



No. 09-059243-00004-0000

Fecha: 2010-12-16 16:24:26 Dep. 2020 DIR.NUEVASCR
 Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
 Act. 400 OPOSIOBSERVTER Folios: 35



No. 09-059243-00004-0000

Fecha: 2010-12-16 16:24:26 Dep. 2020 DIR.NUEVASCR
 Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
 Act. 400 OPOSIOBSERVTER Folios: 34

FORMULARIO ÚNICO DE OPOSICION

①

OPOSICIÓN A:

- | | |
|--|--|
| ☒ Patente de Invención. | ☐ Marca Colectiva. |
| ☐ Patente de Modelo de Utilidad. | ☐ Marca de Certificación. |
| ☐ Diseño Industrial. | ☐ Lema Comercial. |
| ☐ Esquema de Trazado para Circuitos Integrados. | |
| ☐ Marcas de Productos o Servicios. | |

②

SOLICITUD
PUBLICADANúmero del expediente: 09 59243Solicitante: BIOCON LIMITED.Apoderado: EMILIO FERREROTítulo de la nueva creación o denominación del signo: "UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU RECUPERACIÓN Y PURIFICACIÓN"Gaceta: 620

③

OPOSICIÓN PRESENTADA POR:

SOLICITANTE

Nombre: LABORATORIOS SYNTHESIS S.A.S.Dirección: CARRERA 44 N° 20C-73Nacionalidad o Domicilio: COLOMBIALugar de Constitución: BOGOTÁTeléfono: 3 69 22 22 Fax:

E-mail:

IDENTIFICACIÓN

C.C. ☐ NIT ☒C.E. ☐ Otro ☐

Cual

Número 860,000,760-1REPRESENTANTE
O APODERADONombre: ELIANA ISAURA MORENO BOHORQUEZDirección: CARRERA 13 No. 119-95Teléfono: (571)213 12 13 Fax: (571)612 19 79E-mail: negocios@optimum-co.com

IDENTIFICACIÓN

C.C. ☒ NIT ☐C.E. ☐ Otro ☐

Cual

Número 51'975.664 TP 83823

④

FUNDAMENTOS DE LA OPOSICION:

Art.14, 16, 18, 20 y 30 de la Decisión 486 de la CAN

Causal: No cumple con los requisitos de patentabilidad.

Signo opositor:

Justificación:

La presente solicitud no cumple con los requisitos de patentabilidad dado que lo
revelado, ya se encontraba en el estado de la técnica o es posible derivarlo de la
misma tal y como se demostrará en el capítulo "Fundamento de Hechos".

⑤ Comprobante de pago No. _____

Fecha _____

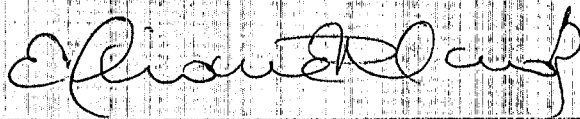
⑥ ANEXOS

- ☒ Comprobante de pago de la tasa.
- ☒ Poderes, si fuere el caso.
- ☒ Pruebas de los fundamentos de la oposición.
- ☐ Documentos que acrediten el legítimo interés, si fuere el caso.
- ☒ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.

⑦

NOMBRE: ELIANA ISAURA MORENO BOHORQUEZ ✓

FIRMA:



C.C. 51'975.664

TP. 83823



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

Señor Jefe,

DIRECCIÓN DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia : Expediente N° 09 59243 ✓
Asunto : Oposición a la solicitud de patente de Invención titulada
"UNA FORMA PURA DE RAPAMICINA Y UN PROCESO
PARA SU RECUPERACION Y PURIFICACION"
Solicitante :BIOCON LIMITED.
Opositora :LABORATORIOS SYNTHESIS S.A.S.

Publicada en la ~~Gaceta~~ de la Propiedad Industrial
N° 620 del 20 de septiembre de 2010

Yo, *ELIANA ISAURA MORENO BOHORQUEZ*, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. *83823* del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad **LABORATORIOS SYNTHESIS S.A.S.** Conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada "UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU RECUPERACION Y PURIFICACION", cuyos titulares son **BIOCON LIMITED**, y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

1. INTERÉS PARA ACTUAR ✓

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legítimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la comercialización de algunos productos de mi representada, que contienen, en particular RAPAMICINA. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

2.1. La solicitud objeto de la presente fue presentada el 08 de junio de 2009 reivindicando prioridad bajo la solicitud IN2006/000502 del 2006-11-10; y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 620 del 20 de **septiembre de 2010**, cuya publicación internacional corresponde a la WO2008056372 del 2008-05-15.

2.2. La solicitud colombiana 09 59243 objeto de la presente oposición se refiere a una forma pura de la rapamicina con un contenido de impurezas total de menos de 1,2%, un proceso de recuperación y purificación de la rapamicina que abarca pasos de (a) tratar el caldo de fermentación, extractos o soluciones que contengan

rapamicina inmiscibles con el agua disolvente y la concentración; (b) la adición de un solvente miscible de agua para efectuar la separación de las impurezas presentes, (c) opcionalmente, la unión del disolvente que contiene el producto de la etapa (b) a un sólido inerte, lavando el sólido con una base y ácido, seguido de elución, (d) someter la elución de la etapa (c) o el disolvente que contiene el producto de la etapa (b) a la cromatografía de gel de sílice (e) de la cristalización del producto obtenido de la etapa (d), (f) imponer una solución del producto de la etapa (e) a la interacción hidrofóbica o la cromatografía en fase reversa, y (g) la recrystalización para obtener rapamicina en su forma sustancialmente pura.

2.3. Con base en el artículo 14 de la Decisión 486 de la CAN que consagra: *“Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial”*, tenemos que las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afectan la patentabilidad de la presente solicitud:

- D1: WO2005019226 publicada el 03 de marzo de 2005.

2.4. Ahora bien, el documento D1, se refiere a un procedimiento para la recuperación de un macrólido en forma sustancialmente pura que comprende: a) tratamiento de los macrólidos con disolvente inmiscible en agua seguido por la concentración, b) mezclar con agua, un disolvente miscible en agua o mezcla de los mismos, c) realizar la cromatografía de interacción hidrofóbica y recoger las fracciones, d) la extracción de la fracción que contiene los macrólidos con disolvente inmiscible en agua seguido por la concentración, e) la adición de solventes miscibles en agua para efectuar la separación de las impurezas del compuesto macrólido, f) realizar



cromatografía de gel de sílice y recoger las fracciones, g) aislar el compuesto macrólico en forma sustancialmente pura. Los macrólidos elegidos son preferiblemente rapamicina, tacrolimus o immunomycin. Dicho documento D1 de forma particular en la página 8 línea 10 revela Rapamicina con una pureza de 99,8%; la solicitud objeto de la presente oposición no es novedosa pues lo reclamado ya era parte de arte anterior

2.5. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana 09 59243 no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con el mas importante de los tres requisitos necesarios para su protección, “La Novedad”.

2.6. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.6.1. **CARENCIA DE NOVEDAD:** Según la decisión 486 de la Comunidad Andina de Naciones, Artículo 16.- *“Una invención se considerará nueva cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.”*. A la fecha de prioridad, 10 de noviembre de 2006 ya era conocido, pues como se demostró, era parte del arte anterior con base en el documento D1, una forma pura de la rapamicina con un contenido de impurezas total de menos de 1,2%, considerando que un producto no puede ser considerado novedoso, simplemente por el hecho de ser producido en base a un nuevo proceso. Sobre lo cual busca protección en el capítulo reivindicatorio.

2.7. Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *“Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial.”*. La solicitud pretendida no cumple con el más importante de los tres requisitos de patentabilidad, “La Novedad”.

2.8. Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada la presente oposición y en consecuencia negar el registro a la solicitud de patente titulada “UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU RECUPERACION Y PURIFICACION”, cuyos titulares son BIOCON LIMITED, solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 620 del 20 de septiembre de 2010.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: WO2005019226 publicada el 03 de marzo de 2005.



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

- 4.1. Numeral 1 del artículo 586 del Código de Comercio.
- 4.2. Artículo 42 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.
- 4.3. En las demás normas concordantes.

5. ANEXOS

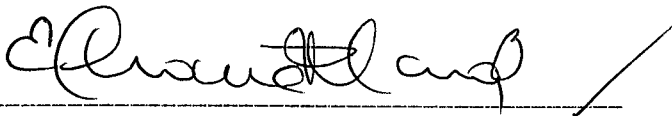
Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de **LABORATORIOS SYNTHESIS S.A.S.**
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:
 - D1: **WO2005019226** publicada el 03 de marzo de 2005.

6. NOTIFICACIONES

Para efectos de notificaciones que por disposición del artículo 50 del Decreto 01 de 1984 debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor Superintendente,



ELIANA ISaura MORENO BOHORQUEZ

C.C. 51'975.664 de Bogotá

T.P.A. 83823 del C.S. de la J.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-059243-00004-0000

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 Act. 400 OPOSIOBSERVTER Folios: 35

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-059243-00004-0000

Fecha: 2010-12-16 16:24:26 Dep. 2020 DIR.NUEVASCR
 Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI
 Act. 400 OPOSIOBSERVTER Folios: 34

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 10 - 107,313
 FECHA : DICIEMBRE 16 DE 2010

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	Vr. PAGO
LAB. PROCAPS	CONSIGNACION	BANCO DE BOGOTA	062754387	382186103	07/12/2010	1,180,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-04 PRESENTACION DE OBSERVACIONES	12 MARCAS Y LEMAS COMERCIALES	295,000.00
	TOTAL :	\$ 295,000.00

SON: DOSCIENTOS NOVENTA Y CINCO MIL PESOS

RESPONSABLE

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

PODER ESPECIAL

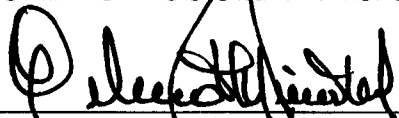
Yo, **ORLANDO MUÑOZ MARTÍNEZ**, mayor de edad y vecino de la ciudad de Bogotá (Colombia), identificado como aparece al pie de mi firma, quien en el presente documento actúa en calidad de Subgerente de la sociedad **LABORATORIOS SYNTHESIS S.A.S.** sociedad legalmente constituida y domiciliada en la ciudad de Bogotá (Colombia), por el presente documento otorgo a la doctora **ELIANA ISAURA MORENO BOHÓRQUEZ**, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del C.S. de la J., poder especial, amplio y suficiente, para formular oposiciones u observaciones a la solicitud de patente No. **09 059.243**, titulada "**UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU RECUPERACIÓN Y PURIFICACIÓN**", cuyo titular es **BIOCON LIMITED**, publicada en la gaceta No. 620 del 20 de Septiembre de 2010.

Nuestro apoderado queda facultado, de manera expresa, para realizar todo tipo de actos necesarios para llevar a cabo el trámite de la oposición, incluyendo, sin limitación, preparar y presentar solicitudes, hacer declaraciones, pagar tasas, contribuciones e impuestos, recabar los títulos o certificados. Asimismo quedan autorizados para impugnar administrativa todo lo que fuere resuelto en el expediente a que se hace expresa referencia en el presente poder, con todas las facultades generales y específicas que fuera requerido para ella.

De la misma manera, además de las facultades que le confiere la naturaleza del presente mandato, se encuentran facultados para recibir, desistir, transigir, sustituir, y reasumir el presente poder, incoar acciones, interponer recursos y demás facultades conferidas por la ley.

Dado y Firmado en Bogotá (Colombia) a los ____ días del mes de ____ de 2010.

LABORATORIOS SYNTHESIS S.A.S.

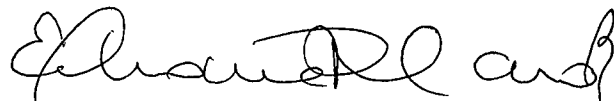


C.C. 8.317.044 de Medellín.

NIT: 860.000.760-1

Subgerente

Acepto:



ELIANA ISAURA MORENO BOHÓRQUEZ

C.C. 51'975.664 de Bogotá

T.P.A. 83823 del C.S. de la J.



5105.

NOTARIA DIECIOCHO DEL
CIRCULO DE BOGOTÁ D.C.
LA HUELLA SE AUTENTICA POR
LA FIRMA DEL INTERESADO

DILIGENCIA DE RECONOCIMIENTO

REPUBLICA DE COLOMBIA

111200

Compareció ante el Notario 18 del Circuito
de Bogotá.

MARCELO MARTINEZ
8317 044
Medellin

quien exhibió la C.C.

Expedida en

declaró que la firma y huella que
aparecen en el presente documento
son suyas y que el contenido del mismo
es cierto.

4 DIC 2010

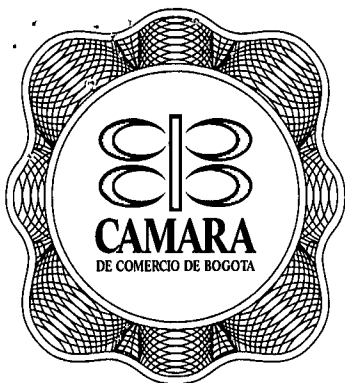
BOGOTÁ, D.C.

[Signature]



HUELLA DEL
INDICE DERECHO

REPUBLICA DE COLOMBIA
ALBERTO VARGAS ALLO
NOTARIO 18 (E) DEL CIRCULO DE BOGOTÁ D.C.



01



* 9 8 7 7 1 4 0 0 *

12

CAMARA DE COMERCIO DE BOGOTA

SEDE PALOQUEMAO

25 DE NOVIEMBRE DE 2010 HORA 09:33:10

R029935134

PAGINA: 1 de 5

* * * * *

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O INSCRIPCION DE DOCUMENTOS

LA CAMARA DE COMERCIO DE BOGOTA, CON FUNDAMENTO EN LAS MATRICULAS E INSCRIPCIONES DEL REGISTRO MERCANTIL

CERTIFICA: ✓

NOMBRE : LABORATORIOS SYNTHESIS S A S

N.I.T. : 860000760-1

DOMICILIO : BOGOTA D.C.

CERTIFICA:

MATRICULA NO: 00008059 DEL 23 DE MARZO DE 1972

CERTIFICA:

DIRECCION DE NOTIFICACION JUDICIAL : CRA 44 NO 20C 73

MUNICIPIO : BOGOTA D.C.

EMAIL DE NOTIFICACION JUDICIAL : synthe@synthesis.com.co

DIRECCION COMERCIAL : CRA 44 NO 20C 73

MUNICIPIO : BOGOTA D.C.

EMAIL COMERCIAL : synthe@synthesis.com.co

CERTIFICA:

CONSTITUCION: ESCRITURA PUBLICA NO.2078, NOTARIA 8 BOGOTA, DEL 27 DE JULIO DE 1.950, INSCRITA EL 10 DE AGOSTO DE 1.950, BAJO EL NO. 21.766 DEL LIBRO RESPECTIVO ACLARADA POR ESCRITURA NO. 3608 DEL 8 DE OCTUBRE DE 1.956 NOTARIA 8 DE BOGOTA INSCRITA EN ESTA - CAMARA DE COMERCIO EL 19 DE OCTUBRE DE 1.956 BAJO EL NO. 35.547 DEL LIBRO RESPECTIVO, SE CONSTITUYO LA SOCIEDAD DENOMINADA: "LABORATORIOS BALBAG".

CERTIFICA:

QUE POR ESCRITURA PUBLICA NUMERO 3997 OTORGADA EN LA NOTARIA 8 DE BOGOTA EL 24 DE NOVIEMBRE DE 1.952, INSCRITA EN ESTA CAMARA DE COMERCIO EL 1 DE DICIEMBRE DE 1.952, BAJO EL NUMERO 26.158 DEL LIBRO RESPECTIVO, LA SOCIEDAD CAMBIO SU NOMBRE DE: "LABORATORIOS --- BALBAG" POR EL DE "LABORATORIO SYNTHESIS" E INTRODUJO OTRAS REFORMAS.

CERTIFICA :

QUE POR ESCRITURA PUBLICA NUMERO 6491 OTORGADA EN LA NOTARIA 1. - DE BOGOTA EL 24 DE DICIEMBRE DE 1.981, INSCRITA EN ESTA CAMARA DE COMERCIO EL 12 DE MARZO DE 1.982, BAJO EL NUMERO 113.297 DEL LIBRO IX, LA SOCIEDAD CAMBIO SU NOMBRE DE "LABORATORIOS SYNTHESIS - BALAVOINE Y CIA. S.C.A" POR EL DE "LABORATORIOS SYNTHESIS LTDA. Y CIA. S.C.A" E INTRODUJO OTRAS REFORMAS.

CERTIFICA:

QUE POR ESCRITURA PUBLICA NUMERO 8580 OTORGADA EN LA NOTARIA 3 DE BOGOTA EL 4 DE DICIEMBRE DE 1.973, INSCRITA EN ESTA CAMARA DE COMERCIO EL 7 DE ENERO DE 1.974, BAJO EL NUMERO 14.478 DEL LIBRO IX, LA SOCIEDAD SE TRASFORMO DE LIMITADA EN COMANDITA POR ACCIONES BAJO EL NOMBRE DE LABORATORIOS SYNTHESIS BALAVOINE Y CIA. S.C.A.

CERTIFICA:

QUE POR ACTA NO. 117 DE LA ASAMBLEA DE ACCIONISTAS, DEL 30 DE ABRIL DE 2010, INSCRITA EL 10 DE JUNIO DE 2010 BAJO EL NÚMERO 1390261 DEL LIBRO IX, LA SOCIEDAD DE LA REFERENCIA SE TRANSFORMO DE SOCIEDAD EN COMANDITA POR ACCIONES A SOCIEDAD POR ACCIONES SIMPLIFICADA BAJO EL NOMBRE DE: LABORATORIOS SYNTHESIS S.A.S.

CERTIFICA:

QUE POR E.P. NO.5.435 DEL 17 DE SEPTIEMBRE DE 1.996, NOTARIA PRIMERA DE SANTAFE DE BOGOTA, INSCRITA EL 30 DE SEPTIEMBRE DE 1.996, BAJO EL NO.556.909 DEL LIBRO IX, ADICIONADA POR LOS REGISTROS 557 146 DEL 1 DE OCTUBRE DE 1.996, Y 557.301 DEL 2 DE OCTUBRE DE 1996 DEL LIBRO IX, LA SOCIEDAD DE LA REFERENCIA COMUNICO QUE SE FUSIONO, ABSORBIENDO A LA SOCIEDAD "SYNTHESIS VETERINARIA LTDA Y CIA S C A"

CERTIFICA:

QUE POR E.P. NO.1024 DEL 28 DE FEBRERO DE 1997, DE LA NOTARIA 1. DE SANTAFE DE BOGOTA, INSCRITA EL 31 DE MARZO DE 1997, BAJO EL NUMERO 579245 DEL LIBRO IX, CELEBRO ACUERDO DE FUSIÓN CON LA SOCIEDAD PRODUCTOS DIETÉTICAS DIETESYN S.A, DONDE LA SOCIEDAD DE LA REFERENCIA FUE LA ABSORBENTE.

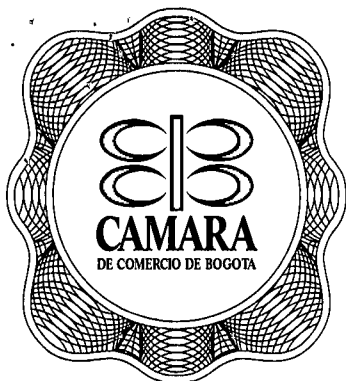
CERTIFICA:

QUE POR ACTA NO. 120 DE LA ASAMBLEA DE ACCIONISTAS DEL 29 DE JULIO DE 2010, INSCRITA EL 19 DE NOVIEMBRE DE 2010 BAJO EL NUMERO 01429837 DEL LIBRO IX, LA SOCIEDAD DE LA REFERENCIA (ABSORBENTE) ABSORBE MEDIANTE FUSIÓN A LAS SOCIEDADES INMUNOSYN SAS Y GYNOPHARM SAS LAS CUALES SE DISUELVEN SIN LIQUIDARSE.

CERTIFICA:

REFORMAS:

ESCRITURAS NO.	FECHA	NOTARIA	INSCRIPCION
4471	18-XII-1.956	8 BTA.	22-XII-1.956 NO. 36.077
3322	31-XII-1.958	8 BTA.	12-I-1.959 NO. 42.034
2322	22-IX-1.961	8 BTA.	29-IX-1.961 NO. 51.161
290	2-II-1.967	6 BTA.	24-II-1.967 NO. 71.304
8545	16-XII-1.967	6 BTA.	26-I-1.968 NO. 75.041
4679	20-XII-1.967	8 BTA.	26-I-1.968 NO. 75.042
3010	14-V-1.968	6 BTA.	30-V-1.968 NO. 76.244
2931	4-V-1.970	6 BTA.	18-V-1.970 NO. 84.983
5656	13-VII-1.970	6 BTA.	13-VIII-1.970 NO. 86.126
3487	15-VII-1.971	3 BTA.	29-VII-1.971 NO. 90.980
4638	10-VII-1.984	11 BTA.	27-VIII-1.984 NO.157.001
1443	14-III-1.985	11 BTA.	26- III-1.985 NO.167.627
724	11- II-1.985	1 BTA.	21- II-1.985 NO.165.940
4836	11-VIII-1986	1 BTA.	23- IX -1.986 NO.197.784
5659	15- IX -1986	1 BTA.	23- IX -1.986 NO.197.784
6326	16- X -1987	1 BTA.	23- X -1.987 NO.221.604
5164	21-VIII-1990	1 BTA.	3- IX -1.990 NO.303.579
9244	23-XII- 1991	1 STAFE.BTA.	8-I -1.992 NO.351.255
8145	15- X - 1992	1 STAFE.BTA.	16- X-1.992 NO.382.488
1374	26- III-1993	1 STAFE.BTA.	19- III-1993 NO.399972
3120	01-VI-1.995	1A.STAFE BTA	23-VI- 1.995 NO.498.060
5435	17-IX-1.996	1A.STAFE BTA	30-IX- 1.996 NO.556.909
5435	17-IX-1.996	1A.STAFE BTA	01- X- 1.996 NO.557.146
5435	17-IX-1.996	1A.STAFE BTA	02- X- 1.996 NO.557.301
5942	7-X -1.996	1A.STAFE BTA	11- X- 1.996 NO.558.222
6305	24-X--1.996	1A.STAFE BTA	07-XI--1.996 NO.561.207
6929	25-XI-1.996	1A.STAFE BTA	12-XII-1.996 NO.565.961
787	19-II-1.997	1A.STAFE BTA	25-II -1.997 NO.575.141



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CAMARA DE COMERCIO DE BOGOTA

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28-II-1.997 1A.STAFE BTA 31-III-1.997 NO.579.245

CERTIFICA:

REFORMAS:

E.P. NO.	FECHA	NOTARIA	CIUDAD	FECHA	NO. INSC.
0007110	1997/10/28	0001	BOGOTA D.C.	1997/11/06	00609204
0001896	1998/03/16	0001	BOGOTA D.C.	1998/04/01	00628368
0002924	1998/04/21	0001	BOGOTA D.C.	1998/05/18	00634309
0002377	2000/06/14	0001	BOGOTA D.C.	2000/06/20	00733626
0000SIN	2004/06/22	0000	BOGOTA D.C.	2004/06/28	00940785
117	2010/04/30	0000	BOGOTA D.C.	2010/06/10	01390261
120	2010/08/18	0000	BOGOTA D.C.	2010/08/26	01409241
120	2010/08/30	0000	BOGOTA D.C.	2010/09/13	01413659
120	2010/07/29	0000	BOGOTA D.C.	2010/11/19	01429837

CERTIFICA:

VIGENCIA: QUE LA SOCIEDAD NO SE HALLA DISUELTA. DURACION HASTA EL 27 DE JULIO DE 2040

CERTIFICA:

OBJETO SOCIAL: EL OBJETO DE LA SOCIEDAD ES EL EJERCICIO DE TODAS LAS ACTIVIDADES COMERCIALES PARA: 1) LA IMPORTACIÓN, EXPORTACIÓN, FABRICACIÓN, COMERCIALIZACIÓN, DISTRIBUCIÓN Y VENTA DE PRODUCTOS FARMACÉUTICOS, HIGIÉNICOS, VETERINARIOS E IMPLEMENTOS MÉDICOS. 2) CUALQUIER OTRA ACTIVIDAD LÍCITA DE COMERCIO RELACIONADA CON SU OBJETO SOCIAL PRINCIPAL Y TODO LO QUE SE RELACIONA CON LA IMPORTACIÓN DE LOS BIENES Y EQUIPOS NECESARIOS PARA EL DESARROLLO DE SU OBJETO SOCIAL. 3) TENER LA REPRESENTACIÓN EN COLOMBIA DE EMPRESAS O SOCIEDADES ESTABLECIDAS FUERA DEL TERRITORIO COLOMBIANO, CUYO OBJETO SEA SIMILAR O COMPLEMENTARIO. EN DESARROLLO DE SU OBJETO SOCIAL PRINCIPAL, LA SOCIEDAD PODRÁ ADELANTE Y CELEBRAR LOS SIGUIENTES ACTOS Y CONTRATOS: A) EXPORTAR E IMPORTAR, AGENCIAR, DISTRIBUIR, COMERCIALIZAR, COMPRAR O VENDER A COMISIÓN O CONSIGNACIÓN O DE CUALQUIER OTRA FORMA O MODALIDAD, TODA CLASE DE EQUIPOS, APARATOS, BIENES AL POR MAYOR O AL DETAL, Y SERVICIOS RELACIONADOS CON SU ACTIVIDAD. B) GRAVAR EN CUALQUIER FORMA LOS BIENES DE SU PROPIEDAD MUEBLES O INMUEBLES, DAR EN PRENDA LOS PRIMEROS O HIPOTECAR LOS SEGUNDOS PARA GARANTIZAR OBLIGACIONES DE LA SOCIEDAD. C) CONSTITUIR APODERADOS QUE LE REPRESENTEN, ASOCIARSE CON OTRAS COMPAÑÍAS DE LA MISMA O PARECIDA NATURALEZA Y EN GENERAL REALIZAR TODA CLASE DE OPERACIONES COMERCIALES O CIVILES Y EJECUTAR O CELEBRAR TODA CLASE DE CONTRATOS O ACTOS DEL MISMO ORDEN, YA SEAN INDUSTRIALES, COMERCIALES O FINANCIEROS ENCAMINADOS A ALCANZAR LOS FINES QUE SE PROPONEN. D) ADQUIRIR, ENAJENAR, ADMINISTRAR, RECIBIR O DAR EN ARRENDATARIO O A CUALQUIER TÍTULO, TODA CLASE DE BIENES MUEBLES E INMUEBLES. E) INTERVENIR ANTE TERCEROS O ANTE LOS ACCIONISTAS MISMOS COMO ACREEDORA O COMO DEUDORA EN TODA CLASE DE OPERACIONES DE CRÉDITO, DANDO O RECIBIENDO LAS GARANTÍAS DEL CASO, CUANDO HAYA LUGAR A ELLAS; F) EFECTUAR CUALQUIER

CLASE DE OPERACIONES DE CRÉDITO ACTIVO O PASIVO, TALES COMO CONSTITUIR DEPÓSITOS, EFECTUAR PRESTAMOS. OTORGAR O RECIBIR DOCUMENTOS NEGOCIABLES, GIRAR, ACEPTAR, ENDOSAR, ASEGURAR Y NEGOCIAR EN GENERAL, TÍTULOS VALORES RECIBIRLOS EN PAGO; G) CELEBRAR Y EJECUTAR EN GENERAL TODOS LOS ACTOS Y CONTRATOS PREPARATORIOS, COMPLEMENTARIOS O ACCESORIOS DE TODOS LOS ANTERIORES, LOS QUE SE RELACIONEN CON LA EXISTENCIA Y FUNCIONAMIENTO DE LA SOCIEDAD, Y LOS QUE SEAN CONDUCTENTES AL BUEN LOGRO DE LOS FINES SOCIALES.

