: 350 52 20

E-mail: info@sic.gov.co

www.sic.gov.co

Bogotá, D.C., Colombia

PATENTE

DE

INVENCION

09-10531

7

3

/

4

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
CADENA SARMIENTO JUAN PABLO

REFERENCIA	Radicación No.	9 10531
	Solicitud internacional	PCT/US2007/015762
	Fecha inicio fase nacional	10/02/2009
	Trámite	11
	Evento	378
	Actuación	430
	Oficio No.	3778

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.

2. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante, conforme a lo establecido por el artículo 26 literal k) de la Decisión 486 de la Comisión de la Comunidad Andina. Cuando se trate de documentos otorgados en el extranjero, estos deberán legalizarse de conformidad con lo dispuesto por los artículos 259 y 260 del Código de Procedimiento Civil. Cuando se trate de las declaraciones a que hace referencia la Regla 4.17 ii) del Tratado de Cooperación en Materia de Patentes PCT., el alcance de las tales declaraciones se determinará según indiquen claramente una cesión de derechos del inventor al solicitante o su causante, de conformidad con lo dispuesto en el numeral 5.2.15 del Capítulo Quinto, Título X de la Circular Única de Superintendencia de Industria y Comercio.

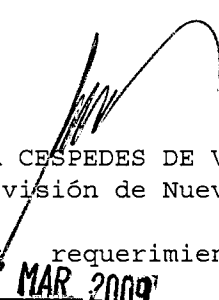
En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No.10 de 2001.

Continúa en la página siguiente...

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Radicación No.:9 10531


ALIX CARMENZA CESPEDES DE VERGEL

Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de
fecha 27 MAR 2009
jfernand