CERTIFICA:

CAPITAL:

**** CAPITAL AUTORIZADO ****

VALOR : \$20,000,000,000.00
NO. DE ACCIONES : 20,000,000.00
VALOR NOMINAL : \$1,000.00

**** CAPITAL SUSCRITO ****

VALOR : \$17,207,141,000.00
NO. DE ACCIONES : 17,207,141.00
VALOR NOMINAL : \$1,000.00

**** CAPITAL PAGADO ****

VALOR : \$17,207,141,000.00
NO. DE ACCIONES : 17,207,141.00
VALOR NOMINAL : \$1,000.00

CERTIFICA:

REPRESENTACION LEGAL: GERENCIA LA CUAL SERÁ ASUMIDA POR UN GERENTE Y UN SUBGERENTE QUIENES TENDRÁN REPRESENTACIÓN LEGAL DE LA SOCIEDAD.

CERTIFICA:

**** NOMBRAMIENTOS ****

QUE POR ACTA NO. 127 DE ASAMBLEA DE ACCIONISTAS DEL 12 DE NOVIEMBRE DE 2010, INSCRITA EL 23 DE NOVIEMBRE DE 2010 BAJO EL NUMERO 01430759 DEL LIBRO IX, FUE (RON) NOMBRADO (S):

NOMBRE	IDENTIFICACION
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GERENTE

PALACIOS LUIS ALBERTO

C.E. 000000000319788

QUE POR ACTA NO. 117 DE ASAMBLEA DE ACCIONISTAS DEL 30 DE ABRIL DE 2010, INSCRITA EL 10 DE JUNIO DE 2010 BAJO EL NUMERO 01390261 DEL LIBRO IX, FUE (RON) NOMBRADO (S):

NOMBRE	IDENTIFICACION
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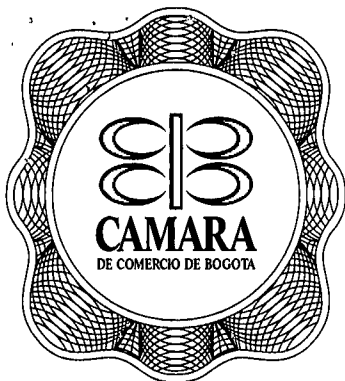
SUBGERENTE

MUÑOZ MARTINEZ ORLANDO

C.C. 000000008317044

CERTIFICA:

FACULTADES DEL REPRESENTANTE LEGAL: EL GERENTE DE LA SOCIEDAD TIENE A SU CARGO LA ADMINISTRACIÓN INMEDIATA DE LA SOCIEDAD, ASÍ COMO LA REPRESENTACIÓN LEGAL DE LA MISMA Y EN TAL VIRTUD LE ESTÁN ASIGNADAS LAS SIGUIENTES FUNCIONES Y ATRIBUCIONES: A) LLEVAR LA REPRESENTACIÓN DE LA ENTIDAD, TANTO JUDICIAL COMO EXTRAJUDICIALMENTE. B) EJECUTAR LOS ACUERDOS Y DECISIONES DE LA ASAMBLEA GENERAL DE ACCIONISTAS. C) CONSTITUIR, PARA CASOS ESPECIALES, APODERADOS JUDICIALES O EXTRAJUDICIALES. D) CELEBRAR LOS ACTOS, OPERACIONES Y CONTRATOS CONDUCTENTES AL BUEN LOGRO DEL OBJETO DE LA SOCIEDAD. E) CUIDAR DE LA RECAUDACIÓN E INVERSIÓN DE LOS FONDOS DE LA SOCIEDAD. F) PRESENTAR A LA REUNIÓN ORDINARIA ANUAL DE LA ASAMBLEA GENERAL DE ACCIONISTAS, EL INVENTARIO Y LOS ESTADOS FINANCIEROS DE PROPÓSITO GENERAL DE FIN DE EJERCICIO, JUNTO CON UN INFORME ESCRITO RELACIONADO CON LA SITUACIÓN Y LA MARCHA DE LA ENTIDAD. G) CREAR LOS EMPLEADOS NECESARIOS PARA LA



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DEBIDA MARCHA DE LA SOCIEDAD, SEÑALAR SUS FUNCIONES Y ASIGNACIONES Y HACER LOS NOMBRAMIENTOS CORRESPONDIENTES. H) CONVOCAR A LA ASAMBLEA GENERAL CUANDO PROCEDA HACERLO CONFORME A LA LEY O A ESTOS ESTATUTOS. I) PRESENTAR A LA ASAMBLEA GENERAL DE ACCIONISTAS, CUANDO ASÍ LO SOLICITE, ESTADOS FINANCIEROS MENSUALES DE PRUEBA E INFORMES. J) EJERCER LAS FUNCIONES QUE LE DELEGUE LA ASAMBLEA GENERAL DE ACCIONISTAS. K) CUMPLIR Y HACER QUE SE CUMPLAN EN OPORTUNIDAD Y DEBIDAMENTE TODAS LAS EXIGENCIAS DE LAS LEYES EN RELACIÓN CON EL FUNCIONAMIENTO Y LAS ACTIVIDADES DE LA INSTITUCIÓN. L) LAS DEMÁS QUE LE CORRESPONDAN CONFORME A LA LEY Y A ESTOS ESTATUTOS. EL GERENTE DE LA SOCIEDAD DEBERÁ CONTAR CON AUTORIZACIÓN PREVIA DE LA ASAMBLEA GENERAL DE ACCIONISTAS, CUANDO PRETENDA SUSCRIBIR EN NOMBRE DE LA SOCIEDAD (I) ACTOS O CONTRATOS PARA ADQUIRIR, ENAJENAR, RECIBIR O DAR EN ARRENDAMIENTO O A CUALQUIER OTRO TÍTULO, TODA CLASE DE BIENES INMUEBLES DE LA SOCIEDAD O BIENES MUEBLES DIFERENTES A LOS PRODUCTOS COMERCIALIZADOS ORDINARIAMENTE POR LA SOCIEDAD, ASÍ COMO (II) ACTOS O CONTRATOS, INDEPENDIENTE DE SU OBJETO, QUE SUPERE EN CUANTÍA EL EQUIVALENTE A 300 SMLMV (TRESCIENTOS SALARIOS MÍNIMOS MENSUALES LEGALES VIGENTES).

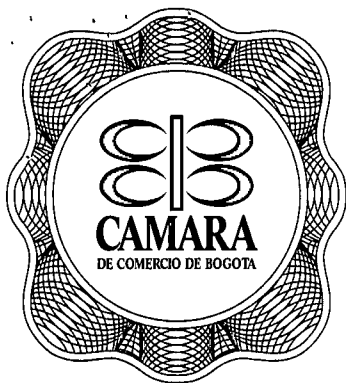
CERTIFICA:

QUE POR ESCRITURA PUBLICA NO. 654 DE LA NOTARIA 01 DE BOGOTA D.C., DEL 20 DE FEBRERO DE 2009, INSCRITA EL 02 DE MARZO DE 2009 BAJO EL NO. 15209 DEL LIBRO V, COMPARECIO ORLANDO MUÑOZ MARTINEZ IDENTIFICADO CON CEDULA DE CIUDADANIA NO. 8.317.044 EN SU CALIDAD DE DELEGADO GENERAL SUPLENTE DEL SOCIO GESTOR LABORATORIOS SYNTHESIS LTDA., POR MEDIO DE LA PRESENTE ESCRITURA PUBLICA, CONFIERE PODER GENERAL AL SEÑOR ANDRES FERNANDO MOLLAJOLI CENTURION, IDENTIFICADO CON CÉDULA DE EXTRANJERÍA NO. 351.283 QUE EN NOMBRE DE LA SOCIEDAD QUE REPRESENTO, ACTUÉ CON LAS MÁS AMPLIAS FACULTADES EN LA REPÚBLICA DE COLOMBIA, EN LOS SIGUIENTES ASUNTOS 1. PARTICIPAR EN CALIDAD DE SOCIO, ACCIONISTA O EMPRESARIO EN PERSONAS JURÍDICAS COLOMBIANAS, CONSTITUIDAS O POR CONSTITUIRSE, EN EL PORCENTAJE QUE LA APODERADA AQUÍ NOMBRADA ESTIME CONVENIENTE A NUESTROS INTERESES, CON FACULTAD PARA ENTREGAR LAS SUMAS CORRESPONDIENTES AL PAGO DEL MISMO; 2. DILIGENCIAR Y SUSCRIBIR LOS DOCUMENTOS NECESARIOS PARA EL REGISTRO DE INVERSIONES EXTRANJERAS ANTE LAS AUTORIDADES COLOMBIANAS; 3. REPRESENTAR A LABORATORIOS SYNTHESIS LTDA & CIA S.C.A., EN LAS REUNIONES ASAMBLEA GENERAL O JUNTAS EN LAS CUALES TENGA INTERÉS; 4. EFECTUAR CUALQUIER CLASE DE OPERACIONES DE CRÉDITO ACTIVO O PASIVO, TALES COMO CONSTITUIR DEPÓSITOS, EFECTUAR PRÉSTAMOS DANDO O RECIBIENDO LAS GARANTÍAS DEL CASO, CUANDO HAYA LUGAR A ELLAS, OTORGAR O RECIBIR DOCUMENTOS NEGOCIABLES, GIRAR, ACEPTAR, ENDOSAR, ASEGURAR, Y NEGOCIAR EN GENERAL TÍTULOS VALORES Y RECIBIRLOS EN PAGO; 5. CELEBRAR ASÍ MISMO OPERACIONES RELACIONADAS CON LA PROTECCIÓN DE SUS BIENES, NEGOCIOS Y PERSONAS A SU SERVICIO; 6. CELEBRAR CONTRATOS DE CUENTAS EN

PARTICIPACIÓN, SEA COMO PARTICIPE ACTIVA O COMO PARTICIPE INACTIVA; 7. TRANSIGIR, DESISTIR Y APELAR DECISIONES DE ÁRBITROS O DE AMIGABLES COMPONEDORES EN LAS CUESTIONES EN QUE TENGA INTERÉS FRENTE A TERCEROS, A LOS ASOCIADOS MISMOS O A SUS ADMINISTRADORES; 8. ADQUIRIR MARCAS, PATENTES, PROCEDIMIENTOS DE FABRICACIÓN, EXPLOTADAS EN CUALQUIER FORMA, YA SEA UTILIZÁNDOLOS DIRECTAMENTE O PERMITIENDO SU EXPLOTACIÓN POR OTRAS PERSONAS NATURALES O JURÍDICAS CONTRA EL PAGO DE REGALÍAS O PARTICIPACIONES. 9. CELEBRAR Y EJECUTAR EN GENERAL TODOS LOS ACTOS PREPARATORIOS, COMPLEMENTARIOS O ACCESORIOS DE TODOS LOS ANTERIORES, LOS QUE SE RELACIONEN CON LA EXISTENCIA Y FUNCIONAMIENTO DE LA SOCIEDAD, Y LOS QUE SEA CONDUCTENTES AL BUEN LOGRO DE LOS FINES SOCIALES; 10. RECIBIR NOTIFICACIONES DE CUALESQUIERA TIPO DE ACTOS EMANADOS DE LAS AUTORIDADES ADMINISTRATIVAS O JUDICIALES. 11. NOTIFICARSE VÁLIDAMENTE DE AQUELLOS ACTOS DEL ORDEN ADMINISTRATIVO O JUDICIAL QUE SE FORMULEN CONTRA LA SOCIEDAD, ASÍ COMO ABSOLVER INTERROGATORIOS CON CAPACIDAD PARA CONFESAR; 12. ADMINISTRAR LOS BIENES MUEBLES O INMUEBLES DE PROPIEDAD DE LA SOCIEDAD LABORATORIOS SYNTHESIS LTDA & CIA S.C.A. 12. ADMINISTRAR LOS BIENES MUEBLES O INMUEBLES DE PROPIEDAD DE LA SOCIEDAD LABORATORIOS SÍNTESIS LTDA. & CIA S.C.A., RECAUDAR O RECIBIR POR SUS FRUTOS Y CELEBRAR TODA CLASE DE CONTRATOS RELACIONADOS CON LA ADMINISTRACIÓN. 13. ENAJENAR, ADQUIRIR, PERMUTAR, GRAVAR, ARRENDAR BIENES MUEBLES, PRESENTES O FUTUROS DE LA SOCIEDAD LABORATORIOS SYNTIIESIS LTDA & CIA S.C.A, SIN LÍMITE DE CUANTIA. 14. ENAJENAR, PERMUTAR, ADQUIRIR, GRAVAR, ARRENDAR BIENES MUEBLES E INMUEBLES PRESENTES O FUTUROS DE LA SOCIEDAD LABORATORIOS SYNTHESIS LTDA & CIA S.C.A., CUANDO LA CUANTÍA DE CADA OPERACIÓN INDIVIDUALMENTE CONSIDERADA NO EXCEDA DE SEISCIENTAS VECES EL SALARIO MÍNIMO LEGAL MENSUAL MAS ALTO EN LA FECHA DE CELEBRACIÓN Y TENGA RELACIÓN DIRECTA CON EL DESARROLLO SOCIAL, ASÍ COMO LAS QUE EXCEDAN DE ESTA SUMA CON LA EXPRESA AUTORIZACIÓN DE LA SOCIEDAD GESTORA. 15. TOMAR DINERO EN MUTUO, CON FACULTAD PARA ESTIPULAR TODO TIPO DE INTERESES, PLAZOS Y DEMÁS CONDICIONES Y CELEBRAR CONTRATOS DE CUENTA CORRIENTE; 16. GIRAR, ORDENAR GIRAR, ENDOSAR, PROTESTAR, OTORGAR, ACEPTAR ORDENAR AVALÚOS Y HACER TODA CLASE DE NEGOCIOS RELACIONADOS CON TÍTULOS VALORES CUANDO LA CUANTÍA DE CADA OPERACIÓN INDIVIDUALMENTE CONSIDERADA NO EXCEDA DE SEISCIENTAS VECES EL SALARIO MÍNIMO LEGAL MENSUAL MÁS ALTO EN LA FECHA DE SU CELEBRACIÓN, SÍ COMO LAS QUE EXCEDAN DE ESTA SUMA CON LA EXPRESA AUTORIZACIÓN DEL REPRESENTANTE LEGAL DE LA SOCIEDAD GESTORA. 17. PRESENTAR DECLARACIÓN DE RENTA, PATRIMONIO, IMPUESTOS A LAS VENTAS EN ADMINISTRACIÓN DE IMPUESTOS NACIONES Y MUNICIPALES E INTERPONER LOS RECURSOS Y DECLARACIONES PERTINENTES CONTRA ACTOS DE DICHA ADMINISTRACIÓN CON FACULTAD PARA DEFENDER LOS INTERESES DE LA SOCIEDAD EN ÉSTA MATERIA. 18. DESIGNAR APODERADO ESPECIAL PARA CADA PROCESO JUDICIAL O ADMINISTRATIVO. 19. PRÉSENTAR RECURSOS POR VÍA ADMINISTRATIVA O JUDICIAL, RENUNCIAR A TÉRMINOS, DESISTIR, CONCILIAR, TRANSIGIR Y MODIFICAR 20. ELEVAR PETICIONES Y SOLICITAR INFORMACIÓN A LAS AUTORIDADES ADMINISTRATIVAS Y JURISDICCIONALES 21. PRESENTAR DEMANDAS ANTE LAS AUTORIDADES ADMINISTRATIVAS Y JUDICIALES 22. SUSTITUIR EL PRESENTE PODER Y REVOCAR TAL SUSTITUCIÓN DENTRO DE LA REPÚBLICA DE COLOMBIA

CERTIFICA:

QUE POR ESCRITURA PUBLICA NO. 3511 DE LA NOTARIA 18 DE BOGOTA D.C., DEL 8 DE SEPTIEMBRE DE 2010, INSCRITA EL 1 DE OCTUBRE DE 2010 BAJO EL NO. 00018604 DEL LIBRO V, COMPARECIO JOSE ANTONIO JAIME ESCOBAR IDENTIFICADO CON CEDULA DE CIUDADANÍA NO. 19.262.407 DE



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BOGOTA, EN SU CALIDAD DE GERENTE DE LABORATORIOS SYNTHESIS LTDA., POR MEDIO DE LA PRESENTE ESCRITURA PUBLICA, CONFIERE PODER GENERAL AL A OLGA VICTORIA PRIETO ZAMORA, IDENTIFICADA CON CEDULA DE CIUDADANÍA NO. 52.780.308 DE BOGOTA PARA QUE EN NOMBRE DE LA SOCIEDAD LABORATORIOS SYNTHESIS S.A.S., PROMUEVA Y DEFIENDA LOS INTERESES DE LA SOCIEDAD JUDICIAL Y EXTRAJUDICIALMENTE, EN TODA ESPECIE DE GESTIONES Y ACTUACIONES ANTE LAS AUTORIDADES ADMINISTRATIVAS O JURISDICCIONALES Y EN ESPECIAL PARA: A. DILIGENCIAR Y SUSCRIBIR LOS DOCUMENTOS NECESARIOS PARA EL REGISTRO DE INVERSIONES EXTRANJERAS ANTE LAS AUTORIDADES COLOMBIANAS. B. TRANSIGIR, DESISTIR Y APELAR A DECISIONES DE ÁRBITROS O DE AMIGABLES COMPONEDORES EN LAS CUESTIONES EN QUE TENGA INTERÉS FRENTE A TERCEROS, A LOS ASOCIADOS MISMOS O A SUS ADMINISTRADORES. C. ADELANTAR CUALQUIER TIPO DE TRÁMITE ANTE EL INSTITUTO DE VIGILANCIA DE MEDICAMENTOS Y ALIMENTOS INVIMA O A LA ENTIDAD QUE HAGA SUS VECES. D. RECIBIR NOTIFICACIÓN DE TODAS LAS PROVIDENCIAS QUE SE EXPIDAN DENTRO DE LOS DIFERENTES TRÁMITES E INTERPONER LOS RECURSOS PROPIOS DE LA VÍA GUBERNATIVA. E. REPRESENTAR Y VIGILAR ANTE LOS FUNCIONARIOS, OFICINAS Y DEPENDENCIAS DEL INSTITUTO DE VIGILANCIA DE MEDICAMENTOS Y ALIMENTOS INVIMA O A LA ENTIDAD QUE HAGA SUS VECES, TODAS LAS SOLICITUDES QUE CONTENGAN TRÁMITES A NOMBRE DE LABORATORIOS SYNTHESIS S.A.S. F. SOLICITAR, OBTENER, REVISAR Y MODIFICAR REGISTROS SANITARIOS. G. OBTENER ANTE LAS OFICINAS Y AUTORIDADES NACIONALES QUE CORRESPONDAN Y EN ESPECIAL DE LA DIVISIÓN DE PROPIEDAD INDUSTRIAL DE LA SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO, REGISTROS DE MARCAS, RENOVACIÓN DE REGISTROS MARCARIOS, ANOTACIÓN DE TRASPASOS Y CAMBIO DE NOMBRE, PRESENTACIÓN Y TRÁMITE DE OPOSICIONES, PRESENTACIÓN Y TRÁMITE DE RECURSOS POR LA VÍA GUBERNATIVA Y DEMÁS DILIGENCIAS RELACIONADAS, A CUYO EFECTO LA FACULTA PARA DAR ANTE DICHAS AUTORIDADES, PARA ELEVAR SOLICITUDES, FORMULAR DESCRIPCIONES, APELACIONES Y RECLAMOS, SOLICITAR TESTIMONIO, RECIBIR, DESISTIR, SUSTITUIR, CANCELAR, TRANSIGIR, RENUNCIAR, ACEPTAR NOTIFICACIONES Y NOMBRAR APODERADOS JUDICIALES Y EXTRAJUDICIALES. H. ADQUIRIR MARCAS, PATENTES, PROCEDIMIENTOS DE FABRICACIÓN, EXPLOTADAS EN CUALQUIER FORMA, YA SEA UTILIZÁNDOLOS DIRECTAMENTE O PERMITIENDO SU EXPLOTACIÓN POR OTRAS PERSONAS NATURALES O JURÍDICAS CONTRA EL PAGO DE REGALÍAS O PARTICIPACIONES. I. CELEBRAR Y EJECUTAR EN GENERAL TODOS LOS ACTOS Y CONTRATOS PREPARATORIOS, COMPLEMENTARIOS O ACCESORIOS DE TODOS LOS ANTERIORES, LOS QUE SE RELACIONEN CON LA EXISTENCIA Y FUNCIONAMIENTO DE LA SOCIEDAD, Y LOS QUE SEAN CONDUCTENTES AL BUEN LOGRO DE LOS FINES SOCIALES. J. RECIBIR NOTIFICACIONES DE CUALESQUIERA TIPO DE ACTOS EMANADOS DE LAS AUTORIDADES ADMINISTRATIVAS O JUDICIALES. K. PRESENTAR RECURSOS POR LA VÍA ADMINISTRATIVA O JUDICIAL, RENUNCIAR A TÉRMINOS, DESISTIR, CONCILIAR, TRANSIGIR Y MODIFICAR. I. ELEVAR PETICIONES Y SOLICITAR INFORMACIÓN A LAS AUTORIDADES ADMINISTRATIVAS Y JURISDICCIONALES. M. PRESENTAR DEMANDAS ANTE LAS AUTORIDADES ADMINISTRATIVAS Y JUDICIALES.

N. SUSTITUIR EL PRESENTE PODER Y REVOCAR TAL SUSTITUCIÓN DENTRO DE LA REPÚBLICA DE COLOMBIA.

CERTIFICA:

** REVISOR FISCAL **

QUE POR DOCUMENTO PRIVADO DE REVISOR FISCAL DEL 28 DE JUNIO DE 2010, INSCRITA EL 8 DE JULIO DE 2010 BAJO EL NUMERO 01397034 DEL LIBRO IX, FUE (RON) NOMBRADO (S):

NOMBRE	IDENTIFICACION
REVISOR FISCAL PRINCIPAL	
AGUILLON ROJAS SANDRA MILENA	C.C. 000000047436442

REVISOR FISCAL SUPLENTE	
PRADA SANCHEZ NUBIA MARCELA	C.C. 000001032358923

QUE POR ACTA NO. 119 DE ASAMBLEA DE ACCIONISTAS DEL 22 DE JUNIO DE 2010, INSCRITA EL 8 DE JULIO DE 2010 BAJO EL NUMERO 01397026 DEL LIBRO IX, FUE (RON) NOMBRADO (S):

NOMBRE	IDENTIFICACION
REVISOR FISCAL PERSONA JURIDICA	
DELOITTE & TOUCHE LTDA	N.I.T. 000008600058134

CERTIFICA:

PERMISO DE FUNCIONAMIENTO: QUE POR RESOLUCION NO.05952 DEL 11 DE JUNIO DE 1.985, INSCRITA EN ESTA CAMARA DE COMERCIO EL 8 DE JULIO DE 1.985 BAJO EL NO. 172.879 DEL LIBRO IX, LA SUPERINTENDENCIA DE SOCIEDADES CONCEDIO PERMISO DEFINITIVO DE FUNCIONAMIENTO A LA SOCIEDAD.

CERTIFICA:

QUE POR RESOLUCION NO.09006 DEL 19 DE DICIEMBRE DE 1.986 SUPERINTENDENCIA DE SOCIEDADES INSCRITA EL 16 DE ENERO DE 1.987 BAJO EL NO.204.133 DEL LIBRO IX, SE AUTORIZO A LA SOCIEDAD PARA COLOCAR SETENTA Y CUATRO MIL (\$74.000) ACCIONES DE LAS QUE TIENE EN RESERVA, A SU VALOR NOMINAL DE CIENTOS PESOS (\$100.00) M/CTE., CADA UNA.

CERTIFICA:

QUE LA SOCIEDAD TIENE MATRICULADOS LOS SIGUIENTES ESTABLECIMIENTOS:

NOMBRE : CARDIOPHARM

MATRICULA NO : 00537358 DE 3 DE MARZO DE 1993

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : GENERICS

MATRICULA NO : 00664623 DE 18 DE SEPTIEMBRE DE 1995

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : MASCOTT

MATRICULA NO : 00664622 DE 18 DE SEPTIEMBRE DE 1995

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : ONCOPHARM

MATRICULA NO : 00625764 DE 13 DE DICIEMBRE DE 1994

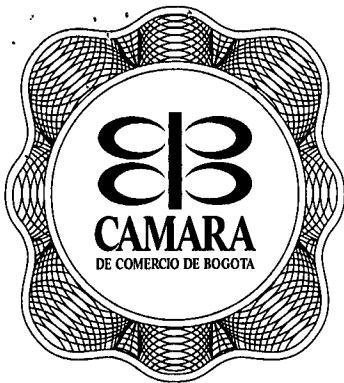
RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : GASTROPHARM

MATRICULA NO : 00621442 DE 2 DE NOVIEMBRE DE 1994

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010



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ULTIMO AÑO RENOVADO : 2010

NOMBRE : GYNECOPHARM

MATRICULA NO : 00621245 DE 1 DE NOVIEMBRE DE 1994

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : ENTEROPHARM

MATRICULA NO : 00621244 DE 1 DE NOVIEMBRE DE 1994

RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

ULTIMO AÑO RENOVADO : 2010

NOMBRE : EQUIPHARMA

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RENOVACION DE LA MATRICULA : EL 29 DE MARZO DE 2010

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CERTIFICA:

DE CONFORMIDAD CON LO ESTABLECIDO POR LA LEY 962 DE 2005, LOS ACTOS DE REGISTRO AQUI CERTIFICADOS QUEDAN EN FIRME CINCO (5) DIAS HABILES DESPUES DE LA FECHA DE INSCRIPCION, SIEMPRE QUE NO SEAN OBJETO DE RECURSOS EN LA VIA GUBERNATIVA.

* * * EL PRESENTE CERTIFICADO NO CONSTITUYE PERMISO DE
* * * FUNCIONAMIENTO EN NINGUN CASO

* * *
* * *

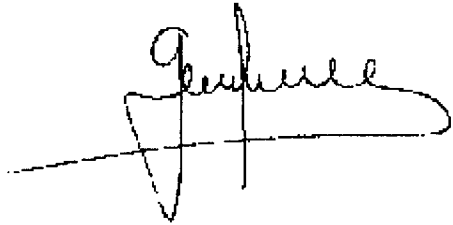
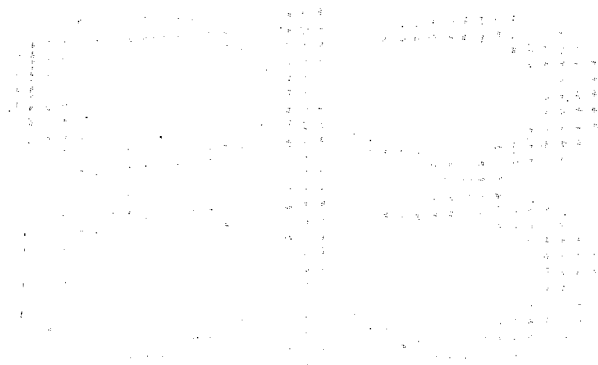
SEÑOR EMPRESARIO, SI SU EMPRESA TIENE ACTIVOS INFERIORES A 30.000 SMLMV Y UNA PLANTA DE PERSONAL DE MENOS DE 200 TRABAJADORES, USTED TIENE DERECHO A RECIBIR UN DESCUENTO EN EL PAGO DE LOS PARAFISCALES DE 75% EN EL PRIMER AÑO DE CONSTITUCION DE SU EMPRESA, DE 50% EN EL SEGUNDO AÑO Y DE 25% EN EL TERCER AÑO. LEY 590 DE 2000 Y DECRETO 525 DE 2009.

RECUERDE INGRESAR A www.supersociedades.gov.co PARA VERIFICAR SI SU EMPRESA ESTA OBLIGADA A REMITIR ESTADOS FINANCIEROS. EVITE SANCIONES.

EL SECRETARIO DE LA CAMARA DE COMERCIO,

**** CERTIFICADO SIN COSTO PARA AFILIADO ****

DE CONFORMIDAD CON EL DECRETO 2150 DE 1995 Y LA AUTORIZACION IMPARTIDA
POR LA SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO, MEDIANTE EL OFICIO
DEL 18 DE NOVIEMBRE DE 1996, LA FIRMA MECANICA QUE APARECE A
CONTINUACION TIENE PLENA VALIDEZ PARA TODOS LOS EFECTOS LEGALES

A handwritten signature in dark ink, appearing to be 'Garcia', with a long horizontal stroke extending to the left.

ANEXO

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

(54) Title: A PROCESS FOR THE RECOVERY OF SUBSTANTIALLY PURE TRICYCLIC MACROLIDE

(57) Abstract: Process for the recovery of a macrolide in substantially pure form comprising: a) treating the macrolide with wa-
ter immiscible solvent followed by concentration, b) mixing with water, water miscible solvent or mixture thereof, c) performing
hydrophobic interaction chromatography and collecting the fractions, d) extracting the fraction containing macrolide with water im-
miscible solvent followed by concentration, e) adding water miscible solvent to effect separation of impurities from the macrolide
compound, f) performing silica gel chromatography and collecting the fractions, g) isolating the macrolide compound in substan-
tially pure form. The macrolide is preferably rapamycin, tacrolimus or immunomycin.

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A PROCESS FOR THE RECOVERY OF SUBSTANTIALLY PURE TRICYCLIC MACROLIDE FIELD OF THE INVENTION

This invention relates to recovery of tricyclic macrolides.

10 BACKGROUND OF THE INVENTION

In 1975, Vezina et al., identified
(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-
9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-Hexadecahydro-
9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclo
15 hexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-
hexamethyl-23,27-epoxy-3H-pyrido[2,1-
c][1,4]oxaazacyclhentriacontine-1,5,11, 28,29(4H,6H,31H)-
pentone, also known by the synonyms rapamycin and sirolimus as
an antifungal antibiotic harvested from a *Streptomyces*
20 *hygroscopicus* culture, which was isolated from an Easter Island
soil sample. (J. Antibiot. 28, 721-726 (1975); and U.S. Pat. No.
3,929,992, was issued to Sehgal, et. al. Dec. 30, 1975). Martel, R.
et al. (1977) described the ability of this compound to inhibit the
immune response (Can. J. Physiol. Pharmacol., 55, 48-51). More
25 recently, Calne, R. Y. et al. (1989), has described rapamycin to be
immunosuppressive in rats given heterotopic heart allografts.
(Lancet vol. 2, p. 227). A compound, 15,19-Epoxy-3H-pyrido[2,1-
c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone,
5,6,8,11,12, 13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-
30 5,19-dihydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-
methoxycyclohexyl]-1-methyl ethenyl]-14,16-dimethoxy-

5 4,10,12,18-tetramethyl-8-(2-propenyl)-,
(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19R,26aS), also known by
the synonyms FK506 and tacrolimus disclosed by EP 184162 and
US 4,894,366 is also useful as immunosuppressant. Another
compound, 15,19-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-
10 1,7,20,21 (4H,23H)-tetrone,8-ethyl-
5,6,8,11,12,13,14,15,16,17,18,19,24,25,26, 26a-hexadecahydro-
5,19-dihydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-
methoxycyclohexyl]-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-
tetramethyl-, (3S,4R,5S,8R,9E,12S,14S, 15R,16S,18R,19R,26aS)-,
15 also known by the synonyms immunomycin and FK 520, disclosed
in EPO Publication No. 0184162 is also useful as an
immunosuppressant. Many other derivatives of these compounds
as well as structural analogues have immunosuppressant property.

US 5,508,398 discloses a process for separating a neutral
20 non-polypeptide macrolide from acidic, basic and non-polar neutral
impurities present in a concentrate of fermentation broth extracts
or mother liquors containing said neutral macrolide which
comprises in any order, extraction step (a) and optionally one or
both of steps (b) and (c), wherein (a) involves extraction with
25 aqueous base, (b) involves extraction with aqueous acid and (c)
involves treatment with non-aromatic hydrocarbon solvent.

US 5,616,595 discloses a process for recovering water
insoluble compounds (including FK506, FK520 and rapamycin) from
a fermentation broth which includes sequential steps of
30 concentrating, solubilizing and diafiltering the compound of

5 interest, all through a single closed recirculation system to recover the compound for further downstream purification.

The prior art methods for the recovery of macrolide compounds are tedious, does not result in pure product or require special setup for filtration.

10 The instant invention relates to a novel process for recovery of macrolide compounds.

SUMMARY OF THE INVENTION

The instant invention relates to a novel process for recovery of macrolide compounds.

15 The novel process of the instant invention comprises:

- a) treatment of an impure macrolide with water immiscible solvent and concentration and/or mixing, the impure macrolide or concentrate, with water, water miscible solvent or mixture thereof,
- 20 b) performing hydrophobic interaction chromatography,
- c) extraction into water immiscible solvent and concentration,
- d) addition of a water miscible solvent to effect separation of impurities present with the macrolide compound
- e) performing silica gel chromatography
- 25 f) isolating the macrolide compound in substantially pure form.

The product thus obtained is of pharmaceutically acceptable quality.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned earlier, The instant invention relates to a novel process for the production and recovery of macrolide compounds.

30 The novel process of the instant invention comprises:

- 5 a) treatment of a macrolide with water immiscible solvent and concentration and/or mixing, the impure macrolide or concentrate with water, water miscible solvent or mixture thereof,
- b) performing hydrophobic interaction chromatography,
- 10 c) extraction into water immiscible solvent and concentration,
- d) addition of a water miscible solvent to effect separation of impurities present with the macrolide compound,
- e) performing silica gel chromatography
- f) isolating the macrolide compound in substantially pure form.

15 The macrolide of the present invention can be produced by fermentation, chemical synthesis or combination of both. The broth obtained by fermentation can be directly extracted by water immiscible solvent. Any crude material in solid, semisolid or liquid form obtained either from broth, synthetic process or combination

20 thereof can be treated with water immiscible solvent to effect solubilization of the macrolide into the water immiscible solvent. The water immiscible solvent containing the macrolide can be concentrated. The concentration can be affected by methods known per se. The concentration can be affected by vaporization of

25 the solvent. The vaporization of the solvent can be carried out by heating without or with reduced pressure. The concentrate thus obtained can be diluted with water, water miscible solvent or mixture thereof. The water miscible solvent can be selected from alcohol, ketone or dielectric aprotic solvent. Preferably, the water

30 miscible solvent can be selected from one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile. The

5 mixture can be then subjected to hydrophobic interaction chromatography. The hydrophobic interaction chromatography can be carried out on matrix selected from a group comprising polystyrene divinyl benzene, methacrylates, polystyrene, resin linked or coated with hydrocarbons or any other suitable

10 hydrophobic matrix. The fraction containing the macrolide can be collected and extracted with water immiscible solvent. The water immiscible solvent can be selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters. Preferably, the water immiscible solvents can be selected from

15 benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate, n-butyl acetate or t-butyl acetate. The solution can be subjected to charcoalization. The solution can be concentrated by method discussed earlier. The concentrate can be treated with a water miscible solvent to effect separation of

20 impurities present with the macrolide. The impurities may be present in form of solids or liquid, immiscible with the solvent or both. The impurities can be separated out by filtration, phase separation or both. Preferably, the water miscible solvent can be selected from acetone, methanol, or acetonitrile. The filtrate or

25 product containing phase can be concentrated and the concentrate can be subjected to silica gel chromatography. The elute from the chromatography can be mixed with water immiscible solvent. The water immiscible solvent can be selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters. Preferably

30 the solvent can be selected from hydrocarbon, ether or ester. The solvent used can be a mixture of two or more solvents. Pure

5 macrolide can be isolated from the water immiscible solvent or mixture thereof.

In particular, the novel process of the instant invention comprises:

- 10 a) treatment of an impure macrolide in broth with water immiscible solvent, preferably toluene or ethyl acetate and concentration,
- b) mixing with water, water miscible solvent or mixture thereof, preferably a mixture of acetone and water,
- c) performing hydrophobic interaction chromatography,
- 15 d) extraction into water immiscible solvent, preferably ethyl acetate and concentration,
- e) addition of a water miscible solvent, preferably acetonitrile, to effect separation of impurities from the macrolide compound,
- f) performing silica gel chromatography,
- 20 g) crystallization from one or more solvents selected from ether, ethyl acetate and hexane to afford the macrolide compound in substantially pure form.

In particular, the novel process of the instant invention also comprises:

- 25 a) mixing the impure macrolide with water, water miscible solvent or mixture thereof, preferably a mixture of acetone and water,
- b) performing hydrophobic interaction chromatography,
- c) extraction into water immiscible solvent and concentration,
- 30 d) addition of a water miscible solvent to effect separation of impurities from the macrolide compound,

- 5 e) performing silica gel chromatography,
f) crystallization from one or more solvents selected from ether, ethyl acetate and hexane to afford the macrolide compound in substantially pure form.

The following examples further illustrate the invention, it being
10 understood that the invention is not intended to be limited by the details disclosed therein.

EXAMPLES

Example 1: RECOVERY OF RAPAMYCIN

The fermentation broth (31Kg) containing rapamycin was
15 twice extracted with 31 L of toluene. The toluene extract was concentrated to obtain 52 g of residue. The residue was dissolved in 5 L of acetone. 15 L of water was added to this. The solution was passed through a 1.2L column packed with Sepabeads SP 825 resin. The column was washed with 3-column volume of water and
20 3-column volume of 25% acetone in water. The product was eluted with 75% acetone in water. The product containing fractions were pooled and diluted with water to make 50% acetone in the solution. The diluted solution was extracted with ethyl acetate. 4 g of activated charcoal was added to the ethyl acetate extract and
25 mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 15 g of oily residue. 100 ml of acetonitrile was added to the residue. The precipitate obtained after acetonitrile addition was filtered. The filtrate was concentrated to obtain 14 g of oily residue. The residue was
30 applied to a silica gel column. The column was washed with 2 column volumes of 15% acetone in hexane and 2 column volumes

5 of 30% acetone in hexane. The product was eluted with 40% acetone in hexane. The product containing fractions were pooled and concentrated to obtain 5.5 g oily residue. To the residue 10 ml of diethyl ether was added and the product was crystallized at 4 °C. The product was filtered and dried. The product yield was 54%.
10 The chromatographic purity was 99.8%

EXAMPLE 2: RECOVERY OF TACROLIMUS

The fermentation broth (5Kg) containing tacrolimus was twice extracted with 5 L of ethyl acetate. The ethyl acetate extract was concentrated to obtain 22 g of residue. The residue was
15 dissolved in 1 L of acetone. 4 L of water was added to this. The solution was passed through a 0.6L column packed with Sepabeads SP 825 resin. The column was washed with 3-column volumes of water and 3-column volumes of 25% acetone in water. The product was eluted with 75% acetone in water. The product
20 containing fractions were pooled and concentrated. The concentrated solution was extracted with ethyl acetate. 1.5 g of activated charcoal was added to the ethyl acetate extract and mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 5.8 g of oily residue. The
25 residue was twice extracted with acetonitrile. The acetonitrile extract was concentrated to obtain 3.9 g of oily residue. The residue was applied to a silica gel column. The column was washed with 3 column volumes of 25% ethyl acetate in hexane and 3 column volumes of 50% ethyl acetate in hexane. The product was
30 eluted with 75% ethyl acetate in hexane. The product containing fractions were pooled and concentrated to obtain 1 g oily residue.

- 5 To the residue 2.5 ml of ethyl acetate was added. The product was crystallized at 4 °C by slow addition of hexane. The product was filtered and dried. The product yield was 60%. The chromatographic purity was greater than 99.5%

EXAMPLE 3: RECOVERY OF IMMUNOMYCIN

- 10 The fermentation broth (16Kg) containing immunomycin was extracted with 16 L of ethyl acetate. The ethyl acetate extract was concentrated to obtain 36 g of residue. The residue was dissolved in 2 L of acetone. 8 L of water was added to this. The solution was passed through a 1.2L column packed with Sepabeads SP 825
- 15 resin. The column was washed with 3-column volumes of water and 3-column volumes of 25% acetone in water. The product was eluted with 75% acetone in water. The product containing fractions were pooled and concentrated. The concentrated solution was extracted with ethyl acetate. 15 g of activated charcoal was added
- 20 to the ethyl acetate extract and mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 62 g of oily residue. The residue was twice extracted with acetonitrile. The acetonitrile extract was concentrated to obtain 43 g of oily residue. The residue was applied to a silica gel column. The column
- 25 was washed with 3 column volumes of 25% ethyl acetate in hexane and 3 column volumes of 50% ethyl acetate in hexane. The product was eluted with 75% ethyl acetate in hexane. The product containing fractions were pooled and concentrated to obtain 12 g oily residue. To the residue 12.5 ml of ether was added. The
- 30 product was crystallized at 4 °C by slow addition of hexane. The

5 product was filtered and dried. The product yield was 56%. The chromatographic purity was greater than 99.6%

5 We claim:

1. A novel process for the recovery of a macrolide in substantially pure form comprising:
 - a) treating the macrolide with water immiscible solvent followed by concentration,
 - 10 b) mixing with water, water miscible solvent or mixture thereof,
 - c) performing hydrophobic interaction chromatography and collecting the fractions,
 - d) extracting the fraction containing macrolide with water immiscible solvent followed by concentration,
 - 15 e) adding water miscible solvent to effect separation of impurities from the macrolide compound,
 - f) performing silica gel chromatography and collecting the fractions,
 - 20 g) isolating the macrolide compound in substantially pure form.
2. A process as in claim 1, wherein the macrolide is selected from rapamycin, tacrolimus or immunomycin.
3. A process as in claim 1, wherein the water immiscible solvent
25 is selected from a group comprising hydrocarbons, heterocyclic compounds, ethers, esters or a mixture of two or more of these.
4. A process as in claim 3, wherein the water immiscible solvents is selected from benzene, toluene, hexane; butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate,
30

- 5 n-butyl acetate, t-butyl acetate or a mixture of two or more of these.
5. A process as in claim 1, wherein the water miscible solvent is selected from one or more among alcohol, ketone or dielectric aprotic solvent.
- 10 6. A process as in claim 5, wherein the water miscible solvent is selected one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile.
7. A process as in claim 1, wherein the hydrophobic interaction chromatography is performed using matrix selected from
- 15 polystyrene divinyl benzene, methacrylates, polystyrene or matrix linked or coated with hydrophobic groups.
8. A process as in claim 1 step (e), wherein the solvent is selected from acetone, methanol or acetonitrile.
9. A process as in claim 1, wherein the macrolide compound is
- 20 isolated by crystallization.
10. A process as in claim 9, wherein the crystallization is carried out in one or more solvents selected from ethyl acetate, diethyl ether or hexane.
11. A process as in claim 1, wherein the macrolide is obtained by
- 25 fermentation.
12. A process as in claim 1, wherein the macrolide is obtained by synthetic process.
13. A novel process for the recovery of the macrolide in substantially pure form as in claim 1 from crude macrolide
- 30 comprising:

- 5 a) mixing with water, water miscible solvent or mixture thereof,
- b) performing hydrophobic interaction chromatography and collecting the fractions,
- c) extracting the fraction containing macrolide with water
- 10 immiscible solvent followed by concentration,
- d) adding water miscible solvent to effect separation of impurities from the macrolide compound,
- e) performing silica gel chromatography and collecting the fractions,
- 15 f) isolating the macrolide compound in substantially pure form.
14. A process as in claim 13, wherein the macrolide is selected from rapamycin, tacrolimus or immunomycin.
15. A process as in claim 13, wherein the water immiscible
- 20 solvent is selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters.
16. A process as in claim 15, wherein the water immiscible solvents is selected from benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate
- 25 or butyl acetate.
17. A process as in claim 13, wherein the water miscible solvent is selected from one or more among alcohol, ketone or dielectric aprotic solvent.
18. A process as in claim 17, wherein the water miscible solvent
- 30 is selected one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile.

- 5 19. A process as in claim 13, wherein the hydrophobic interaction chromatography is performed using matrix selected from polystyrene divinyl benzene, methacrylates, polystyrene or matrix linked or coated with hydrophobic groups.
- 20 A process as in claim 13 step (d), wherein the solvent is
10 selected from acetone, methanol or acetonitrile.
21. A process as in claim 13, wherein the macrolide compound is isolated by crystallization.
22. A process as in claim 21, wherein the crystallization is carried out in one or more solvents selected from ethyl acetate,
15 diethyl ether or hexane.
23. A process as in claim 13, wherein the macrolide is obtained by fermentation.
24. A process as in claim 13, wherein the macrolide is obtained by synthetic process.
- 20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2003/000283

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: C07D 498/18

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)

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)

STN: File Medline, CA, WPIDS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US A 5508398 (GLETSOS) 16 April 1996 Whole document	1-24
A	US A 5616595 (CHU et al) 1 April 1997 Whole document	1-24
	US 4734492 (TANBA et al) 29 March 1988 Whole document	1-24



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 but 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
1 December 2003

Date of mailing of the international search report

22 JAN 2004

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2003/000283

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Sanglier JJ et al, "Sanglifehrins A, B, C and D, novel cyclophilin-binding compounds isolated from streptomyces sp. A92-308110. I. Taxonomy, fermentation, isolation and biological activity" J Antibiot (Tokyo). 1999 May;52(5):466-73 Whole document	1-24

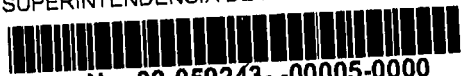
INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2003/000283

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
US	5508398	AU	60714/98	AU	77595/94
		BR	9404265	CA	2134844
		CZ	9402674	EP	0652219
		HU	71122	IL	111424
		JP	7184674	NZ	264852
		SK	130494	ZA	9408641
US	5616595	CA	2222810	EP	0832108
US	4734492	FR	2585022	JP	62019599
					END OF ANNEX



No. 09-059243 - 00005-0000

Fecha: 2010-12-17 17:42:13 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 400 OPOSIOBSERVTER Folios: 113

**FORMULARIO UNICO DE OPOSICION
2000-12**

1

OPOSICION A:

- | | |
|---|---|
| ☒ Patente de Invención | ☐ Marca Colectiva |
| ☐ Patente de Modelo de Utilidad | ☐ Marca de Certificación |
| ☐ Diseño Industrial | ☐ Lema Comercial |
| ☐ Esquema de Trazado para Circuitos Integrados | |
| ☐ Marcas de Productos o Servicios | |

2

**SOLICITUD
PUBLICADA**

Número del expediente: 09 059.243

Solicitante: BIOCON LIMITED.

Apoderado: EMILIO FERRERO.

Título de la nueva creación o denominación del signo:

**UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU
RECUPERACIÓN Y PURIFICACIÓN.**

Gaceta 620 de 20/09/2010, N° de publicación 647.

3

OPOSICION PRESENTADA POR:

OPOSITOR

Nombre: Lafrancol S.A. ✓

Dirección: Carrera 1 No. 46-84

Teléfono: (092) 449.30.51

Fax:

Domicilio: Cali - Valle ✓

IDENTIFICACION

C.C. ☐ NIT ☒

C.E. ☐ Otro ☐

Número: 890 301 463 -8

**REPRESENTANTE
O APODERADO
(74)**

Nombre: José Luis Reyes Villamizar

Dirección: Cra. 17 No. 88-23, Of. 205

Teléfono: 621.26.31

E-mail: info@reyes-abogados.com

Fax: 621.25.42

Domicilio: Bogotá, D.C.

IDENTIFICACION

C.C. ☒ NIT ☐

C.E. ☐ Otro ☐

Número: 79'152.473 de Usaquén
TP 44655

4

FUNDAMENTOS DE LA OPOSICION:

Causal: Incumplimiento de requisitos de patentabilidad (Título II, Capítulo I, Decisión 486 de 2000). Se violan los artículos 14, (las patentes de invención deben ser nuevas, tener nivel inventivo y ser susceptibles de aplicación industrial), 16 y 18 (una invención tiene nivel inventivo si no es obvia para una persona versada en la materia).

Justificación: VER ANEXO

5

Comprobante de pago No. 10 107 863

(Oposición) Fecha

17 / 12 / 2010

(Prórroga) Fecha

6

Anexos:

Comprobante de pago de la tasa



Poderes, si fuere el caso



Pruebas de los fundamentos de la oposición



Certificado de existencia y representación legal de las partes, cuando sean personas jurídicas



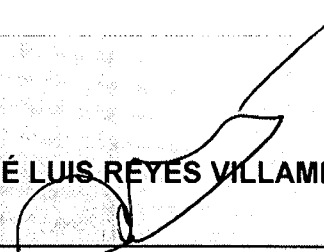
Documentos que acrediten el legítimo interés, si fuere el caso



Copia de la oposición para el traslado al solicitante.

7

NOMBRE: JOSÉ LUIS REYES VILLAMIZAR

FIRMA: 

C.C.No. 79 152.473 T.P. No. 44655

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E. S. D.

REF: Expediente **09 059.243**

TÍTULO SOLICITADO: UNA FORMA PURA DE RAPAMICINA Y UN PROCESO PARA SU RECUPERACIÓN Y PURIFICACIÓN.

PUBLICACIÓN: Gaceta 619 de 20/09/2010, N° de publicación 647.

PRIORIDAD: IN 2079/CHE/2006 de 10/11/2006.

SOLICITANTE: BIOCON LIMITED.

APODERADO: EMILIO FERRERO.

TRÁMITE: PRESENTACIÓN DE OPOSICION

JOSÉ LUIS REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **LAFRANCOL S.A.**, sociedad constituida y vigente de conformidad con las leyes colombianas, con domicilio en Cali, Valle, en ejercicio de lo dispuesto por el artículo 42 de la Decisión 486 de 2000, muy respetuosamente me permito formular la siguiente

I. PETICIÓN

Negar el beneficio patentario a la solicitud contenida en el expediente **09 059.243**, por no cumplir con los requisitos de patentabilidad a que se refieren los artículos 14, 16 y 18 de la Decisión 486 de la Comunidad Andina de Naciones, con base en los fundamentos de hecho y de derecho que me permito exponer a continuación.

II. LEGÍTIMO INTERÉS

Asiste a **LAFRANCOL S.A.**, legítimo interés para presentar esta oposición, pues la eventual concesión de la solicitud restringiría, en forma contraria al Régimen Común Andino de Propiedad Industrial, el empleo de ciertas sustancias y composiciones farmacéuticas, en detrimento de sus legítimos intereses comerciales y de la libre competencia.

III. FUNDAMENTO DE LA OPOSICIÓN

La solicitud colombiana en trámite **09 059.243**, que se refiere a una forma pura de rapamicina con un contenido de impureza total de menos de 1,2% y un proceso para la recuperación y purificación de dicha rapamicina; incumple con los requisitos fundamentales para poder otorgársele el privilegio de patente de invención, tal y como se demuestra a continuación:

En primer lugar, y dado el hecho que la solicitud en cuestión menciona la formación de cristales de rapamicina, específicamente en la reivindicación 5, es relevante señalar que cualquier persona medianamente versada en la materia debe reconocer que los sólidos pueden existir, de acuerdo con su estructura u ordenamiento interno, en formas cristalinas o amorfas. En las formas amorfas las partículas componentes no presentan una disposición interna ordenada, sino que se encuentran distribuidas al azar. Por el contrario, en las formas cristalinas se presenta un arreglo interno ordenado y regular de las moléculas, que implica la apreciación de formas geométricas definidas. Un polimorfo es una fase -o arreglo- cristalino sólido de un compuesto conocido, dado por el sometimiento de la molécula a diversas condiciones de ambiente, temperatura, tiempo o acción de solventes, otras como presión, velocidad de cristalización, concentración de saturación, e inclusive, la influencia de sustancias extrañas y campos de fuerza exteriores al cristal¹. A esta característica de algunas sustancias, que pueden organizar de manera diversa sus moléculas en estado sólido, se le reconoce como polimorfismo. El polimorfismo, en los términos de la Farmacia Práctica de Remington, es "la capacidad de cualquier elemento o compuesto de cristalizar en más de un sistema cristalino"; así mismo se reconoce que el descubrimiento del polimorfismo como propiedad de las entidades químicas se atribuye al

¹ J. Garrido. Forma y estructura de los Cristales, (obra citada), capítulo V, página 204

farmacéutico Martín Enrique Klaproth en 1798, y la tesis según la cual los mismos elementos químicos pueden adoptar formas sólidas diversas – polimorfos - y mostrar propiedades físicas diferentes, se atribuye a Louis Jacques Thénard (1777-1857) y Jean Baptiste Biot (1774-1862)².

También se reconoce que el polimorfismo es una característica para tener en cuenta en el desarrollo de una formulación farmacéutica (en especial para los principios activos), puesto que los diferentes polimorfos de una sustancia pueden llegar a tener diferente densidad, capacidad para fluir, compresibilidad y solubilidad, entre otras características fisicoquímicas, con lo cual pueden traer variaciones considerables en la parte tecnológica y biofarmacéutica al momento de usar uno u otro polimorfo de la misma sustancia en el proceso de elaboración del medicamento. Por esta razón es una característica que se evalúa dentro de los ensayos de preformulación y formulación, especialmente dentro de lo que se conoce como las consideraciones fisicoquímicas de la etapa de preformulación³.

Teniendo en cuenta lo anterior se puede afirmar lo siguiente:

- ❖ Una vez conocida la posibilidad del hábito cristalino de una sustancia, es tarea fácil reportar, mediante la aplicación de métodos analíticos comunes (tales como espectroscopia de difracción de rayos X o la microscopia de platina caliente que son los que se utilizan en la solicitud colombiana en trámite), un gran número de variaciones morfológicas de dicho hábito, sin que su caracterización implique modificaciones en la disposición geométrica esencial del cristal, ni alteración de las propiedades químicas del mismo.
- ❖ Las alteraciones en el hábito cristalino que no suponen variaciones en la red cristalina interna de la molécula no reportan avance técnico alguno, en la medida en que simplemente muestran una estructura u organización diferente.
- ❖ El polimorfismo es una propiedad inherente a la materia en su estado sólido y su reporte es el simple hallazgo de algo que ya se sabía podía ser hallado y no la invención de algo nuevo.

² A. R. Verna, P. Krishna – “Polytypism in crystals”. John Wiley and Son Inc., New York 1966.

³ Vila Jato J.L. Tecnología Farmacéutica. Volumen I. Páginas 42 a 49.

En el mismo sentido, la reivindicación de los isómeros A, B y C que específicamente se hace en las reivindicaciones 29 y 30 de la solicitud colombiana en trámite, carece de nivel inventivo.

Cualquier persona medianamente versada en la materia debe reconocer que la isomería es una propiedad de ciertos compuestos químicos que con igual fórmula química, es decir, iguales proporciones relativas de los átomos que conforman su molécula, presentan estructuras moleculares diferentes y que dichos compuestos reciben la denominación de isómeros. Dichos isómeros son predecibles de manera teórica, con el conocimiento previo de la fórmula química del compuesto. Esto aplica tanto a la isomería estructural (cuando moléculas con la misma fórmula molecular, tienen un arreglo diferente en enlaces entre átomos) como en la estereoisomería (compuestos que tienen fórmulas moleculares idénticas y sus átomos presentan la misma distribución pero su disposición en el espacio es distinta).

Dicha persona medianamente versada en la materia también debe reconocer que la isomerización es el proceso por el cual una molécula es transformada en otra que tiene exactamente el mismo número de átomos pero dichos átomos están reorganizados⁴, y que dichas isomerizaciones suelen darse de manera espontánea bajo las condiciones apropiadas, especialmente cuando los isómeros de dicho compuesto tienen una igual o muy semejante energía de enlace, lo que les permite interconvertirse de manera relativamente espontánea⁵. Por esta razón poseer isómeros es una propiedad inherente a los compuestos químicos y su reporte es el simple hallazgo de algo que ya se sabía podía ser hallado y no la invención de algo nuevo.

En otro aspecto, y esta vez refiriéndonos a las técnicas a través de las cuales se lleva a cabo la obtención y purificación de la rapamicina en la solicitud colombiana en cuestión, específicamente refiriéndonos a la cromatografía líquida de alto rendimiento (HPLC)⁶, cromatografía en gel de sílice y cromatografía de

⁴ <http://goldbook.iupac.org/I03295.html>

⁵ <http://en.wikipedia.org/wiki/Isomer>

⁶ Csaba Horváth's instrument was without question the first modern, high-pressure liquid chromatograph and as such, it essentially represented the start of the present-day instruments one can find today worldwide in almost every laboratory. However, by the middle of the 1960s.

<http://chromatographyonline.findanalytichem.com/lcgc/article/articleDetail.jsp?id=172945>

interacción hidrófoba y/o de fase inversa⁷, son todas técnicas utilizadas rutinariamente como herramientas en diversos tipos de procesos de separación y purificación de diversos compuestos. La selección de la fase móvil (sistema de elución o mezclas de solventes) así como de las fases estacionarias, dependen de las características de la sustancia a separar y del medio en que se encuentra mezclada, y cualquier persona medianamente versada en la materia está en la capacidad de hacer este tipo de elecciones.

En otras palabras, no se puede atribuir nivel inventivo al hecho de aplicar técnicas cromatográficas específicamente diseñadas para la separación y purificación de compuestos, ni tampoco en la realización de los ajustes básicos que son inherentes a dichas técnicas y que se deben aplicar a cualquier proceso de separación/purificación.

Para finalizar podemos hacer evidente que con anterioridad se habían aplicado procesos de purificación con las mismas características descritas en la solicitud colombiana en trámite para rapamicina y/o compuestos estrechamente relacionados, veamos:

En primer lugar es importante indicar que existen documentos como la patente americana US 3993749 publicada el 23 de Noviembre de 1976 y la patente europea EP 0652219 publicada el 20 de junio de 2001, publicadas con anterioridad a la prioridad reivindicada por la solicitud en cuestión, que revelan la obtención de Rapamicina a partir del cultivo de microorganismos (*Streptomyces hygroscopicus*).

En relación con lo anterior, la publicación WO/2004/089958 publicada el 21 de Octubre de 2004 revela métodos de cristalización de macrólidos estrechamente relacionados a rapamicina, en dónde dicho proceso se puede hacer a partir de un tratamiento del caldo de cultivo de fermentación (reivindicación 6) y en donde se hacen mención de diversos solventes con las mismas características que los utilizados en la solicitud colombiana en trámite:

⁷ The name "reversed phase" has a historical background. In the 1970s most liquid chromatography was done on non-modified silica or alumina with a hydrophilic surface chemistry and a stronger affinity for compounds polar- hence it was considered "normal". The introduction of alkyl chains bonded covalently to the support surface reversed the elution order. http://en.wikipedia.org/wiki/Reversed-phase_chromatography

The present invention relates to a method for crystallization and purification of macrolides, especially tacrolimus, sirolimus, pimecrolimus, and everolimus, including the steps of: providing a combination of a macrolide starting material; a polar solvent, especially a polar solvent that is an alkyl ester of an alkanoic acid, an alcohol, an ether, an aliphatic ketone, an aliphatic nitrile, or a dipolar aprotic solvent; a hydrocarbon solvent, especially an acyclic or cyclic aliphatic hydrocarbon or an aromatic hydrocarbon (e.g. toluene); and water; at a pH of about 7 or above, especially about 8 or above; maintaining the combination at a temperature of between about -15°C to about 50°C, preferably between about -5°C to about 40°C, most preferably between about -2°C to about 35°C for at least about 1 hour, preferably between about 48 to about 100 hours; and isolating crystalline macrolide.

(...)

In another aspect, the present invention relates to a method for crystallization and purification of a macrolide, especially tacrolimus, sirolimus, pimecrolimus, or everolimus including the steps of: providing a concentrate residue from whole-broth extraction of macrolide-containing biomatter in a polar solvent, especially a polar solvent that is an alkyl ester of an alkanoic acid, an alcohol, an ether, an aliphatic ketone, an aliphatic nitrile, or a dipolar aprotic solvent; combining the solution, in any order, with water and a hydrocarbon solvent, especially an acyclic or cyclic aliphatic hydrocarbon or an aromatic hydrocarbon (e.g. toluene), wherein the pH is about 7 or above, especially about 8 or above; maintaining the combination at a crystallization temperature for a crystallization time; and isolating crystalline macrolide.

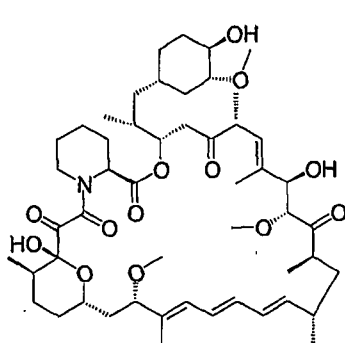
La publicación internacional WO/2006/093745 publicada el 08 de septiembre de 2006 y titulada "PURIFICATION OF RAPAMYCIN" revela:

This invention relates to a chemical process for the purification of rapamycin.

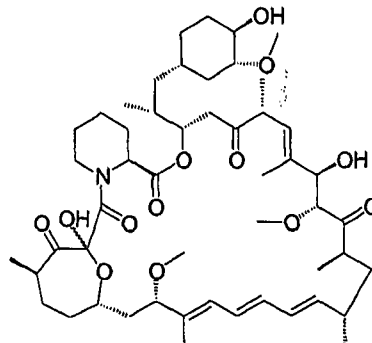
Rapamycin is a macrocyclic triene antibiotic produced naturally by *Streptomyces hygroscopicus*. It has been found useful in an array of applications based on its

(...)

Isomers of rapamycin are known which have structures below, referred to as Isomer B and Isomer C:



Isomer B



Isomer C

Conventional production of rapamycin is by way of fermentation. The

(...)

charcoal treatment methods. The product obtained by these methods has a purity of approximately 94% (based on the sum of the individual purities of isomers B and C), and

(...)

The invention provides a chemical process as an alternative method for the purification of rapamycin. This method is useful for crude rapamycin or second crop recovered from mother liquors during the isopropanol recrystallization. This material in general is dark brown in color with low purity and low B:C isomeric ratio. The purification process includes treatment of rapamycin with chlorotrimethylsilane to yield 31,42-bis-trimethylsilyl ether, heptane extraction, and de-protection of the 31,42-bis-trimethylsilyl ether to yield a purified rapamycin product.

In another aspect, a rapamycin generated is buffered in order to increase the ratio of B isomer to C isomer in the drug substance.

(...)

1. Rapamycin having a purity of greater than 98% and a yellow color index less than 1.

(...)

18. The process according to any one of claims 7 to 17, wherein the suitable base in step (a) is imidazole.

19. The process according to any one of claims 7 to 18, wherein the acid in step (e) is sulfuric acid.

Como se puede apreciar, en esta anterioridad se revelan los isómeros B y C de rapamicina, así como un proceso de cristalización que involucra lavados con base y ácido tal como en el proceso de la solicitud en cuestión.

La publicación WO/2005/019226 publicada el 03 de Marzo de 2005 y titulada "A PROCESS FOR THE RECOVERY OF SUBSTANTIALLY PURE TRICYCLIC MACROLIDE" revela:

The instant invention relates to a novel process for recovery of macrolide compounds.

The novel process of the instant invention comprises:

- a) treatment of an impure macrolide with water immiscible solvent and concentration and/or mixing, the impure macrolide or concentrate, with water, water miscible solvent or mixture thereof,
- b) performing hydrophobic interaction chromatography,
- c) extraction into water immiscible solvent and concentration,
- d) addition of a water miscible solvent to effect separation of impurities present with the macrolide compound
- e) performing silica gel chromatography
- f) isolating the macrolide compound in substantially pure form

The product thus obtained is of pharmaceutically acceptable quality.

(...)

°C. The product was filtered and dried. The product yield was 54%.
The chromatographic purity was 99.8%

(...)

The macrolide of the present invention can be produced by fermentation, chemical synthesis or combination of both. The broth
(...)

The water immiscible solvent containing the macrolide can be concentrated. The concentration can be affected by methods
(...)

chromatography. The hydrophobic interaction chromatography can be carried out on matrix selected from a group comprising polystyrene divinyl benzene, methacrylates, polystyrene, resin linked or coated with hydrocarbons or any other suitable hydrophobic matrix. The fraction containing the macrolide can be
(...)

immiscible solvent can be selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters. Preferably, the water immiscible solvents can be selected from benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate, n-butyl acetate or t-butyl acetate.

(...)

separation or both. Preferably, the water miscible solvent can be selected from acetone, methanol, or acetonitrile. The filtrate or

(...)

14. A process as in claim 13, wherein the macrolide is selected from rapamycin, tacrolimus or immunomycin.

(...)

Como se puede apreciar, en esta anterioridad se revelan absolutamente todas las características de un proceso de purificación de macrólidos, que incluye a rapamicina, que al igual que el proceso descrito por la solicitud en cuestión parte de un caldo de cultivo de fermentación, utiliza solventes inmiscibles en agua y solventes miscible en agua para efectuar la separación de impurezas presentes y así mismo cromatografía de fase inversa. Además se expresa literalmente que la

pureza alcanzada por dicho método es, inclusive, mayor que la referida en la solicitud colombiana (99.8% vs. 98.8%), con lo cual se hace mucho mas evidente que con anterioridad ya se había resuelto el mismo problema técnico a partir de las mismas estrategias tecnológicas.

La publicación internacional WO/1993/011130 publicada el 10 de Junio de 1993 y titulada "RAPAMYCIN DERIVATIVE AND ITS MEDICINAL USE" revela:

**Preparation of 14-methylene rapamycin
from a culture of Streptomyces by fermentation**

A culture producing 14-methylene rapamycin has been classified as *Streptomyces* sp. and has been deposited in the national Collection of Industrial and Marine Bacteria, 23, St. Machar Drive, Aberdeen AB2 1RY, Scotland, UK. under the accession number NCIB 40319.

(...)

Isolation Procedure

Solvent Extraction

After 158hr fermentation 1000 litres of whole broth were removed from the fermenter and adjusted to pH4 with sulphuric acid. 500 litres of MIBK was added and the mixture stirred for 2 hours.

The solvent phase was recovered using a Westfalia SA7-03-076 liquid/solid centrifugal separator (Westfalia Separator Ltd; Oelde Germany). After concentration in vacuo to 20L, the concentrate was stored at 5°C.

After 202hrs fermentation the remaining 2100 litres of whole broth was removed from the fermenter and adjusted to pH4 with sulphuric acid. 1050l of MIBK were added and the mixture stirred for 1 hour.

The solvent, phase was recovered as above and combined with the stored concentrate from above. The combined, rich MIBK extracts were concentrated to give a final volume of 68L.

Solvent Partition

125L of methanol and 125L of hexane were added to the concentrate and the mixture stirred for 1/2 hour. Solvent phases were allowed to separate by gravity overnight. The upper phase was then recovered (190L) and concentrated in vacuo to give 7kg of oil.

(...)

Silica chromatography

2.5L hexane was added to the stored acetone solution and the result loaded on a silica (Sorbisil C60 silica 40-60µm, Rhone-Poulenc) column 33 x 30cm packed in 85:15, hexane-acetone. Elution was with a step gradient of acetone in hexane. Fractions eluted with up to 70:30 hexane-acetone contained 14-methylene rapamycin, these were combined and concentrated in vacuo. 34g of oil was obtained.

Amberchrom CG71 chromatography

2L of Amberchrom CG71 50µm-100µm (Tosohaas, Stuttgart, Germany) were packed in a medium pressure liquid chromatograph (Jobin Yvon, France, 8cm diameter) and equilibrated in hexane. After dissolving 34g oil in toluene, elution continued at up to 8 bar pressure with 4L of 80:20 hexane-ethyl acetate followed by 7.2L 75:25 hexane-ethyl acetate. Fractions containing 14-methylene rapamycin were combined and evaporated in vacuo to give 1.0g of solid.

Preparative hplc

800mg of the above solid was dissolved in 76:24 methanol-H₂O at up to 75mg/ml and 2ml portions injected separately onto a reverse phase 5µm C₁₈ column and pre column (21.4mm x 25cm and 21.4mm x 5cm) (Rainin Instruments USA). After injection elution continued with 76:24 methanol-H₂O, at 15ml/minute and was monitored for UV absorbance at 275nm. Fractions containing the object compound from a total of six injections were pooled and concentrated in vacuo to yield 83mg of white solid.

Fractions containing the object compound were analysed by reverse phase hplc using a Microsorb 5 μ m C₁₈ column 4.6 x 250mm (Rainin Instruments USA) and an Upchurch pre column (2.0 x 20mm). This column was operated at 30°C and monitored by ultraviolet absorbance at 275nm. The column was eluted with methanol-water 74:26 at 1ml/minute. Under these conditions the object compound designated 14-methylene rapamycin had a retention time of 31.4 minutes differing from that of rapamycin.

14-methylene rapamycin was characterised by ultra violet absorbance UV max 268,277,289nm by mass spectroscopy FAB $[M+Na]^+ = 922$ and by proton and ¹³C nuclear magnetic resonance spectroscopy.

Como se puede apreciar en esta anterioridad se revela un proceso de purificación de 14-metilen-rapamicina, un derivado estrechamente relacionado de rapamicina. Dicho proceso inicia desde el caldo de cultivo de fermentación, seguido de un aislamiento de dicho derivado de rapamicina por extracción con solvente (hexano), una primera cromatografía en sílica-gel, seguida de una cromatografía de fase reversa y una cromatografía preparativa en HPLC. Luego de una evaporación, el derivado de rapamicina es obtenido.

Cualquier persona medianamente versada en la materia podría tomar la información presentada en este documento así como en los anteriores, específicamente el método de cristalización de la publicación WO/2005/0192226 junto al método de la publicación WO/1993/011130 para obtener la rapamicina con la pureza reivindicada por la solicitud colombiana en trámite.

En conclusión, la solicitud colombiana en trámite carece de los requisitos fundamentales para otorgársele el privilegio de patente de invención, en primer lugar porque utiliza estrategias tecnológicas convencionales para la separación y purificación de compuestos, además, absolutamente todas las características de dicho proceso de purificación ya se habían aplicado con anterioridad en procesos de purificación del mismo compuesto (rapamicina y sus isómeros) o compuestos estrechamente relacionados (un derivado de rapamicina y otros macrólidos), con resultados en la solución del problema técnico (la pureza de la rapamicina) inclusive superiores al resultado de la solicitud colombiana en cuestión.

Así las cosas, y con fundamento en los argumentos expuestos a lo largo del presente escrito, respetuosamente reitero la solicitud para que su despacho se abstenga de conceder el privilegio de patente solicitado, y ordene en consecuencia el archivo del expediente respectivo.

IV. FUNDAMENTOS DE DERECHO

Son fundamentos de derecho el Capítulo I, artículos 14, 16 y 18 (Requisitos de Patentabilidad) y el artículo 42 (Oposiciones) de la Decisión 486 de 2000.

V. PRUEBAS

Ruego que se tengan como tales, sin perjuicio de las que puedan presentarse al vencimiento de la prórroga solicitada, y de aquellas que el Despacho considere oficiosamente, las siguientes:

- ❖ Todas las publicaciones mencionadas en el acápite de Fundamentos de la Oposición.

VI. ANEXOS

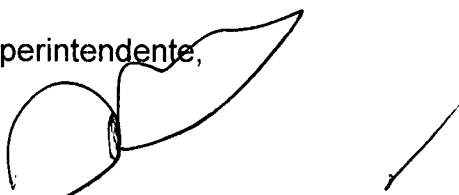
Para efectos del trámite correspondiente, me permito anexar al presente escrito los siguientes anexos

1. Certificado de existencia y representación legal de Lafrancol S.A.
2. Poder para actuar.
3. Constancia de pago de las tasas de tramitación de oposición.
4. Copia para correr traslado al solicitante.
5. El mencionado en el acápite de pruebas.

VII. NOTIFICACIONES

El suscrito recibirá notificaciones en la secretaría de su Despacho o en mi oficina localizada en la Carrera 17 No. 88-23, Oficina 205, Fax (1) 621.25.42 de esta ciudad. Lafrancol S.A., las recibirá en sus oficinas localizadas en la Carrera 1 N° 46-84 de Cali.

Señor Superintendente,

A handwritten signature in black ink, consisting of a large, stylized 'J' followed by a series of loops and a final downward stroke.

JOSÉ LUIS REYES VILLAMIZAR

C.C. 79'152.473 de Usaquén

T.P.A. 44.655 del C.S.J.

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
21 October 2004 (21.10.2004)

PCT

(10) International Publication Number
WO 2004/089958 A2

- (51) International Patent Classification⁷: **C07D 498/00**
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(54) Title: CRYSTALLIZATION AND PURIFICATION OF MACROLIDES

(57) Abstract: Provided is a method for crystallization and purification of a macrolide such as tacrolimus, sirolimus, pimecrolimus, or everolimus that includes the step of providing a combination of a macrolide, and a polar solvent, dipolar aprotic solvent, or hydrocarbon solvent at pH of 7 or above.

CRYSTALLIZATION AND PURIFICATION OF MACROLIDES

FIELD OF THE INVENTION

The present invention relates to the crystallization and purification of macrolides, especially tacrolimus, sirolimus (rapamycin), pimecrolimus, and everolimus.

RELATED APPLICATIONS

The present application claims the benefit of the filing date of the following United States Provisional Patent Applications: U.S. provisional application Serial No. 60/512,887, filed October 20, 2003, U.S. provisional application Serial No. 60/461,707, filed April 9, 2003, and U.S. provisional application Serial No. 60/459,591, filed March 31, 2003, the contents of all of which are incorporated herein.

SUMMARY OF THE INVENTION

The present invention relates to a method for crystallization and purification of macrolides, especially tacrolimus, sirolimus, pimecrolimus, and everolimus, including the steps of: providing a combination of a macrolide starting material; a polar solvent, especially a polar solvent that is an alkyl ester of an alkanoic acid, an alcohol, an ether, an aliphatic ketone, an aliphatic nitrile, or a dipolar aprotic solvent; a hydrocarbon solvent, especially an acyclic or cyclic aliphatic hydrocarbon or an aromatic hydrocarbon (e.g. toluene); and water; at a pH of about 7 or above, especially about 8 or above; maintaining the combination at a temperature of between about -15°C to about 50°C, preferably between about -5°C to about 40°C, most preferably between about -2°C to about 35°C for at least about 1 hour, preferably between about 48 to about 100 hours; and isolating crystalline macrolide.

In another aspect, the present invention relates to a method for crystallization and purification of a macrolide, especially tacrolimus, sirolimus, pimecrolimus, or everolimus including the steps of: providing a concentrate residue from whole-broth extraction of macrolide-containing biomatter in a polar solvent, especially a polar solvent that is an alkyl ester of an alkanoic acid, an alcohol, an ether, an aliphatic ketone, an aliphatic nitrile, or a dipolar aprotic solvent; combining the solution, in any order, with water and a

hydrocarbon solvent, especially an acyclic or cyclic aliphatic hydrocarbon or an aromatic hydrocarbon (e.g. toluene), wherein the pH is about 7 or above, especially about 8 or above; maintaining the combination at a crystallization temperature for a crystallization time; and isolating crystalline macrolide.

5 In a further aspect, the present invention relates to a method of crystallizing and purifying a macrolide, especially tacrolimus, sirolimus, pimecrolimus, or everolimus including the steps of: combining, in any order, an oil that is a concentrate obtained by concentrating a solution obtained by extracting macrolide-containing biomatter with a hydrophobic extraction solvent, e.g. butyl acetate; with a polar solvent, especially a polar
10 solvent that is an alkyl ester of an alkanolic acid, an alcohol, an ether, an aliphatic ketone, an aliphatic nitrile, or a dipolar aprotic solvent; a hydrocarbon solvent, especially an acyclic or cyclic aliphatic hydrocarbon or an aromatic hydrocarbon (e.g. toluene); and water; wherein the pH is about 7 or above, especially 8 or above; maintaining the combination at a first crystallization temperature for a first crystallization time; and
15 isolating crystalline macrolide.

In any of the forgoing aspects, the combination can be, but need not be, maintained at a second crystallization temperature for a second crystallization time.

DETAILED DESCRIPTION OF THE INVENTION

As used herein in connection with a measured quantity, "about" refers to that
20 variation in the measured quantity as would be expected by the skilled artisan performing or interpreting the measurement and exercising a level of care commensurate with the objective of the measurement and the precision of the measuring equipment being used.

As used herein, ambient temperature refers to a temperature of about 18°C to about 25° C.

25 As used herein, "RN" refers to the registry number assigned to a chemical compound by the Chemical Abstracts Service, Columbus OH, USA).

The method of the present invention is applied to the crystallization and purification of macrolides from macrolide-containing starting material. The macrolides are multi-membered lactone rings having one or more deoxy sugars as substituents.
30 Erythromycin, azithromycin, and clarithromycin are macrolides that have bacteriostatic and / or bactericidal activity. The macrolides tacrolimus (FK 506) and sirolimus

(rapamycin) are preferred macrolides for use in the practice of the present invention. The macrolides pimecrolimus (the 33-epichloro derivative of ascomycin; RN = 137071-32-0) and everolimus (40-O-(2-hydroxyethyl)-rapamycin; RN=159351-69-6) are also preferred macrolides for use in the practice of the present invention.++

5 The macrolides are typically obtained by fermentation, although synthetic routes to some are known. The macrolide starting material for use in the practice of the present invention can be from any source. Concentrate residue from concentrating the extract of the entire fermentation broth ("whole broth method") from macrolide-containing biomatter can be used as the macrolide starting material for the present method. Use of
10 hydrophobic extraction solvent in the extraction to obtain solution to be concentrated results in an efficient extraction yield, leaving behind most water-soluble impurities, with removal of mycelium in one step. Concentration under reduced pressure at $T > 25^{\circ}\text{C}$ and reduced pressure results in a high evaporation rate of solvent without precipitation or decomposition of macrolide and provides a macrolide starting material for use in the
15 practice of the present invention. Concentrate residue for use as macrolide starting material in the practice of the present invention can be obtained as described in United States Patent Application 10/366,266, published as U.S. 2003/01666924 A1 and incorporated herein in its entirety by reference.

20 Oily residue from macrolide-producing processes can also be used as starting macrolide starting material.

25 Preferred macrolide-containing biomatter that can be a source of macrolide starting material for the practice of the present invention includes tacrolimus-containing biomatter, particularly fermentation broth obtainable by fermentation using a tacrolimus-producing microorganism, for example, *Streptomyces tsukubaensis*, new and mutated strains thereof, *Streptomyces hygroscopicus*, and *Streptomyces lividans*, as described in United States Patent numbers 4,894,366, 5,116,756, 5,624,842, 5,496,727, and 5,622,866, all of which are incorporated herein by reference. Sirolimus-containing (rapamycin-containing) biomatter is also a preferred macrolide-containing biomatter. Sirolimus (rapamycin) can be produced by fermentation of *Streptomyces hygroscopicus*, NRRL
30 5491, as described in United States Patent 3,993,749, incorporated herein by reference. Pimecrolimus-containing biomatter and everolimus-containing biomatter are also examples of preferred macrolide-containing biomatter for use in the practice of the

method of the present invention. Ascomycin-containing biomatter is also a preferred macrolide-containing biomatter for use in the practice of the present invention

The method of the present invention employs, among other things, polar solvents, hydrocarbon solvents, and bases (alkali).

5 Polar solvents are organic compounds, normally liquid at ambient temperature, that dissolve a macrolide, especially tacrolimus, sirolimus, pimecrolimus, or everolimus. Polar solvents useful in the practice of the present invention include esters, alcohols, aliphatic nitriles, acyclic and cyclic aliphatic ethers, aliphatic ketones, and dipolar aprotic solvents.

10 Esters useful in the practice of the present invention have the general formula $R_1-C(O)O-R_2$, wherein R_1 is H or linear or branched C1-6 alkyl, and R_2 is linear or branched C1-6 alkyl. Examples of esters include methyl acetate, ethyl acetate, *n*-propyl acetate, *iso*-propyl acetate, *n*-butyl acetate, *iso*-butyl acetate, methyl formate, *n*-propyl formate, *iso*-propyl formate, *n*-butyl formate, and *iso*-butyl formate, to mention just a few. Alcohols
15 (alkanols, glycols, and aromatic alcohols) useful in the practice of the present invention include methanol, ethanol, *n*-propanol, *iso*-propanol, ethylene glycol, propylene glycol, polyethylene glycol, polypropylene glycol, amyl alcohol and benzyl alcohol, to mention just a few.

Aliphatic ketones useful in the practice of the present invention have the general
20 formula $R_1-C(O)-R_2$, wherein R_1 and R_2 are, independently, linear or branched alkyl groups, each having from 1 to 4 carbon atoms. Examples of aliphatic ketones include acetone, methyl ethyl ketone, and methyl *iso*-butyl ketone, to mention just three.

Examples of aliphatic nitriles useful in the practice of the present invention include acetonitrile, propionitrile, and butyronitrile, to mention just three.

25 Ethers useful in the practice of the present invention include both acyclic and cyclic aliphatic ethers. Acyclic aliphatic ethers have the general formula R_1-O-R_2 , wherein R_1 and R_2 are as defined above. Examples of acyclic aliphatic ethers include diethyl ether, di-*n*-propyl ether, and ethyl *n*-propyl ether, to mention just a few. Tetrahydrofuran and the dioxanes are examples of cyclic aliphatic ethers useful in the
30 practice of the present invention.

Dipolar aprotic solvents are well known to the skilled artisan. Dimethyl acetamide (DMAC), dimethyl formamide (DMF), N-methyl-2-pyrrolidone (NMP), acetamide, dioxane and dioxalane are examples of dipolar aprotic solvents useful in the practice of the present invention. ++

5 Hydrocarbon solvents are organic compounds, normally liquid at ambient temperature, that are poor solvents for macrolides. The hydrocarbon solvents can be aliphatic hydrocarbon solvents, or they can be aromatic hydrocarbon solvents.

The aliphatic hydrocarbon solvents can be acyclic or they can be cyclic. Acyclic hydrocarbon solvents can be linear or branched and have the general formula C_nH_{2n+2} ,
10 where n is from about 5 to about 10. *n*-Hexane, *n*-heptane, octane and *iso*-octane are examples of preferred acyclic aliphatic hydrocarbon solvents. Cyclohexane and methylcyclohexane are examples of cyclic aliphatic hydrocarbon solvents. Examples of aromatic hydrocarbon solvents include benzene, toluene, the xylenes, and the tetralins, to mention just a few.

15 Any base, organic or inorganic, can be used in the practice of the present invention. Examples of inorganic bases include ammonia, alkali and alkaline earth metal hydroxides, bicarbonates, and carbonates, to mention just a few. The amines are examples of organic bases that can be used in the practice of the present invention.

The present invention provides a method for crystallization and purification of a
20 macrolide, preferably tacrolimus, sirolimus, pimecrolimus, or everolimus including the steps of: providing, in a crystallization vessel, a combination of a macrolide starting material, a polar solvent, a hydrocarbon solvent, and water, whereby a water rich phase is formed. A water-rich phase is a phase in which the majority of the solvent is water and can contain other solvents and solutes. The pH of the water-rich phase is or is adjusted to be
25 about 7 or above, preferably about 8 or above. The pH can be adjusted by addition of base.

The combination is provided, preferably with agitation, and is maintained at a temperature of between about -15°C to about 50°C, preferably between about -5°C to about 40°C, most preferably between about -2°C to about 35°C for at least about 1 hour,
30 preferably between about 48 to about 100 hours, whereby a macrolide-rich phase forms.

The manner in which the provided combination is assembled is irrelevant to the practice of the present invention. The components of the combination can be assembled in any order, or they can be assembled simultaneously.

5 The combination of macrolide, polar solvent, hydrocarbon solvent, and water is provided in a crystallization vessel (crystallization space) provided with an agitator. The design and peculiar characteristics of the crystallization vessel are unimportant and the skilled artisan will know to select the crystallization vessel and agitator based on, among other things, the volume of the combination and the process variables.

10 At the start of the first crystallization time, the combination provided will include two or more phases, at least one of which is water-rich. The pH of the water-rich phase is about 7 or above, preferably about 8 or above. The pH of the water-rich phase can be constant throughout the total crystallization time, or it can be varied in the course of the crystallization time, provided the pH is always at least about 7 or above.

15 The desired pH is established with the use of any available inorganic or organic base and the desired pH can be established in any manner or sequence. For example, the pH of the water used to assemble the combination can be adjusted, prior to assembly of the combination, with an inorganic or organic base. Thus, as used herein in connection with the combination provided, "water" will be understood to include dilute aqueous solutions (water solutions) of inorganic or organic bases, e.g., N/10 NaOH_{aq}, N/10 KOH, 20 N/10 Ca(OH)₂, N/10 NH_{3aq}, N/10 (C₂H₅)₃N_{aq}, N/10 diethylamine or triethyl amine, N/10 pyridine etc. Base can be added before the water-rich phase is established by, for example, admitting a low-boiling amine, e.g. methylamine, before water is introduced. The skilled artisan will recognize a plethora of alternatives to establishing the desired pH of the water-rich phase.

25 The pH can be adjusted after the combination is assembled by adding inorganic base, neat, especially as a gas, or in solution in a suitable solvent, e.g. water. The pH can be adjusted in increments. For example, the pH of the water used to assemble the combination can be adjusted to, e.g., ca. 7 before the combination is assembled and, after assembly, the pH of the water-rich phase can be further adjusted, e.g. to pH 8, by the 30 addition of base, neat or in solution.

During the course of the total crystallization time, at least one macrolide-rich phase develops, from which the macrolide crystallizes, substantially free of impurities. At the end of the total crystallization time, crystalline macrolide is isolated by any of the common methods, for example filtration (gravity or pressure-assisted) or centrifugation, to mention just two. The purity of the isolated crystalline macrolide rivals that of macrolide purified by multiple-pass chromatography.

In one embodiment, the combination provided is assembled by the steps of providing macrolide starting material that is a solution of macrolide, or a concentrate from macrolide extraction, preferably tacrolimus, sirolimus, pimecrolimus, or everolimus in a polar solvent and combining the solution, in any order, with hydrocarbon solvent and water.

The solution provided can be made by any means or method. The concentration of the solution provided is not critical and will generally be between about 0.05 g/mL (g macrolide per mL polar solvent) and about 0.3 g/mL. The macrolide can come from any source and can be a solid, semi-solid, or an oil (especially an oil that is a residue from concentration of extract from a whole-broth extraction of macrolide-containing biomatter).

The relative volumes of solution, water, and hydrocarbon solvent are not critical. Typically, the ratio of the volume of solution to the volume of hydrocarbon solvent will be between about 1:2 and about 1:10. The ratio of the volume of solution to the volume of water will typically be between about 1:8 to about 1:25.

The pH of the water-rich phase can be adjusted and the combination treated as described above.

In another embodiment, the combination provided is assembled by combining, in any order, macrolide starting material, preferably tacrolimus, sirolimus, pimecrolimus, or everolimus starting material, hydrocarbon solvent, polar solvent, and water, wherein the tacrolimus starting material is an oily phase that is a concentrate obtained by concentrating a solution obtained by extracting macrolide-containing biomatter with a hydrophobic extraction solvent, especially wherein the hydrophobic extraction solvent is selected from the group consisting of C2-C6 linear and branched esters of acetic acid or formic acid, C3-C6 linear or branched aliphatic ketones, halogenated methanes, and

aromatic hydrocarbons that are liquid at 25° C and that have a boiling point at atmospheric pressure less than about 150°C, wherein the extraction is at a temperature between about 2° C to about 70° C, especially between about 30°C and about 70°C, and at a pH of between about 5.5 and about 13, especially between about 7.5 and about 13, to
5 obtain the solution of the macrolide in the hydrophobic extraction solvent.

The oil (macrolide starting material) can first be combined with polar solvent or hydrocarbon solvent or water. The order is irrelevant to the practice of the present invention. The base required to establish the desired pH can be introduced at any point, or at several points prior to or during the crystallization time. The base can be introduced
10 neat, or as a solution, e.g. a solution in water.

The present invention, in certain of its embodiments, is illustrated by the following non-limiting examples.

Example 1:

Extraction:

15 Fermentation broth (22.2 m³) containing tacrolimus (3.42 kg) was extracted with 6.4 m³ iso-butyl acetate at pH between 9.0-9.5. The iso-butyl acetate solution was washed with water at pH between 6.0-8.0. The washed iso-butyl acetate phase was concentrated to oily-like residue under reduced pressure at temperature between 40-45 °C.

20 The oily-like residue was dissolved with iso-butyl acetate to a volume of 31 L. This concentrate was diluted with 167.5 L methanol and 18.6 L water. The water-methanol solution was washed with 139.6 L n-Hexane. The water-methanol phase was concentrated under reduced pressure to volume of 44 L, and the concentrate was diluted with 44 L water.

25 The obtained mixture was extracted with 88 L ethyl acetate. The ethyl acetate extract was concentrated to volume of 22.4 L.

Crystallization:

This concentrate of ethyl acetate extract was combined with 158.4L 0.1 M aqueous triethyl amine solution and with 67.3 L n-Hexane. The mixture was stirred at

20° - 25 °C for 3 hours. The mixture was let to stand at 0° - 25 °C for 48 hours (1 minute stirring every hour).

The crystals formed were isolated by filtration and were suspended first in 83 L 0,1 M aqueous triethyl amine solution and, second, in 83 L n-Hexane. The crystals were isolated by filtration.

The crystals were dried at 40 °C under reduced pressure. The dried crude tacrolimus had an assay 83 %. Crude product contains 1.9 kg tacrolimus.

The yield of the crystallization step was 91 % .

Example 2:

In the following example, a macrolide (tacrolimus), as an oily concentrate from whole-broth extraction of macrolide-containing biomatter, was combined with polar solvent, hydrocarbon solvent, and water containing a base. The combination was held at a crystallization temperature for a total crystallization time. At the end of the total crystallization time, the crystalline macrolide was isolated. The proportions of components, the process variables, and the results are collected in Table I.

Table I

Number of experiment	Concentrate	Tacrolimus content	Polar solvent	Hydrocarbon solvent	Water	Total t _c (hr)	T _c (°C)	Yield	Assay
1	15,23 g	1,42 g	Ethyl acetate 30,3 ml	n-Hexane 60,7 ml	0,1 N NaOH 273 ml	24	+50 - +20	41,82 %	84,65 %
2	14,36 g	1,42 g	Ethyl acetate 12,3 ml	n-Hexane 73,7 ml	0,1 N NaOH 172 ml	20	+25 - 0	78,48 % ‡	81,68 %
3	13,67 g	1,42 g	Ethyl acetate 7,5 ml	n-Hexane 74,5 ml	0,1 N NaOH 164 ml	20	+25 - 0	79,06 %	81,74 %
4	12,35 g	1,42 g	Ethyl acetate 10,6 ml	n-Hexane 63,4 ml	0,1 N NaOH 148 ml	11	+20 - -10	82,8 %	82,85 %
5	11,47 g	1,42 g	Ethyl acetate 9,8 ml	n-Hexane 59 ml	0,1 N NH ₃ 137,6 ml	62	+25 - 0	79,19 %	82,55 %
6	11,72 g	1,42 g	Ethyl acetate 6,4 ml	n-Hexane 63,9 ml	0,1 N NH ₃ 140,64 ml	62	+25 - 0	82,84 %	79,20 %
7	12,17 g	1,42 g	Ethyl acetate 10,4 ml	n-Hexane 62,6 ml	0,1 N (C ₂ H ₅) ₃ N 144,2 ml	62	+25 - 0	85,76 %	85,03 %
8	12,94 g	1,42 g	Ethyl acetate 7 ml	Cyclohexane 70,6 ml	0,1 N (C ₂ H ₅) ₃ N 155,3 ml	62	+25 - 0	82,98 %	82,18 %
9	13,28 g	1,42 g	Ethyl acetate 11,4 ml	n-Hexane 68,3 ml	0,1 N (C ₂ H ₅) ₃ N 159,4 ml	50	+25 - 0	72,84 %	75,64 %
10	14,72 g	1,42 g	Ethyl acetate 12,6 ml	n-Hexane 75,8 ml	0,1 N (C ₂ H ₅) ₃ N 176 ml	32	+25 - 0	74,74 %	84,81 %
11	11,36 g	1,42 g	Ethyl acetate 9,7 ml	n-Hexane 58,4 ml	0,1 N (C ₂ H ₅) ₃ N 136,3 ml	50	+25 - 0	71,64 %	80,89 %

Number of experiment	Concentrate	Tacrolimus content	Polar solvent	Hydrocarbon solvent	Water	Total t_c (hr)	T_c (°C)	Yield	Assay
12	11,39 g	1,42 g	Ethyl acetate 9,8 ml	n-Hexane 58,6 ml	0,1 N (C ₂ H ₅) ₃ N 136,7 ml	50	+25 - 0	88,32 %	83,68 %
13	20,93 g	2,23 g	Ethyl acetate 17,9 ml	n-Hexane 107,8 ml	0,1 N (C ₂ H ₅) ₃ N 251,2 ml	62	+25 - 0	91,2 %	86,49 %
14	20,17 g	2,23 g	Ethyl acetate 17,3 ml	n-Hexane 103,7 ml	0,1 N (C ₂ H ₅) ₃ N 242 ml	62	+25 - 0	62,7 %	83,34 %
15	19,15 g	2,23 g	Ethyl acetate 16,4 ml	n-Hexane 98,5 ml	0,1 N (C ₂ H ₅) ₃ N 229,8 ml	62	+25 - 0	91,2 %	88,05 %
16	20,4 g	2,23 g	Ethyl acetate 8,7 ml	n-Hexane 52,5 ml	0,1 N (C ₂ H ₅) ₃ N 122,4 ml	62	+25 - 0	91,2 %	88,06 %
17	18,78 g	2,23 g	Ethyl acetate 4 ml	n-Hexane 24,2 ml	0,1 N (C ₂ H ₅) ₃ N 56 ml	62	+25 - 0	86,64 %	86,90 %
18	4,56 g	0,557 g	Acetonitrile 3,9 ml	n-Hexane 23,45 ml	0,1 N (C ₂ H ₅) ₃ N 54,7 ml	18	+25 - +20	79,92 %	83,46 %
19	4,62 g	0,557 g	n-Butanol 3,96 ml	n-Hexane 23,76 ml	0,1 N (C ₂ H ₅) ₃ N 55,44 ml	18	+25 - +15	63,12 %	88,72 %
20	4,58 g	0,557 g	Acetone 3,93 ml	n-Hexane 23,55 ml	0,1 N (C ₂ H ₅) ₃ N 54,96 ml	18	+30 - +20	87,07 %	82,56 %
21	4,62 g	0,557 g	Isobutanol 3,75 ml	n-Hexane 22,5 ml	0,1 N (C ₂ H ₅) ₃ N 52,44 ml	18	+25 - +10	67,34 %	89,78 %
22	4,84 g	0,557 g	Isopropanol 4,15 ml	n-Hexane 24,9 ml	0,1 N (C ₂ H ₅) ₃ N 58,08 ml	18	+25 - +20	80,26 %	83 %
23	4,54 g	0,557 g	Ethanol 3,89 ml	n-Hexane 23,35 ml	0,1 N (C ₂ H ₅) ₃ N 54,48 ml	18	+35 - +20	76,92 %	82,13 %
Number of experiment	Concentrate	Tacrolimus content	Polar solvent	Hydrocarbon solvent	Water	Total t_c (hr)	T_c (°C)	Yield	Assay
24	4,43 g	0,525 g	n-Propanol 3,79 ml	n-Hexane 22,78 ml	0,1 N (C ₂ H ₅) ₃ N 53,16 ml	18	+25 - +15	75,6 %	84,79 %

25	4,34 g	0,525 g	Methanol 3,72 ml	n-Hexane 22,32 ml	0,1 N (C ₂ H ₅) ₃ N 52,08 ml	18	+25 - +20	77,16 %	78,18 %
26	3,84 g	0,525 g	Diisopropyl ether 3,29 ml	n-Hexane 19,74 ml	0,1 N (C ₂ H ₅) ₃ N 52,08 ml	18	+25 - +10	59,52 %	72,35 %

Example 3:

Fermentation broth containing ascomycin was processed according to example 1. The process resulted in 60 % yield for crude ascomycin.

CLAIMS

1. A method of crystallizing a macrolide from a macrolide starting material comprising the steps of:
 - a) combining a macrolide starting material, a polar solvent, a hydrocarbon solvent, and water, whereby at least two phases are formed, at least one of which is a water-rich phase, and wherein the pH of the water-rich phase is at least about 7,
 - b) maintaining the combination at for at least 1 hour, whereby a macrolide-rich phase is formed from which the macrolide crystallizes.
2. The method of claim 1 further comprising the step of isolating the macrolide that crystallizes.
3. The method of claim 1 wherein the combination of step b is maintained at a temperature of from about -15°C to about 50°C.
4. The method of claim 3 wherein the combination of step b is maintained at a temperature of from about -5°C to about 40°C.
5. The method of claim 4 wherein the combination of step b is maintained at a temperature of from about -2°C and about 35°C.
6. The method of claim 1 wherein the combination of step b is maintained for between 48 and 100 hours.
7. The method of claim 1 wherein the polar solvent is selected from the group consisting of alcohols, esters, nitriles and ethers.
8. The method of claim 7 wherein the polar solvent is selected from the group consisting of ethyl acetate, acetonitrile, methanol, ethanol, *n*-propanol, *iso*-propanol, *n*-

butanol, *iso*-butanol, acetone, diisopropyl ether, dimethyl formamide, and dimethyl acetamide.

9. The method of claim 8 wherein the polar solvent is ethyl acetate.

5

10. The method of claim 1 wherein the hydrocarbon solvent is selected from the group consisting of *n*-hexane, *n*-heptane, octane, *iso*-octane cyclohexane, methylcyclohexane, benzene, toluene, and xylene.

10 11. The method of claim 10 wherein the hydrocarbon solvent is *n*-hexane.

12. The method of claim 1 wherein the pH of the water-rich phase is about 8 or higher.

15 13. The method of claim 1 wherein the water comprises a base selected from NaOH, KOH, Ca(OH)₂, NH₃, Et₃N, diethylamine and pyridine.

14. The method of claim 1 wherein the macrolide is selected from the group consisting of tacrolimus, sirolimus, pimecrolimus, and everolimus.

20

15. A method of crystallizing a macrolide from a macrolide starting material comprising the steps of:

a) combining a concentrate residue from whole-broth extraction of macrolide-containing biomatter in a polar solvent with a hydrocarbon solvent, and water, whereby at least two phases are formed, at least one of which is a water-rich phase, and wherein the pH of the water-rich phase is at least about 7,

25

b) maintaining the combination at for at least 1 hour, whereby a macrolide-rich phase is formed from which the macrolide crystallizes.

16. The method of claim 15 further comprising the step of isolating the macrolide that crystallizes.

17. The method of claim 15 wherein the combination of step b is maintained at a
5 temperature of from about -15°C to about 50°C.

18. The method of claim 17 wherein the combination of step b is maintained at a temperature of from about -5°C to about 40°C.

10 19. The method of claim 18 wherein the combination of step b is maintained at a temperature of from about -2°C and about 35°C.

20. method of claim 15 wherein the combination of step b is maintained for between 48 and 100 hours.

15

21. The method of claim 15 wherein the polar solvent is selected from the group consisting of alcohols, esters, nitriles and ethers.

22. The method of claim 21 wherein the polar solvent is selected from the group
20 consisting of ethyl acetate, acetonitrile, methanol, ethanol, *n*-propanol, *iso*-propanol, *n*-butanol, *iso*-butanol, acetone, diisopropyl ether, dimethyl formamide, and dimethyl acetamide.

23. The method of claim 22 wherein the polar solvent is ethyl acetate.

25

24. The method of claim 15 wherein the hydrocarbon solvent is selected from the group consisting of *n*-hexane, *n*-heptane, octane, *iso*-octane cyclohexane, methylcyclohexane, benzene, toluene, and xylene.

25. The method of claim 24 wherein the hydrocarbon solvent is *n*-hexane.
26. The method of claim 15 wherein the pH of the water-rich phase is about 8 or higher.
- 5 27. The method of claim 15 wherein the water comprises a base selected from NaOH, KOH, Ca(OH)₂, NH₃, Et₃N, diethylamine and pyridine.
- 10 28. The method of claim 15 wherein the macrolide is selected from the group consisting of tacrolimus, sirolimus, pimecrolimus, and everolimus.
29. A method of crystallizing a macrolide from a macrolide starting material comprising the steps of:
- 15 a) combining, at a temperature of about 20° to about 25°C, macrolide starting material, ethyl acetate, *n*-hexane, and a water solution of a base selected from NaOH, KOH, Ca(OH)₂, NH₃, (C₂H₅)₃N, diethylamine and pyridine whereby at least two phases are formed, one of which is a water-rich phase, wherein the pH of the water-rich phase is > about 7,
- 20 b) maintaining the combination at a temperature of about 20°C to about 25°C for at least 1 hour, whereby a macrolide-rich phase is formed from which macrolide crystallizes,
- c) maintaining the combination at a temperature of about 0°C to about 20°C for at least 1 hour, and
- 25 d) recovering the macrolide that crystallizes.
30. The method of claim 29 wherein the macrolide is selected from the group consisting of tacrolimus, sirolimus, pimecrolimus, and everolimus.
31. The method of claim 29 wherein the pH of the water-rich phase is about 8 or higher.
- 30

32. A method of crystallizing a macrolide from a macrolide starting material comprising the steps of:

- a) combining, at a temperature of about 20° to about 25°C, a concentrate residue
5 from whole-broth extraction of macrolide-containing biomatter in ethyl acetate, *n*-hexane, and a water solution of a base selected from NaOH, KOH, Ca(OH)₂, NH₃, (C₂H₅)₃N, diethylamine and pyridine whereby at least two phases are formed, one of which is a water-rich phase, wherein the pH of the water-rich phase is > about 7,
- b) maintaining the combination at a temperature of about 20°C to about 25°C for
10 at least 1 hour, whereby a macrolide-rich phase is formed from which macrolide crystallizes,
- c) maintaining the combination at a temperature of about 0°C to about 20°C for at least 1 hour, and
- d) recovering the macrolide that crystallizes.

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33. The method of claim 32 wherein the macrolide is selected from the group consisting of tacrolimus, sirolimus, pimecrolimus, and everolimus.

34. The method of claim 32 wherein the pH of the water-rich phase is about 8 or
20 higher.

35. In a method for crystallizing a macrolide from a macrolide starting material, the step of combining the macrolide starting material, a polar solvent, a hydrocarbon solvent, and water, whereby at least two phases are formed, at least one of which is water rich,
25 wherein the pH of the water-rich phase is at least about 7.

36. In a method for crystallizing a macrolide from a concentrate residue from whole-broth extraction of macrolide-containing biomatter in a polar solvent, the step of combining the macrolide concentrate in the polar solvent, a hydrocarbon solvent, and
30 water, whereby at least two phases are formed, at least one of which is water rich, wherein the pH of the water-rich phase is at least about 7.

[54] **RAPAMYCIN AND PROCESS OF PREPARATION**

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[60] Division of Ser. No. 460,665, April 12, 1974, Pat. No. 3,929,992, which is a continuation-in-part of Ser. No. 293,699, Sept. 29, 1972, abandoned.

[52] U.S. Cl. **424/122**

[51] Int. Cl.² **A61K 35/74**

[58] Field of Search **424/122**

[56] **References Cited**

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Millev, *The Pfizer Handbook of Microbial Metabolites*, McGraw-Hill Book Co., Inc., N.Y., N.Y., 1961, p. 580.

Primary Examiner—Jerome D. Goldberg

[57] **ABSTRACT**

Antibiotic rapamycin is producible by culturing *Streptomyces hygroscopicus* NRRL 5491 in an aqueous nutrient medium. Rapamycin has antifungal properties. Methods for its preparation and use are disclosed.

2 Claims, 2 Drawing Figures

RAPAMYCIN AND PROCESS OF PREPARATION

This application is a division of application Ser. No. 460,665, filed Apr. 12, 1974, now U.S. Pat. No. 3,929,992, granted Dec. 30, 1975, which, in turn, is a continuation in part of Ser. No. 293,699, filed Sept. 29, 1972, now abandoned.

BACKGROUND OF THE INVENTION

A. Field of Invention

This invention relates to an antibiotic, a new composition of matter called rapamycin, and to a process for its preparation.

B. Description of Prior Art

The antibiotic of this invention is readily distinguished from prior art compounds of its class by its profound antifungal activity and its relatively low order of toxicity.

More explicitly, the ultra violet spectrum of rapamycin, noted herein, indicates that this compound belongs to the class of antibiotics known as triene antibiotics. In this particular class there are only five compounds reported previously, Trienine, A. Aszalos et al., J. Antibiotics, 21, 611 (1968) is a triene antibiotic with antitumor activity which also shows marked activity against gram positive organisms and only marginal activity against *Candida* strains. The antifungal triene reported by J. J. Armstrong, et al., Nature, 206, 399 (1965) and Mycotrienin reported by C. Coronelli et al., J. Antibiotics, 20, 329 (1967) are probably identical. Both have low antifungal activity (MIC against *C. albicans*: 5 µg/ml) and high toxicity (LD₅₀ in mice: 15 mg/kg). The remaining two antibiotics — Resistaphilin, S. Azeiva et al., J. Antibiotics, 14, 393 (1971) and Proticin, G. Nesemann et al., Naturwissenschaften, 59, 81 (1972) — are readily distinguished from the compound of the present invention in that they exhibit antibacterial without any antifungal activity.

BRIEF SUMMARY OF THE INVENTION

Rapamycin is a chemical compound producible by culturing a rapamycin-producing organism in an aqueous nutrient medium. The compound has the property of adversely affecting the growth of fungi, for example, *Candida albicans* and *Microsporum gypseum*. Accordingly, rapamycin may be used to prevent the growth of or reduce the number of certain fungi in various environments.

The rapamycin — producing organism used for this invention, *Streptomyces hygroscopicus* NRRL 5491, was obtained from Easter Island soils and samples thereof have been deposited without restrictions with the Northern Utilization and Research Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

It is to be understood that the invention is not limited to the use of the particular organism herein described, but includes variations and mutants obtained by natural selection or by treatment of the microorganism with, for instance, ultraviolet rays, X-rays, N-methyl-N'-nitro-N-nitroso-guanidine, manganese chloride, camphor, nitrogen mustards, and the like, as well as polyploids of the various mutants.

Streptomyces hygroscopicus NRRL 5491 develops abundantly in culture media usually employed for cultivation of other organisms of the same genus. It is capable of growing at temperatures ranging from 20° to 35° C., preferably at about 28° C, on Czapek's agar, glu-

cose asparagine agar, glycerol asparagine agar, starch agar and peptone beef agar. Also, the organism grows very well on yeast extract agar, malt extract agar, starch-inorganic salts agar, oatmeal agar, oatmeal-tomato agar and Bennet's agar. On potato slices there is no aerial mycelium, but substrate growth is well developed and buff in color. On all media, the aerial growth is at first white then grayish with black spots. Sporophores are often compact, forming a spiral of more than ten spores. Substrate growth is light yellow to almost colorless and in some media pale brown. Occasionally a yellowish pigment is produced. The organism is H₂S- and melanine-negative.

Carbohydrate utilization by *Streptomyces hygroscopicus* NRRL 5491 was studied in carbon utilization agar (ISP Medium 9) according to the procedure standardized by the International Streptomyces Project (ISP).

The best utilized carbohydrates were D-glucose, inositol, D-fructose and D-mannitol; less well utilized carbohydrates were rhamnose, raffinose, xylose, starch and arabinose. Carbohydrates not utilized were sucrose and cellulose.

The environment and nutritional requirements for the fermentation of *Streptomyces hygroscopicus* NRRL 5491 are similar to those necessary for the production of antibiotics by other aerobic microorganisms. Thus, aerobiosis can be sustained in a liquid nutrient medium inoculated with a sterile culture incubated in flasks placed on shaking machines. For industrial production, metal tanks with internal aeration and agitation by means of paddles can be substituted. Rapamycin is also produced by surface cultivation. The microorganism requires as nutrient elements assimilable carbon and organic nitrogenous substances. The presence of mineral salts is desirable. Cultivation is best effected when the initial pH of the culture medium is between 6.5 and 7.5; the optimum pH being around 6.8-7.3.

The utilizable sources of assimilable carbon for the production of the antibiotic are very diverse, there being included sugars (for example, glucose, D-fructose, D-mannitol, maltose, arabinose, rhamnose, raffinose, xylose, and the like), dextrin, starches of different types of origina, glycerol (and other polyalcohols), inositol and animal and vegetable fats, as well as esters thereof. The sources of organic assimilable nitrogen which actively stimulate growth and favor production of rapamycin are substances such as soybean meal, cotton meal and other vegetable meals (whole or partially or totally defatted), meat flours or animal viscera, various peptones, casein hydrolysates, soybean hydrolysates, yeast hydrolysates, lactalbumin, wheat glutins, distillers solubles, corn steepes, molasses, urea and amino acids.

Mineral salts, such as the chlorides, nitrates, sulfates, carbonates and phosphates of sodium, potassium, ammonium and calcium, should be included in appropriate concentrations. The nutritive medium should contain a number of trace elements such as magnesium, iron, manganese, and zinc.

The inoculum of the above medium for the fermentation is provided with a fresh slant of *Streptomyces hygroscopicus* NRRL 5491.

Under the described conditions and with the temperature of cultivation at about 20°-35° C, preferably at about 25° C, maximum production of rapamycin in tanks is obtained in from about two to about eight days. Alternatively, the pH may be controlled during ferment-

tation in tanks and maintained at about pH 6.0, and glucose may be added continuously from about 2 days after beginning to the end of fermentation, thus obtaining maximum yields in about four to five days.

Thereafter a variety of procedures may be employed in the isolation and purification of rapamycin, for example, solvent extraction, partition chromatography, silica gel chromatography, liquid-liquid distribution in a Craig apparatus, and crystallization from solvents. Solvent extraction procedures are preferred for commercial recovery inasmuch as they are less time consuming and less expensive.

Generally speaking, rapamycin may be harvested by one of the following methods.

a. The fermentation mixture is extracted with a substantially water-immiscible solvent, preferably a lower alkanol, for example n-butanol, n-pentanol or the commercial mixture of pentanols known as "Pentanol" or n-hexanol, or a substantially water-immiscible lower alkyl lower alkanoate, for example, ethyl acetate, butyl acetate, amyl acetate or the commercially available mixture of amyl acetates, or a substantially water-immiscible halogenated aliphatic hydrocarbon, for example, chloroform, methylene dichloride or dichloroethane. The extracts are dried and concentrated under reduced pressure to yield an oily residue which is in turn extracted with a water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. Said last-named extracts are filtered through diatomaceous earth ("Celite"), and the filtrate concentrated under reduced pressure to yield an oily residue containing crude rapamycin.

b. The fermentation mixture is filtered through a pad of diatomaceous earth ("Celite") and the filter cake containing the mycelium is extracted as described below under (c). The filtrate, i.e. the mycelium-free fermentation mixture, is extracted several times with a substantially water-immiscible solvent, for example, a lower alkanol, lower alkyl lower alkanoate or halogenated aliphatic hydrocarbon as exemplified above in section (a). The extracts are dried and concentrated under reduced pressure to yield an oily residue which is extracted with a water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. Said last-named extracts are treated in the same manner as described above under (a) to yield an oily residue containing crude rapamycin.

c. The mycelium is separated from the fermentation mixture and extracted with a suitable water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. The extract is concentrated by evaporation to the aqueous phase, which in turn is extracted with a substantially water-immiscible solvent, such as a lower alkyl lower alkanoate, halogenated aliphatic hydrocarbon or a substantially water-immiscible lower alkanol as described above or an aromatic hydrocarbon, for example benzene or toluene. The latter extract is evaporated under reduced pressure to yield an oily residue containing crude rapamycin.

The crude rapamycin obtained by any of the processes described in sections (a), (b), or (c) is then purified by a variety of methods, for example, see above. Preferred methods include absorption of the crude rapamycin on an absorbent, for instance charcoal or silica gel from a solution in a substantially non-polar, first solvent, followed by elution therefrom with a second solvent, more polar than said first solvent.

DETAILS OF THE INVENTION

Rapamycin is useful as an antifungal agent against a number of pathogenic fungi; for example, *Candida albicans*, and other candida species, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Aspergillus sp.*, and *Sporotrichum sp.*

The inhibitory activity of rapamycin is especially pronounced against *Candida albicans* and said last organism may be used advantageously for assay purposes.

The antifungal activity of this compound is demonstrable in standard tests used for this purpose, for example, in the tests described in "Antiseptics, Disinfectants, Fungicides and Sterilization", G. F. Reddish, Ed., 2nd ed., Lea and Febiger, Philadelphia, 1957 or by D. C. Grove and W. A. Randall in "Assay Methods of Antibiotics", Med. Encycl. Inc., New York 1955.

When the antibiotic of this invention is employed as an antifungal agent in warm-blooded animals, e.g. rats, it may be used alone or in combination with pharmaceutically acceptable carriers, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard biological practice. For example, an antifungally effective amount of the antibiotic may be administered orally in solid form containing such excipients as starch, sugar, certain types of clay and so forth. Similarly, such an amount may also be administered orally in the form of solutions or suspensions, or the antibiotic may be injected parenterally. For parenteral administration the antibiotic may be used in the form of a sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monooleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

The dosage of the present antibiotic will vary with the form of administration and the particular compound chosen. Furthermore, it will vary with the particular host under treatment. Generally, treatment is initiated with small dosages substantially less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. In general, the compound of this invention is most desirably administered at a concentration level that will generally afford antifungally effective results without causing any harmful or deleterious side effects and preferably at a level that is in a range of from about 1.0 mg to about 250 mg per kilo per day, although as aforementioned variations will occur. However, a dosage level that is in the range of from about 10 mg to about 100 mg per kilo per day is most desirably employed in order to achieve effective results.

In addition, the agent may be employed topically. For topical application it may be formulated in the form of solutions, creams, or lotions in pharmaceutically acceptable vehicles containing 0.1 - 5 percent, preferably 2 percent of the agent, and may be administered topically to the infected area of the skin.

Rapamycin may also be used for cleaning and disinfecting laboratory equipment, surgical instruments, locker rooms, or shower rooms of sensitive fungus organisms. For such purposes it is preferred to use 0.1 - 10% solutions of rapamycin in a lower alkanol, preferably methanol, diluted with 10 - 100 volumes of water containing 0.001 - 0.1% of a non-ionic surface-

active agent, for example, polysorbate 80 U. S. P., immediately before applying it to the objects to be cleaned and disinfected.

PREPARATION

In one embodiment of this invention rapamycin is prepared in the following manner:

A suitable fermenter is charged with production medium 8KM (see Example 1). After sterilization* and cooling, the medium is inoculated with a first stage inoculum preparation of *Streptomyces hygroscopicus* NRRL 5491.

A maximum titre of 20 to 100 $\mu\text{g/ml}$ of the antibiotic is reached in the fermentation mixture after 2-8 days, usually after about 5 days, as determined by the cup plate method and *Candida albicans* as the test organism. The mycelium is harvested by filtration with diatomaceous earth. Rapamycin is then extracted from the mycelium with a water-miscible solvent, for example a lower alkanol, preferably methanol or ethanol. The latter extract is then concentrated, preferably under reduced pressure, and the resulting aqueous phase is extracted with a water-immiscible solvent. A preferred water-immiscible solvent for this purpose is methylene dichloride although chloroform, carbon tetrachloride, benzene, n-butanol and the like may also be used. The latter extract is concentrated, preferably under reduced pressure, to afford the crude product as an oil.

The product may be purified further by a variety of methods. Among the preferred methods of purification is to dissolve the crude product in a substantially non-polar, first solvent, for example petroleum ether or hexane, and to treat the resulting solution with a suitable absorbent, for example charcoal or silica gel, so that the antibiotic becomes absorbed on the absorbant. The absorbant is then separated and washed or eluted with a second solvent more polar than the first solvent, for example ethyl acetate, methylene dichloride, or a mixture of methylene dichloride and ether (preferred). Thereafter, concentration of the wash solution or eluate affords substantially pure rapamycin. Further purification is obtained by partial precipitation with a non-polar solvent, for example, petroleum ether, hexane, pentane and the like, from a solution of the rapamycin in a more polar solvent, for example, ether, ethyl acetate, benzene and the like. Still further purification is obtained by column chromatography, preferably employing silica gel, and by crystallization of the rapamycin from ether.

In another preferred embodiment of this invention a first stage inoculum of *Streptomyces hygroscopicus* NRRL 5491 is prepared in small batches in a medium containing soybean flour, glucose, ammonium sulfate, and calcium carbonate incubated at about 25° C at pH 7.1 - 7.3 for 24 hrs. with agitation, preferably on a gyrotary shaker. The growth thus obtained is used to inoculate a number of somewhat larger batches of the same medium as described above which are incubated at about 25° C and pH 7.1 - 7.3 for 18 hrs. with agitation, preferably on a reciprocating shaker, to obtain a second stage inoculum which is used to inoculate the production stage fermenters.

The production stage fermenters are equipped with devices for controlling and maintaining pH at a predetermined level and for continuous metered addition of nutrient. They are charged with a medium containing soybean flour, glucose, ammonium sulfate, and potassium phosphate, sterilized, and the pH is adjusted to pH

5.8 - 6.2. The fermenters are inoculated with the second stage inoculum described above and incubated at about 25° C with agitation and aeration while controlling and maintaining the mixture at approximately pH 6.0 by addition of a base, for example, sodium hydroxide, potassium hydroxide or preferably ammonium hydroxide, as required from time to time. Addition of a source of assimilable carbon, preferably glucose, is started when the concentration of the latter in the broth has dropped to about 0.5% wt/vol, normally about 48 hrs after the start of fermentation, and is maintained until the end of the particular run. In this manner a fermentation broth containing about 60 $\mu\text{g/ml}$ of rapamycin as determined by the assay method described above is obtained in 4 - 5 days, when fermentation is stopped.

Filtration of the mycelium, mixing the latter with a water-miscible lower alkanol, preferably methanol, followed by extraction with a halogenated aliphatic hydrocarbon, preferably trichloroethane, and evaporation of the solvents yields a first oily residue. This first oily residue is dissolved in a lower aliphatic ketone, preferably acetone, filtered from insoluble impurities, the filtrate evaporated to yield a second oily residue which is extracted with a water-miscible lower alkanol, preferably methanol, and the latter extract is evaporated to yield crude rapamycin as a third oily residue. This third oily residue is dissolved in a mixture of a lower aliphatic ketone and a lower aliphatic hydrocarbon, preferably acetone-hexane, an absorbent such as charcoal or preferably silica gel is added to adsorb the rapamycin, the latter is eluted from the adsorbate with a similar but more polar solvent mixture, for example a mixture as above but containing a higher proportion of the aliphatic ketone, the eluates are evaporated and the residue is crystallized from diethyl ether, to yield pure crystalline rapamycin. In this manner a total of 45-58% of the rapamycin initially present in the fermentation mixture is recovered as pure crystalline rapamycin.

CHARACTERIZATION

a. Purified rapamycin is a colourless crystalline compound, m.p. 183°-185° C after recrystallization from ether;

b. rapamycin is soluble in ether, chloroform, acetone, methanol and dimethylformamide; very sparingly soluble in hexane and petroleum ether and substantially insoluble in water;

c. rapamycin shows a uniform spot on thin layer plates of silica gel G (E. Merck A. G., Darmstadt) developed with a variety of thin layer chromatography solvent systems; for example, ether-hexane 40:60 ($R_f = 0.42$), isopropyl alcohol-benzene 15:85 ($R_f = 0.5$) and ethanol-benzene 20:80 ($R_f = 0.43$);

d. rapamycin obtained from four successive fermentation batches gave the following values on repeated elemental analyses:

	AVERAGE				
C%	67.24	66.14	67.26	66.72	66.84
H%	8.93	8.72	8.92	8.9	8.84
N%	1.39	1.37	1.28	1.38	1.37

e. rapamycin exhibits the following characteristic absorption maxima in its ultraviolet absorption spectrum (95% ethanol):

267 nm ($E_{1cm}^{1\%}$ 417), 277 nm ($E_{1cm}^{1\%}$ 541) and 288 nm ($E_{1cm}^{1\%}$ 416);

f. the infrared absorption spectrum of rapamycin in chloroform is reproduced in FIG. 1 and shows characteristic absorption bands at 3560, 3430, 1730, 1705 and 1630-1610 cm^{-1} ;

Further infrared absorption bands are characterized by the following data given in reciprocal centimeters with (s) denoting a strong, (m) denoting a medium, and (w) denoting a weak intensity band. This classification is arbitrarily selected in such a manner that a band is denoted as strong (s) if its peak absorption is more than 2/3 of the background in the same region; medium (m) if its peak is between 1/3 and 2/3 of the background in the same region; and weak (w) if its peak is less than 1/3 of the background in the same region.

2990 cm^{-1} (m)	1158 cm^{-1} (m)
2955 cm^{-1} (s)	1129 cm^{-1} (s)
2919 cm^{-1} (s)	1080 cm^{-1} (s)
2858 cm^{-1} (s)	1060 cm^{-1} (s)
2815 cm^{-1} (m)	1040 cm^{-1} (m)
1440 cm^{-1} (s)	1020 cm^{-1} (m)
1365 cm^{-1} (m)	978 cm^{-1} (s)
1316 cm^{-1} (m)	905 cm^{-1} (m)
1272 cm^{-1} (m)	888 cm^{-1} (w)
1178 cm^{-1} (s)	866 cm^{-1} (w)

g. the nuclear magnetic resonance spectrum of rapamycin in deuterochloroform is reproduced in FIG. 2;

h. the minimum inhibitory concentration of rapamycin against various microorganism is listed below:

Organisms	Rapamycin: ($\mu g/ml$)
<i>Candida albicans</i> (5 strains)	0.02 to 0.1
<i>C. catenulata</i>	< 0.1
<i>C. lipolytica</i>	2.5
<i>C. stellatoidea</i>	< 0.1
<i>C. tropicalis</i>	0.1
<i>C. pseudotropicalis</i>	> 5.0
<i>C. parapsilosis</i>	< 0.1
<i>C. morrera</i>	< 0.1
<i>C. intermedia</i>	< 0.1
<i>M. gypseum</i>	12.5
<i>T. mentagrophytes</i>	> 1000

i. rapamycin exhibits a LD_{50} (i.p., mice) of 597.3 ± 28.1 mg/kg and a LD_{50} (p.o., mice) of $>2,500$ mg/kg.

In protection studies, mice were infected by intravenous injection of *C. albicans* ATCC 11651. At 1, 4 and 24 hours after infection, mice were administered 10 mg/kg (s.c.) of rapamycin. At this dose 50% of the mice were protected. Treatment with 25 mg/kg (s.c.) offered complete protection. When rapamycin was administered orally, at 10 mg/kg 4 out of 10 mice survived, and at 25 mg/kg complete protection was observed.

A 1% suspension (0.2 ml) of rapamycin in water containing 1.5% polysorbate 80 (Tween 80), when injected intradermally into a rabbit's ear caused no irritation. Similarly, two drops of a 0.5% suspension applied to a rabbit's eye caused no irritation.

The following Examples illustrate further this invention.

EXAMPLE I

Microorganism

Streptomyces hygroscopicus NRRL 5491 was grown and maintained on oatmeal-tomato paste agar slants (T. G. Pridham, et al., Antibiotic Annual 1956-1957, Medical Encyclopedia Inc., New York, p. 947) and in Roux bottles containing the same medium. Good growth was obtained after 7 days of incubation at 28°C. Spores from one Roux bottle were washed off and suspended into 50 ml of sterile distilled water. This suspension was used to inoculate the first stage inoculum.

The first-stage inoculum medium consisted of Emerson broth [R. L. Emerson et al., J. Bacteriol., 52, 357 (1946)] 0.4%; peptone, 0.4%; sodium chloride, 0.25%; yeast extract, 0.1%; and glucose, 1%; pH 7.0; flasks containing the above medium were inoculated with 1% of the spore suspension described above. The inoculated flasks were incubated for 30 hrs. at 28°C on a reciprocating shaker set at 65 r.p.m. (4 inch stroke).

Production stage

The production stage was run in 250-liter New Brunswick fermenters Model F-250, equipped with automatic antifoam addition system and pH recorder-controller. The fermenters were charged with 160 liters of an aqueous production medium (8 KM) consisting of the following constituents:

soluble starch	1.0%
$(NH_4)_2SO_4$	0.5%
K_2HPO_4	0.5%
glucose (Cerelease)	1.5%
$MgSO_4$	0.025%
$ZnSO_4$	0.005%
$MnSO_4$	0.001%
$FeSO_4 \cdot 7H_2O$	0.002%
$CaCO_3$	0.2%
"Blackstrap" molasses	0.5%
hydrolyzed casein (NZ-Case, Sheffield Chemical, Norwich, New York)	0.5%
lard oil (Larex No. 1, Swift Canadian Co., Toronto)	0.2%
pH 7.1 to 7.3	

The fermenters were sterilized at 121°C for 45 minutes, cooled and inoculated with one flask (2% inoculum) of first stage inoculum. Incubation temperature: 28°C; aeration: 0.5 vol/vol/min.; agitation: 250 r. p. m.

A titre of ca. 20 $\mu g/ml$, determined by microbiological assay on agar plates seeded with *Candida albicans*, was reached in 5 days. The fermentation was stopped.

Extraction and isolation of the antibiotic was performed by one of the following methods:

EXTRACTION

a. The fermentation mixture was extracted twice with 1 v/v of n-butanol. The combined butanol extracts were washed with 1 v/v of water, dried with anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a residue. The oily residue was extracted three times with 2 litres of methanol. The combined methanol extracts were passed through diatomaceous earth ("Celite") and evaporated to dryness to yield an oily residue containing crude rapamycin.

b. The fermentation mixture was filtered over diatomaceous earth ("Celite"). The filtrate was extracted twice with 1 v/v of ethyl acetate. The ethyl acetate

extracts were washed with 1 volume of water, dried with anhydrous sodium sulfate and evaporated under reduced pressure to dryness. The residue was extracted twice with 1 liter of methanol. The methanol extracts were evaporated under reduced pressure to yield an oily residue containing crude rapamycin.

c. The mycelium obtained as described under section (b) was washed with 1 to 2 volumes of water. The washed mycelium was extracted three times with 5 volumes of methanol per weight of wet mycelium each time. The methanolic extracts were pooled and concentrated under reduced pressure to a small volume of an aqueous phase containing approximately 10% v/v of methanol. This aqueous phase was extracted three times with 1 vol. of methylene chloride; the methylene chloride extracts were combined, dried with anhydrous sodium sulfate and evaporated to yield an oily residue.

The oily residue was diluted with 1 volume of petroleum ether, and 30% w/v of charcoal (Darco G60) was added. The mixture was stirred for half an hour and filtered. The charcoal, which retained substantially all of the product, was washed twice with one volume of petroleum ether. The charcoal was eluted three times with 5 vol. (based on the weight of the charcoal) of a mixture of methylene chloride and ether (50:50). The methylene chloride-ether extracts were evaporated to dryness and the residue dissolved in a small amount of ether. The crude product was obtained by precipitation from the ether solution with cold petroleum ether.

Alternatively, the oily residue obtained by any of the extraction procedures described above was diluted with 1 vol. of hexane and passed through a preparative column of silica gel G. The product was absorbed on the column. The silica gel G containing absorbed product was washed with several volumes of hexane and 50:50 hexane-ether mixtures. The product was eluted from the column with ether. The ether eluant was evaporated to a small volume and crude product obtained by precipitation from the ether solution with cold petroleum ether.

Purification

The aforementioned crude product was purified further by column chromatography on silica gel G Merck (50:1 w/v) in hexane-ether (50:50). The product was eluted from the column with ether. The ether eluate was evaporated to a small volume. Purified rapamycin was precipitated with petroleum ether. The analytical samples were prepared by crystallization from ether, m.p. 183°-185° C.

EXAMPLE 2

Streptomyces hygroscopicus NRRL 5491 is grown and spores are obtained in the same manner as described in Example 1.

First Stage Inoculum

Erlenmeyer flasks (500 ml) are filled with 100 ml of the following medium:

Soybean flour (Archer-Daniels Co., Midland, Mich., "Special X")	= 4%	wt/vol
Glucose (Cerelease)	= 2%	wt/vol
Ammonium sulfate	= 0.3%	wt/vol
Calcium carbonate	= 0.15%	wt/vol
Water to volume, pH 7.1 to 7.3		

The flasks are sterilized at 121° for 35 minutes and cooled to 25°. The flasks are inoculated with 4% (4 ml) of spore suspension described above and incubated on a gyrotary shaker (2 inch stroke) at 240 rpm for 24 hrs at 25° C.

Second Stage Inoculum

Twenty-four liter flat bottom flasks containing 3.2 l of the inoculum medium described above at pH 7.1 - 7.3 are sterilized by autoclaving at 121° for 35 minutes, shaken to resuspend the insoluble material and re-sterilized for another 45 minutes. The flasks are cooled to 25° and inoculated with 64 ml of first stage inoculum, placed on a reciprocating shaker (4 inches stroke) set at 65 rpm and incubated for 18 hrs at 25° C.

Production Stage

The production stage is run in 250 liter New Brunswick fermenters Model F-250 equipped with automatic antifoam addition system and pH recorder-controller. The fermenters are charged with 160 liters of an aqueous production medium consisting of the following constituents:

Soybean flour (Archer-Daniels Co., Midland, Mich., "Special X")	= 3%	wt/vol
Glucose (Cerelease)	= 2%	wt/vol
Ammonium sulfate	= 0.1%	wt/vol
Potassium phosphate (monobasic)	= 0.5%	wt/vol
Antifoaming Agent ("DF-143 - PX" Mazer Chemicals, Inc., Gurnee, Ill.)	= 0.05%	wt/vol

The fermenters are sterilized at 121° C for 30 minutes, cooled, and the pH is adjusted to 5.8 to 6.2 with ammonium hydroxide. They are then inoculated with one flask (2%) of second stage inoculum and fermentation is allowed to proceed at 25° C, with aeration at 0.25 v/v/min and agitation at 200 rpm.

The pH of the fermentation broth starts to drop at 30 - 35 hours and is controlled at 6.0 until the end of fermentation by the automatic, on demand, addition of ammonium hydroxide. At about 48 hrs. of propagation the glucose concentration in the broth drops to about 0.5%, and continuous addition of 40% glucose solution is started at a rate of 3.75% of fermentation mixture volume per day and continued until the end of fermentation. A titer of about 60 µg/ml. determined by microbiological assay on agar plates seeded with *Candida albicans* is reached in 4 to 5 days. The fermentation is stopped at this point.

Extraction and isolation of the antibiotic is performed by the following procedure:

The fermentation mixture is filtered over diatomaceous earth (Celite) to recover the mycelium. A typical 400 liter batch obtained from three fermenters yields about 60 kg of wet mycelium. The wet mycelium is mixed with 1 vol/wt of methanol by agitation and the mixture is extracted twice with 2 vol of trichloroethane (methyl chloroform), yielding about 250 liters of trichloroethane extract containing about 22 - 24 g of rapamycin. The trichloroethane extract is evaporated to dryness under reduced pressure to yield 1 to 1.4 kg of oily residue. This residue is added slowly with agitation to 5 vols of acetone and the resulting precipitate is separated by filtration. The acetone solution is evaporated to dryness under reduced pressure to yield an oily residue. This oily residue is extracted twice with 2 and 1 vols of methanol respectively. The combined methanol extracts are filtered and the remaining oil is discarded. The methanol extract containing rapamycin is

evaporated to dryness under reduced pressure to yield 200 to 300 g of oily residue. This residue is dissolved in 5 v/wt of 15% acetone in hexane. To this solution of the oily residue, silica gel (Merck) is added in an amount equal to twice the weight of the oily residue and the mixture is stirred for about one hr. The mixture is filtered on a sintered glass funnel and the filtrate rejected. The silica gel containing rapamycin is washed with several volumes of 15% acetone in hexane. The washed silica gel is eluted with 30% acetone in hexane. The eluant is evaporated to dryness to yield about 60 to 150 g of dry residue. The dry residue is dissolved in ether and pure rapamycin is separated by crystallization. A typical run containing about 24 g of crude rapamycin yields between 10 and 14 g of pure crystalline product.

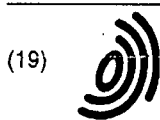
We claim:

1. A pharmaceutical composition which comprises a pharmaceutically acceptable carrier and an antifungally effective amount of the antibiotic rapamycin which

- a. is a colourless, crystalline compound with a melting point of 183° to 185° C, after recrystallization from ether;
- b. is soluble in ether, chloroform, acetone, methanol and dimethylformamide, very sparingly soluble in hexane and petroleum ether and substantially insoluble in water;
- c. shows a uniform spot on thin layer plates of silica gel;
- d. has a characteristic elemental analysis of about C, 66.84%; H, 8.84% N, 1.37%;
- e. exhibits the following characteristic absorption maxima in its ultraviolet absorption spectrum (95% ethanol): 267 nm ($E_{1cm}^{1\%}$ 417), 277 nm ($E_{1cm}^{1\%}$ 541) and 288 nm ($E_{1cm}^{1\%}$ 416);

- f. has a characteristic infrared absorption spectrum shown in accompanying FIG. 1;
 - g. has a characteristic nuclear magnetic resonance spectrum as shown in accompanying FIG. 2;
 - h. has a minimum inhibitory concentration of 0.02 to 0.1 μ g/ml against *Candida albicans*; and
 - i. exhibits a LD_{50} (i.p., mice) of 597.3 ± 28.1 mg/kg and a LD_{50} (p.o., mice) of $>2,500$ mg/kg.
2. A method of inhibiting the growth of pathogenic fungi in a mammal which comprises administering to said mammal an antifungally effective amount of the antibiotic rapamycin which
- a. is a colourless, crystalline compound with a melting point of 183° to 185° C, after recrystallization from ether;
 - b. is soluble in ether, chloroform, acetone, methanol and dimethylformamide, very sparingly soluble in hexane and petroleum ether and substantially insoluble in water;
 - c. shows a uniform spot on thin layer plates of silica gel;
 - d. has a characteristic elemental analysis of about C, 66.84%; H, 8.84%; N, 1.37%;
 - e. exhibits the following characteristic absorption maxima in its ultraviolet absorption spectrum (95% ethanol): 267 nm ($E_{1cm}^{1\%}$ 417), 277 nm ($E_{1cm}^{1\%}$ 541) and 288 nm ($E_{1cm}^{1\%}$ 416);
 - f. has a characteristic infrared absorption spectrum shown in accompanying FIG. 1;
 - g. has a characteristic nuclear magnetic resonance spectrum as shown in accompanying FIG. 2;
 - h. has a minimum inhibitory concentration of 0.02 to 0.1 μ g/ml against *Candida albicans*; and
 - i. exhibits a LD_{50} (i.p., mice) of 597.3 ± 28.1 mg/kg and a LD_{50} (p.o., mice) of $>2,500$ mg/kg.

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(54) New extractive process for the recovery of naturally occurring macrolides

Extraktives Verfahren zur Gewinnung von natürlichen Makroliden

Procédé d'extraction pour la récupération de macrolides naturels

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Description

[0001] This invention relates to an extractive process for the recovery of naturally occurring macrolides, more particularly to a process for separating a neutral macrolide, which is rapamycin or a naturally occurring homolog or analog thereof such as, 32-desmethyrapamycin and 15-deoxorapamycin, from other naturally occurring components that are obtained by fermentation processes. More specifically, this invention relates to a process for recovering the tricyclic macrolide rapamycin from fermentation broth extract concentrate and from mother liquor concentrate.

[0002] The tricyclic macrolides exemplified by rapamycin have immunosuppressant activity as well as antibiotic and other pharmacological activities and are useful in treating graft and transplant rejections, diseases of inflammation, and autoimmune diseases such as lupus, rheumatoid arthritis, diabetes mellitus and multiple sclerosis.

[0003] The tricyclic macrolides rapamycin, 32-desmethyrapamycin and 15-deoxorapamycin and are produced by fermentation of various strains of *Streptomyces* under the proper conditions and are neutral as there is no basic amino group, phenolic or carboxylic acid groups present. Rapamycin is produced by culturing *S. hygroscopicus* NRRL 5491 in an aqueous medium. Mycelia containing the tricyclic macrolide are recovered from the growth medium and extracted with an organic solvent such as methanol to obtain a mixture comprised of the desired tricyclic macrolide, related compounds, acidic compounds such as fatty acids, basic compounds such as alkaloids and peptides, and neutral lipophilic compounds such as fats. Typically, the fermentation broth extract is concentrated to facilitate transportation and/or storage until the macrolide can be isolated. Isolation and purification of the tricyclic macrolides from the fermentation broth extract has been, prior to this invention, a laborious expensive process employing various chemical and chromatographic techniques to obtain purified material. [U.S. 5,091,389; U.S. 3,993,749; WO 93/11130; Sehgal, J. of Antibiotics 28(10)727(1975)]. The fermentation broth extract concentrate for rapamycin contains only 5 to 15% rapamycin and up to about 50% acidic components, for example, and rapamycin must be separated from the other components. Typically, processes for recovery of the tricyclic macrolides from fermentation broth extracts involves adsorption on and desorption from activated carbon, selective solubility procedures, and one or more time consuming and expensive chromatographic procedures using column chromatography and/or high pressure liquid chromatography. Heretofore, acidic or basic conditions have been avoided as tricyclic macrolides such as rapamycin are considered to be unstable under acidic or basic conditions. Rapamycin in water-miscible solution, i.e., in methanol or tetrahydrofuran, undergoes degradation by inorganic bases such as aqueous sodium hydroxide, organic bases such as 4-dimethylaminopyridine (DMAP) or 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) or aqueous mineral acids such as hydrochloric acid and Lewis acids such as zinc chloride [Steffan *et al.*, Tetrahedron Letters 34(23), 3699-3702 (1993), D. Yohanes and S. J. Danishefsky, Tetrahedron Letters 33(49), 7469-7472 (1992); Luengo *et al.*, Tetrahedron Letters 34(6), 991-994 (1993) and D. Yohannes *et al.*, Tetrahedron Letters 34(13), 2075-2078 (1993)].

[0004] This invention provides a relatively fast and efficient process for recovery of rapamycin or a naturally occurring homolog or analog thereof, from fermentation broth extract concentrates and mother liquors or concentrates thereof obtained from recrystallization solvents, triturations and product washings and avoids the time consuming and expensive chromatographic separations exemplified in U.S. 5,091,389; U.S. 3,993,749; WO 93/11130; and Sehgal, J. of Antibiotics 28(10)727(1975).

[0005] Accordingly this invention provides a process for separating a neutral macrolide which is rapamycin or a naturally occurring homolog or analog thereof from acidic, basic and non-polar neutral impurities present in a concentrate of fermentation broth extracts or mother liquors containing said neutral macrolide which comprises in any order extraction step (a) and optionally one or both of steps (b) and (c) as follows:

(a) a solution of said concentrate in a water-immiscible solvent is extracted at a temperature from about -5°C to about 45°C with aqueous base to substantially remove all acidic impurities;

(b) a solution of said concentrate in a water-immiscible solvent is extracted at a temperature from about -5°C to about 45°C with aqueous acid to substantially remove all basic impurities;

(c) a solution of said concentrate is subjected to one or more of the following in order to remove non-polar neutral impurities:

- (i) extraction with a non-aromatic hydrocarbon solvent in which the macrolide is insoluble,
- (ii) treatment with a sufficient quantity of a miscible macrolide non-solvent to cause the macrolide to become insoluble in the resulting solvent mixture and thus separate from the solution whereby the macrolide can be separated from the solvent mixture,
- (iii) trituration with a macrolide crystallizing solvent.

[0006] In one aspect the process comprises separation of the acidic and optionally basic components from the neutral

components by dissolving the macrolide-containing concentrate in a suitable water-immiscible solvent and extracting the acidic and optionally basic components into aqueous base and if desired acid respectively, and employing selective solubility or extraction techniques to separate the macrolide from the non-polar neutral materials present in the concentrate. While the process described herein is particularly suited to concentrates of fermentation broth extracts or mother liquors, the whole extract solution or mother liquor solutions can be used in the process of this invention provided the solvent or solvent mixture used for the fermentation broth extraction or recrystallization, trituration or washings is amenable to the process and the volume of the solvent is not cumbersome. Solvent volume may be reduced by partial concentration. Any of the solutions to which the process of this invention may be applied may be referred to as a macrolide containing solution or concentrate.

[0007] The product obtained by said process can be purified to acceptable purity by standard procedures known to those skilled in the art.

[0008] As used herein, a non-polar solvent is a non-aromatic hydrocarbon solvent such as cyclohexane, cyclohexene, hexane, heptane, pentane. Solvents which are immiscible with the non-aromatic hydrocarbon solvent include but are not limited to acetonitrile and dimethylformamide. The term extraction refers to the procedure of thoroughly mixing one solution with another immiscible solution, allowing the immiscible solutions to separate one from the other and physically removing one layer or phase from the other, and repeating if required. The term wash when referring to a solution refers to the extraction procedure and when referring to a solid, means to rinse the solid with a solvent in which the solid is substantially insoluble. The term mother liquor refers to the organic solvent solutions obtained from crystallization filtrates, washings and back extractions of aqueous extracts and washings and triturations of collected solids. A macrolide solvent is a solvent or solvent mixture which will dissolve the macrolide and accompanying impurities such as the acidic or basic components. A macrolide non-solvent is a solvent or solvent mixture in which the macrolide is substantially insoluble but one in which neutral components such as fats are soluble. A macrolide crystallizing solvent is a solvent or solvent mixture from which the macrolide can be recrystallized or crystallized from an amorphous state upon trituration. Where the process requires the concentration of a solution, it is preferred that the solvent or solvent mixture have sufficient volatility so as to distill off under non-degrading conditions of temperature and pressure.

[0009] This invention also provides an optional step wherein the non-polar neutral components of the macrolide-containing fermentation broth extract concentrate or mother liquor concentrate are removed by extraction of a solution of said concentrate in a first solvent (e.g. acetonitrile, DMF) with a second non-aromatic hydrocarbon solvent immiscible with the first solvent and in which the macrolide is insoluble (e.g. cyclohexane, cyclohexene, hexane, heptane or pentane).

[0010] Also provided is the optional step wherein the water-immiscible solution containing the macrolide after extraction of acid and optionally basic components is either

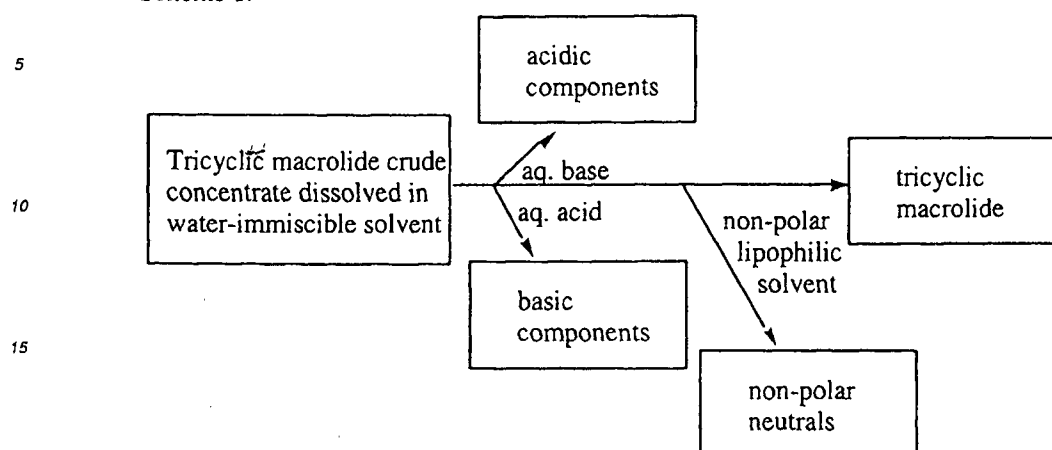
(i) treated with a sufficient quantity of a miscible macrolide non-solvent to cause the macrolide to become insoluble in the resulting solvent mixture and thus separate from the solution whereby the macrolide can be separated from the solvent mixture or

(ii) concentrated and the residue crystallized by admixture with a crystallizing solvent or solvent mixture.

[0011] The water-immiscible solution containing the macrolide after extraction of acid and if desired base components may also be concentrated and the residue dissolved in a first solvent and extracted with a second non-aromatic hydrocarbon solvent immiscible with the first solvent and in which the macrolide is insoluble to remove the neutral non-polar impurities.

[0012] A preferred process of this invention is outlined in the Scheme 1 below:

Scheme 1.



[0013] According to the process, the concentrate containing the macrolide, whether from fermentation broth extract concentrate or concentrates from recrystallization and/or wash solvents, is dissolved in a water-immiscible solvent or solvent mixture selected for ability to dissolve the concentrate and ease of removal, including but not limited to dichloromethane, t-butyl methyl ether, ethyl acetate, toluene, 1-butanol, an ethyl acetate/toluene mixture, a heptane/ethyl acetate mixture, or a hexane/methylene chloride mixture. Aqueous solutions of base, including sodium hydroxide, sodium bicarbonate, sodium carbonate, ammonium hydroxide, preferably sodium hydroxide, in concentrations ranging from 0.1 to 5N, preferably from about 0.1 to 1.0N, most preferably 0.1 to 0.5N, are useful for extracting the acidic components. Organic bases such as triethylamine are not as effective as the stronger aqueous inorganic bases in removing the acidic components. Aqueous solutions of mineral acid, including hydrochloric acid, monopotassium phosphate, monosodium sulfate, preferably hydrochloric acid, in concentrations of from 0.1 to 5N, are useful for extracting the basic components. Organic acids, such as trifluoroacetic acid are not as effective as the mineral acids in removing the basic components. Extractions of acidic and if desired basic components from the solution containing the macrolide are conveniently carried out between -5°C and 45°C, preferably -5°C and 30°C, most preferably in the range of -5 to 10°C. To avoid possible degradation of the macrolide by the acid or base, the extraction process should be completed without delay. Depending on the amounts of basic components present, extraction of bases may not be necessary and extraction of the acidic components alone may be sufficient in removal of enough impurity from the macrolide containing solution to permit isolation of the tricyclic macrolide.

[0014] It is advantageous to remove the acidic (or if desired basic) components from the solution of macrolide-containing concentrate in one time-saving extraction step. The amount of aqueous base (or acid) needed so that excess base (or acid) is available in the extraction process can be determined by extracting an aliquot taken from the solution of the macrolide containing concentrate with aqueous base (or acid) and determining the volume of aqueous base (or acid) required to give an extract of the aliquot having a pH such that a stoichiometric excess of base (or acid) is available. The excess of acid or base is determined by using a pH meter or other means of determining pH. The volume of aqueous base (or acid) needed to extract the solution from which the aliquot was taken is then proportional to the ratio of the volumes of neutral macrolide containing solution to be extracted and the aliquot taken therefrom. Thus extraction of acidic (or basic) components can be done in one operation rather than performing multiple extractions with less than sufficient amounts of aqueous base (or acid). Obviously where both acidic and basic components are to be removed from the solution of the macrolide containing concentrate, separate extractions with aqueous base and aqueous acid respectively will have to be performed. With solutions of rapamycin containing concentrate, for instance, one extraction with aqueous sodium hydroxide solution sufficient to have a final pH of 12 is sufficient to remove substantially all of the acidic components.

[0015] The macrolide recovered from solution using the above processes can be purified to the degree of purity desired by conventional purification techniques known to those skilled in the art. The filtrates and washings may be reworked to recover additional macrolide if desired.

[0016] After performing the extraction procedures to remove the acidic and if desired basic components from the water-immiscible solvent solution containing the macrolide, the macrolide may be separated from the non-polar neutral components by one of the following methods:

(1) A macrolide non-solvent (or solvent mixture), miscible with said macrolide containing water-immiscible solvent solution from which the acidic and if desired basic components were extracted, is added to the macrolide containing solution in sufficient quantity so as to render the macrolide and perhaps macrolide-related products insoluble in the resulting solution and form a separate phase, either an oil or a solid, which may then be separated by ordinary separation techniques known to those skilled in the art.

(2) The water-immiscible solvent solution containing the neutral macrolide from which the acidic and/or polar components were extracted is concentrated and the residual material containing the macrolide is dissolved in a macrolide dissolving solvent such as acetonitrile or dimethylformamide and extracted with a non-aromatic hydrocarbon solvent such as cyclohexane, hexane, heptane or cyclohexene. The macrolide-dissolving solvent layer is separated and concentrated. The residue containing the macrolide is then triturated with a crystallizing solvent to obtain the macrolide if said macrolide is a solid or purified by techniques known to those skilled in the art such as chromatography if the macrolide is an oil. Alternatively, the macrolide-dissolving solvent, after extraction with the non-aromatic hydrocarbon solvent may be treated with a miscible non-macrolide dissolving solvent as in procedure (1) above.

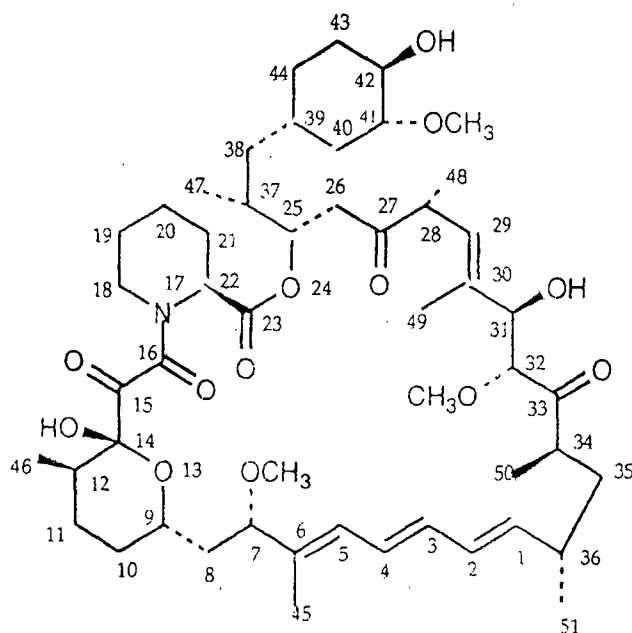
(3) The macrolide containing water-immiscible solvent solution from which the acidic and if desired basic components were extracted is concentrated and the residual material is triturated with macrolide crystallizing solvent such as diethyl ether, diisopropyl ether or t-butyl methyl ether.

[0017] Alternatively, the macrolide containing concentrate can be extracted first according to one of methods (1) to (3) above to remove non-polar components and then the residue containing the macrolide dissolved in a water-immiscible solvent if not already in such a solvent and extracted with aqueous base or acid in either order to remove the acidic or acidic and basic components.

[0018] The above process takes advantage of the unexpected finding that macrolides such as the tricyclic macrolide rapamycin do not decompose when subjected to aqueous acid or base extraction procedures under proper temperature conditions from water-immiscible solvent solutions of macrolide containing concentrates. It was previously thought that degradation would occur, based on the observed degradation of rapamycin that results from acid or base exposure in solution.

[0019] This extraction process greatly shortens the time necessary to recover macrolides from said concentrates and avoids the time-consuming and expensive chromatographic processes.

[0020] The tricyclic macrolide rapamycin is a crystalline solid soluble in methanol, acetone, dimethylformamide, slightly soluble in diethyl ether and is sparingly soluble in hexane or petroleum ether and is insoluble in water. Rapamycin has the structure shown below. The atom numbering system is that used by Chemical Abstracts.



[0021] The structures of 32-desmethylrapamycin and 15-deoxorapamycin can easily be discerned from the above structure of rapamycin.

[0022] The following examples are merely illustrative of the process of the present invention in isolating rapamycin from fermentation broth and mother liquor concentrates and are not to be construed as limiting the scope of this invention in any way. In the following examples, the identity of the product isolated was confirmed as rapamycin through comparison of physical, spectral and chromatographic properties to those of authentic rapamycin. The purity of the product (unrecrystallized) was determined by high pressure liquid chromatographic analysis.

Example 1

[0023] A solution of concentrated fermentation broth extract (157.0 g, 10.4% rapamycin content) taken up in methylene chloride (600 mL) was washed with three 150 mL portions of 0.5 N NaOH solution at 0-5°C, washed with water until the wash is neutral, and then washed with brine. The methylene chloride solution was concentrated and the residue (70.5 g) triturated with diethyl ether (140 mL). The crystalline solid was collected, washed with diethyl ether and dried to obtain rapamycin (6.3 g, 91.7% purity, 35.4% yield). Concentration of the diethyl ether filtrates gave 63.5 g of oil having a 13.1% rapamycin content.

Example 2

[0024] A solution of concentrated fermentation broth extract (206.0 g, 11.8% rapamycin content) was taken up in t-butyl methyl ether (800 mL) and washed with three 400 mL portions of 0.5N NaOH solution at 0-5°C and then washed with water until the wash was neutral. The t-butyl methyl ether solution was concentrated and the residue (75.0 g) triturated with diethyl ether (150 mL). The crystalline solid was collected, washed with diethyl ether, and dried to obtain rapamycin (11.4 g, 92.2% purity, 43.3% yield). Concentration of the diethyl ether filtrates gave 58.7 g of oil having a 11.3% rapamycin content.

Example 3

[0025] A solution of concentrated fermentation broth extract (10.58 g, 10.4% rapamycin content) taken up in acetonitrile (23 mL) was washed with two 23 mL portions of cyclohexane and then concentrated. The residue was dissolved in dichloromethane and washed sequentially with three 23 mL portions of 0.5 N NaOH solution at 0-5°C, two 23 mL portions of 0.5 N HCl at 0-5°C, and then with water until the wash was neutral. The dichloromethane solution was concentrated and the residue (4.07 g) triturated with diethyl ether. The crystalline solid was collected, washed with diethyl ether, and dried to give rapamycin (0.64 g, 89.5% purity, 58.2% yield). The diethyl ether filtrates were concentrated to obtain 3.36 g of oil having a 9.8% rapamycin content.

Example 4

[0026] A solution of concentrated fermentation broth extract (100.0 g, 11.8% rapamycin content) taken up in ethyl acetate (400 mL) was washed with one 200 mL portion and two 100 mL portions of 0.5 N NaOH solution at 0-5°C and then washed with two 200 mL portions of 0.5 N hydrochloric acid solution at 0-5°C and finally washed with water until the wash was neutral. The aqueous washings were back extracted with ethyl acetate (100 mL) and the ethyl acetate solutions combined. The ethyl acetate solution was concentrated and the residue (43.2 g) triturated with diisopropyl ether (45 mL). The crystalline solid was collected, washed with diisopropyl ether and dried to yield 9.5 g of rapamycin (85.3% purity, 68.7% yield). The diisopropyl ether filtrates were concentrated to give an oil (31.6 g) with a 6.6% rapamycin content.

Example 5

[0027] A solution of concentrated fermentation broth extract (25.2 g, 10.4% rapamycin content) taken up in a mixture of toluene (120 mL) and ethyl acetate (25 mL) was washed sequentially with three 50 mL portions of 0.5 N NaOH solution at 0-5°C, twice with two 50 mL portions of 0.5 N hydrochloric acid at 0-5°C, and then with water until the wash was neutral. The toluene/ethyl acetate solution (125 mL) was divided into two equal portions for further treatment by the following methods:

Method A. The toluene/ethyl acetate solution (64 mL) was concentrated and the residue (5.7 g) triturated with diethyl ether (11 mL). The crystalline solid was collected, washed with additional diethyl ether, and dried to give 0.84 g of rapamycin (92.7% purity, 64.1% yield). Concentration of the diethyl ether filtrates gave 4.3 g of an oil

having a 6.8% rapamycin content.

Method B. The toluene/ethyl acetate solution (64 mL) was concentrated and the residue (9.0 g) was dissolved in acetonitrile (50 mL). The acetonitrile solution was washed with two 25 mL portions of cyclohexane. The acetonitrile solution was then concentrated and the residue (4.3 g) triturated with diethyl ether (11 mL). The crystalline solid was collected, washed with diethyl ether, and dried to yield 0.81 g of rapamycin (94.6% purity, 61.8% yield). Concentrations of the diethyl ether filtrates gave 3.0 g of an oil having a 5.9% rapamycin content.

Example 6

[0028] Mother liquor concentrate (996.0 g, 21.6% rapamycin content) was triturated with t-butyl methyl ether (4000 mL). The crystalline solids were collected, washed with t-butyl methyl ether (500 mL), and dried to yield 36.2 g of rapamycin (95.1% purity, 13.8% yield). The filtrates were washed with one 2000 mL portion and two 1000 mL portions of 0.5 N sodium hydroxide solution at 0-5°C. The combined base extract was washed once with t-butyl methyl ether (500 mL). The t-butyl methyl ether filtrate and extract were combined and washed with water until the wash was neutral. The water extracts were combined and extracted with t-butyl methyl ether (500 mL). The t-butyl methyl ether solutions were combined, concentrated, and the residue (377.1 g) triturated with diisopropyl ether (350 mL). The crystalline solids were collected, washed with diisopropyl ether, and dried to give 126.7 g of rapamycin (82.4% purity, 58.9% yield). Thus the total recovery of rapamycin from the mother liquor concentrate was 162.9 g (72.7%). Concentration of the diisopropyl ether filtrates gave 157.9 g of oil having a 20.9% rapamycin content.

Example 7

[0029] A solution of mother liquor concentrate (562.1 g, 21.6% rapamycin content) taken up in dichloromethane (2000 mL) was washed with three 500 mL portions of 0.5 N sodium hydroxide solution at 0-5°C and the combined aqueous basic extracts were extracted with one 200 mL portion of dichloromethane. The organic solutions were combined and washed with two 500 mL portions of 0.5 N hydrochloric acid solution at 0-5°C. The combined aqueous acid extracts were extracted with one 200 mL portion of dichloromethane. The combined organic extracts were washed with water until the wash water was neutral. The organic solution was concentrated and the residue (255.0 g) was triturated with diisopropyl ether (250 mL). The crystalline solids were collected, washed with diisopropyl ether and dried to give 108.6 g of rapamycin (86.6% purity, 77.4% recovery). Concentration of the diisopropyl ether filtrates gave 100.2 g of oil having a 23.1 % rapamycin content.

Example 8

[0030] Aqueous 0.5 N sodium hydroxide (400 mL) at 0-5°C was added to a vigorously stirred chilled (0-5°C) solution of concentrated fermentation broth extract (198.5 g, 8.3% rapamycin content) taken up in 800 mL of t-butyl methyl ether at such a rate so that the temperature could be maintained at 0-5°C. After vigorous stirring for 5 minutes, the lower aqueous basic layer was removed and stored at 0-5°C. The organic layer was re-extracted with two 200 mL portions of 0.5 N sodium hydroxide solution at 0-5°C. The aqueous basic extracts were combined and re-extracted with t-butyl methyl ether (200 mL). The t-butyl methyl ether solutions were combined and washed with water until the wash was neutral (pH=7). The aqueous washes were combined and extracted with t-butyl methyl ether (100 mL). The t-butyl methyl ether solutions were combined, washed with saturated aqueous sodium chloride solution, and concentrated under vacuum at 40°C. The residue was triturated with diisopropyl ether (85 mL) at 20-25°C for a minimum of one hour and the mixture cooled to 0-5°C overnight. The crystalline solid was collected using a sintered glass Buchner funnel and washed with a 4:1 mixture of diisopropyl ether - t-butyl methyl ether at 20-25°C (5 x 20 mL or until filtrate was colorless). The crystalline solid is dried to constant weight to yield 12.0 g of rapamycin (91.2% purity, 66.4% recovery). Concentration of the t-butyl methyl ether filtrates and washing gave 63.9 g of a gum having a 3.63% rapamycin content.

Example 9

[0031] Mother liquor concentrate (200 g, 25% rapamycin content) was dissolved with stirring in t-butyl methyl ether (800 mL) at room temperature. The stirred solution was cooled to 0-5°C and extracted without delay with 270 mL of 0.65 N sodium hydroxide solution, pre-cooled to 0-5°C, while maintaining the temperature of the mixture at 0-5°C. (The amount of aqueous sodium hydroxide necessary to have a final pH of 12 in the extract was determined on an aliquot of the concentrated mother liquor.)

[0032] The aqueous base layer was stored at 0-5°C while the organic layer was washed with 5% sodium chloride solution while maintaining the mixture at 0-5°C. The aqueous basic extract was back extracted with t-butyl methyl ether

(100 mL). The organic layers were combined and washed with three 200 mL portions of 5% sodium chloride solution (pH of final wash solution was 7.4). The organic solution was concentrated under reduced pressure (60-130 mmHg) at a temperature of 25-40°C. Cyclohexene (80 mL) was added slowly over 30 minutes to the residue with stirring at room temperature and stirred until crystallization was complete (3 hours). The mixture was stirred at room temperature for one hour more and the mixture chilled to 0-5°C and stirred overnight. The off-white crystalline solid was then collected on a fritted glass Buchner funnel. The solid was washed five times with 40 mL portions of a 2:3 mixture of t-butyl methyl ether and cyclohexene. The solid was dried to constant weight in a vacuum oven at 35-40°C to obtain 22.7 g of rapamycin (90.6% pure, 41.4% recovery). Concentration of the filtrates and washings (t-butyl methyl ether and cyclohexene) gave 60.1 g of an oil having a 37.5% rapamycin content.

Claims

1. A process for separating a neutral macrolide, which is rapamycin or a naturally occurring homolog or analog thereof, from acidic, basic and non-polar neutral impurities present in a concentrate of fermentation broth extracts or mother liquors containing said neutral macrolide which comprises in any order extraction step (a) and optionally one or both of steps (b) and (c) as follows:
 - (a) a solution of said concentrate in a water-immiscible solvent is extracted at a temperature from about -5°C to about 45°C with aqueous base to substantially remove all acidic impurities;
 - (b) a solution of said concentrate in a water-immiscible solvent is extracted at a temperature from about -5°C to about 45°C with aqueous acid to substantially remove all basic impurities;
 - (c) a solution of said concentrate is subjected to one or more of the following in order to remove non-polar neutral impurities:
 - (i) extraction with a non-aromatic hydrocarbon solvent in which the macrolide is insoluble,
 - (ii) treatment with a sufficient quantity of a miscible macrolide non-solvent to cause the macrolide to become insoluble in the resulting solvent mixture and thus separate from the solution whereby the macrolide can be separated from the solvent mixture,
 - (iii) triturate with a macrolide crystallizing solvent.
2. A process according to Claim 1 in which in step (c) the solution of said macrolide is a solution in a water immiscible solvent.
3. A process according to Claim 1 or Claim 2 in which the extraction of acidic and/or basic impurities is carried out at a temperature from about -5°C to about 10°C.
4. A process according to any one of claims 1 to 3 wherein the non-polar neutral components of the neutral macrolide-containing fermentation broth extract concentrate or mother liquor concentrate are removed by extraction of a solution of said concentrate in a first solvent with a second non-aromatic hydrocarbon solvent immiscible with the first solvent and in which the neutral macrolide is insoluble.
5. A process according to Claim 1 or Claim 2 wherein the water-immiscible solution containing the neutral macrolide after extraction of acid and if desired basic components is treated with a sufficient quantity of a miscible macrolide non-solvent to cause the macrolide to become insoluble in the resulting solvent mixture and thus separate from the solution whereby the neutral macrolide can be separated from the solvent mixture.
6. A process according to Claim 1 or Claim 2 wherein the water-immiscible solution containing the neutral macrolide after extraction of acidic and if desired basic components is concentrated and the residue crystallized by admixture with a crystallizing solvent or solvent mixture.
7. A process according to Claim 1 wherein the fermentation broth extract concentrate or mother liquor concentrate is first dissolved in a first solvent and extracted with a second non-aromatic hydrocarbon solvent immiscible with said first solvent, to remove the non-polar neutral impurities, the solution in the first solvent is then concentrated

and the residue dissolved in a water immiscible solvent and extracted with aqueous base or acid and base in either order to remove acidic or acidic and basic impurities.

8. A process according to any one of Claims 1 to 7 wherein the non-aromatic hydrocarbon solvent is cyclohexane, cyclohexene, hexane, heptane or pentane or a mixture thereof.
9. A process according to claim any one of Claims 1 to 8 in which the water-immiscible solvent is dichloromethane, t-butyl methyl ether, ethyl acetate, toluene, 1-butanol, an ethyl acetate/toluene mixture, a heptane/ethyl acetate mixture, or a hexane/methylene chloride mixture.
10. A process according to claim any one of Claims 1 to 9 in which the aqueous base is sodium hydroxide, sodium bicarbonate, sodium carbonate or ammonium hydroxide.
11. A process according to claim any one of Claims 1 to 10 in which the aqueous acid is hydrochloric acid, monopotassium phosphate or monosodium sulfate.
12. A process according to any one of Claims 1 to 11 wherein the macrolide is rapamycin, 32-desmethylrapamycin or 15-deoxorapamycin.

Patentansprüche

1. Verfahren zum Abtrennen eines neutralen Makrolids, welches Rapamycin oder ein natürlich vorkommendes Homolog oder Analog davon ist, aus sauren, basischen und nicht polaren neutralen Verunreinigungen, welche in einem Konzentrat aus Fermentationsbrüheextrakten oder Stammlösung vorhanden sind, welches besagtes neutrales Makrolid enthält, welches in beliebiger Reihenfolge Extraktionsschritt (a) und gegebenenfalls einen oder beide der Schritte (b) und (c) wie folgt umfasst:
 - (a) eine Lösung des besagten Konzentrats in einem mit Wasser nicht mischbaren Lösungsmittel wird bei einer Temperatur von etwa -5°C bis etwa 45°C mit wässriger Base extrahiert, um im Wesentlichen alle sauren Verunreinigungen zu entfernen;
 - (b) eine Lösung des besagten Konzentrats in einem mit Wasser nicht mischbaren Lösungsmittel wird bei einer Temperatur von etwa -5°C bis etwa 45°C mit wässriger Säure extrahiert, um im Wesentlichen alle basischen Verunreinigungen zu entfernen;
 - (c) eine Lösung des besagten Konzentrats wird einem oder mehreren des Folgenden unterworfen, um unpolare neutrale Verunreinigungen zu entfernen:
 - (i) Extraktion mit einem nicht aromatischen Kohlenwasserstoff-Lösungsmittel, in welchem das Makrolid nicht löslich ist,
 - (ii) Behandlung mit einer ausreichenden Menge eines mischbaren Makrolid-Nicht-Lösungsmittels, um das Makrolid zu veranlassen, in dem sich ergebenden Lösungsmittelgemisch unlöslich zu werden, und sich somit von der Lösung abzutrennen, wodurch das Makrolid aus dem Lösungsmittel abgetrennt werden kann;
 - (iii) Verreiben mit einem Makrolid-kristallisierenden Lösungsmittel.
2. Verfahren gemäß Anspruch 1, wobei in Schritt (c) die Lösung des besagten Makrolids eine Lösung in einem mit Wasser nicht mischbaren Lösungsmittel ist.
3. Verfahren gemäß Anspruch 1 oder Anspruch 2, wobei die Extraktion von sauren und/oder basischen Verunreinigungen bei einer Temperatur von etwa -5°C bis etwa 10°C durchgeführt wird.
4. Verfahren gemäß einem der Ansprüche 1 bis 3, wobei die unpolaren neutralen Bestandteile des neutralen, Makrolid enthaltenden Fermentationsbrüheextraktkonzentrats oder Stammlösungskonzentrats durch Extraktion einer Lösung aus besagtem Konzentrat in einem ersten Lösungsmittel mit einem zweiten, nicht aromatischen Kohlenwasserstoff-Lösungsmittel entfernt werden, welches mit dem ersten Lösungsmittel nicht mischbar ist und in welchem das neutrale Makrolid unlöslich ist.
5. Verfahren gemäß Anspruch 1 oder Anspruch 2, wobei die mit Wasser nicht mischbare Lösung, welche das neutrale

Makrolid nach Extraktion von sauren, und falls gewünscht basischen Bestandteilen enthält, mit einer ausreichenden Menge eines mischbaren Makrolid-Nicht-Lösungsmittels behandelt wird, um das Makrolid zu veranlassen, in dem sich ergebenden Lösungsmittelgemisch unlöslich zu werden und somit aus der Lösung abzutrennen, wobei das neutrale Makrolid aus dem Lösungsmittelgemisch abgetrennt werden kann.

6. Verfahren gemäß Anspruch 1 oder Anspruch 2, wobei die mit Wasser nicht mischbare Lösung, welche das neutrale Makrolid nach Extraktion von sauren, und falls gewünscht basischen Bestandteilen enthält, konzentriert wird, und der Rückstand durch Zusammenmischen mit einem kristallisierenden Lösungsmittel oder Lösungsmittelgemisch kristallisiert wird.
7. Verfahren gemäß Anspruch 1, wobei das Fermentationsbrüheextraktkonzentrat oder Stammlüssigkeitskonzentrat zuerst in einem ersten Lösungsmittel gelöst wird und mit einem zweiten, nicht aromatischen Kohlenwasserstoff-Lösungsmittel, welches mit besagtem ersten Lösungsmittel nicht mischbar ist, extrahiert wird, um die unpolaren neutralen Verunreinigungen zu entfernen, die Lösung in dem ersten Lösungsmittel dann konzentriert und der Rückstand in einem mit Wasser nicht mischbaren Lösungsmittel gelöst, und mit wässriger Base oder Säure und Base in beliebiger Reihenfolge extrahiert wird, um saure oder saure und basische Verunreinigungen zu entfernen.
8. Verfahren gemäß einem der Ansprüche 1 bis 7, wobei das nicht aromatische Kohlenwasserstoff-Lösungsmittel Cyclohexan, Cyclohexen, Hexan, Heptan oder Pentan, oder ein Gemisch daraus ist.
9. Verfahren gemäß einem der Ansprüche 1 bis 8, wobei das mit Wasser nicht mischbare Lösungsmittel Dichlormethan, t-Butylmethylether, Ethylacetat, Toluol, 1-Butanol, ein Ethylacetat/Toluolgemisch, ein Heptan/Ethylacetatgemisch oder ein Hexan/Methylenchloridgemisch ist.
10. Verfahren gemäß einem der Ansprüche 1 bis 9, wobei die wässrige Base Natriumhydroxid, Natriumbicarbonat, Natriumcarbonat oder Ammoniumhydroxid ist.
11. Verfahren gemäß einem der Ansprüche 1 bis 10, wobei die wässrige Säure Salzsäure, Monokaliumphosphat oder Mononatriumsulfat ist.
12. Verfahren gemäß einem der Ansprüche 1 bis 11, wobei das Makrolid Rapamycin, 32-Desmethyrapamycin oder 15-Deoxorapamycin ist.

Revendications

1. Procédé de séparation d'un macrolide neutre qui est la rapamycine ou un homologue ou analogue naturel de celle-ci des impuretés acides, basiques ou neutres non polaires présentes dans un concentré des extraits de bouillon de fermentation ou des liqueurs mères contenant ledit macrolide neutre qui comprend dans n'importe quel ordre l'étape d'extraction (a) et facultativement une ou deux étapes (b) et (c) comme suit :

(a) une solution dudit concentré dans un solvant non miscible à l'eau est soumise à extraction à une température d'environ -5°C à environ 45°C avec une base aqueuse pour éliminer pratiquement toutes les impuretés acides ;

(b) une solution dudit concentré dans un solvant non miscible à l'eau est soumise à extraction à une température d'environ -5°C à environ 45°C avec un acide aqueux pour éliminer pratiquement toutes les impuretés basiques ;

(c) une solution dudit concentré est soumise à une ou plusieurs conditions suivantes en vue d'éliminer les impuretés neutres non polaires :

- (i) extraction avec un solvant hydrocarboné non aromatique dans lequel le macrolide est insoluble ;
- (ii) traitement avec une quantité suffisante d'un non solvant du macrolide miscible pour forcer le macrolide à devenir insoluble dans le mélange de solvant résultant et ainsi séparer de la solution, par lequel le macrolide peut être séparé du mélange de solvant ;
- (iii) trituration avec un solvant cristallisant le macrolide.

2. Procédé selon la revendication 1 dans lequel dans l'étape (c) la solution dudit macrolide est une solution dans un solvant non miscible à l'eau.

3. Procédé selon la revendication 1 ou la revendication 2 dans lequel l'extraction des impuretés acides et/ou basiques est effectuée à une température d'environ -5°C à environ 10°C.
- 5 4. Procédé selon l'une quelconque des revendications 1 à 3 dans lequel les composants neutres non polaires du concentré d'extrait de bouillon de fermentation contenant le macrolide neutre ou le concentré de liqueur mère sont éliminés par extraction d'une solution dudit concentré dans un premier solvant avec un second solvant hydrocarboné non aromatique non miscible avec le premier solvant et dans lequel le macrolide neutre est insoluble.
- 10 5. Procédé selon la revendication 1 ou la revendication 2 dans lequel la solution non miscible à l'eau contenant le macrolide neutre après extraction des composants acides et si souhaité basiques est traitée avec une quantité suffisante d'un non solvant du macrolide miscible pour forcer le macrolide à devenir insoluble dans le mélange de solvants résultant et ainsi séparer de la solution, par lequel le macrolide neutre peut être séparé du mélange de solvant.
- 15 6. Procédé selon la revendication 1 ou la revendication 2 dans lequel la solution non miscible à l'eau contenant le macrolide neutre après extraction des composants acides et si souhaité basiques est concentrée et le résidu cristallisé par mélange avec un solvant ou un mélange de solvants cristallisants.
- 20 7. Procédé selon la revendication 1 dans lequel le concentré d'extrait de bouillon de fermentation ou le concentré de liqueur mère est d'abord dissous dans un premier solvant et soumis à extraction avec un second solvant hydrocarboné non aromatique non miscible avec ledit premier solvant, pour éliminer les impuretés neutres non polaires, la solution dans le premier solvant est ensuite concentrée et le résidu est dissous dans un solvant non miscible à l'eau et soumis à extraction avec une base ou un acide et une base aqueux en vue soit d'éliminer les impuretés acides soit acides et basiques.
- 25 8. Procédé selon l'une quelconque des revendications 1 à 7 dans lequel le solvant hydrocarboné non aromatique est le cyclohexane, le cyclohexène, l'hexane, l'heptane ou le pentane ou un mélange de ceux-ci.
- 30 9. Procédé selon l'une quelconque des revendications 1 à 8 dans lequel le solvant non miscible à l'eau est le dichlorométhane, le t-butylméthyléther, l'acétate d'éthyle, le toluène, le 1-butanol, un mélange acétate d'éthyle/toluène, un mélange heptane/acétate d'éthyle ou un mélange hexane/chlorure de méthylène.
- 35 10. Procédé selon l'une quelconque des revendications 1 à 9 dans lequel la base aqueuse est l'hydroxyde de sodium, le bicarbonate de sodium, le carbonate de sodium ou l'hydroxyde d'ammonium.
- 40 11. Procédé selon l'une quelconque des revendications 1 à 10 dans lequel l'acide aqueux est l'acide chlorhydrique, le phosphate monopotassique ou le sulfate monosodique.
- 45 12. Procédé selon l'une quelconque des revendications 1 à 11 dans lequel le macrolide est la rapamycine, la 32-des-méthylrapamycine ou la 15-désoxorapamycine.
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(54) Title: A PROCESS FOR THE RECOVERY OF SUBSTANTIALLY PURE TRICYCLIC MACROLIDE

(57) Abstract: Process for the recovery of a macrolide in substantially pure form comprising: a) treating the macrolide with wa-
ter immiscible solvent followed by concentration, b) mixing with water, water miscible solvent or mixture thereof, c) performing
hydrophobic interaction chromatography and collecting the fractions, d) extracting the fraction containing macrolide with water im-
miscible solvent followed by concentration, e) adding water miscible solvent to effect separation of impurities from the macrolide
compound, f) performing silica gel chromatography and collecting the fractions, g) isolating the macrolide compound in substan-
tially pure form. The macrolide is preferably rapamycin, tacrolimus or immunomycin.

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**A PROCESS FOR THE RECOVERY OF SUBSTANTIALLY PURE
TRICYCLIC MACROLIDE
FIELD OF THE INVENTION**

This invention relates to recovery of tricyclic macrolides.

10 **BACKGROUND OF THE INVENTION**

In 1975, Vezina et al., identified
(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-
9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-Hexadecahydro-
9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclo
15 hexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-
hexamethyl-23,27-epoxy-3H-pyrido[2,1-
c][1,4]oxaazacyclhentriacontine-1,5,11, 28,29(4H,6H,31H)-
pentone, also known by the synonyms rapamycin and sirolimus as
an antifungal antibiotic harvested from a *Streptomyces*
20 *hygroscopicus* culture, which was isolated from an Easter Island
soil sample. (J. Antibiot. 28, 721-726 (1975); and U.S. Pat. No.
3,929,992, was issued to Sehgal, et. al. Dec. 30, 1975). Martel, R.
et al. (1977) described the ability of this compound to inhibit the
immune response (Can. J. Physiol. Pharmacol., 55, 48-51). More
25 recently, Calne, R. Y. et al. (1989), has described rapamycin to be
immunosuppressive in rats given heterotopic heart allografts.
(Lancet vol. 2, p. 227). A compound, 15,19-Epoxy-3H-pyrido[2,1-
c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone,
5,6,8,11,12, 13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-
30 5,19-dihydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-
methoxycyclohexyl]-1-methyl ethenyl]-14,16-dimethoxy-

5 4,10,12,18-tetramethyl-8-(2-propenyl)-,
(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19R,26aS), also known by
the synonyms FK506 and tacrolimus disclosed by EP 184162 and
US 4,894,366 is also useful as immunosuppressant. Another
compound, 15,19-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-
10 1,7,20,21 (4H,23H)-tetrone,8-ethyl-
5,6,8,11,12,13,14,15,16,17,18,19,24,25,26, 26a-hexadecahydro-
5,19-dihydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-
methoxycyclohexyl]-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-
tetramethyl-, (3S,4R,5S,8R,9E,12S,14S, 15R,16S,18R,19R,26aS)-,
15 also known by the synonyms immunomycin and FK 520, disclosed
in EPO Publication No. 0184162 is also useful as an
immunosuppressant. Many other derivatives of these compounds
as well as structural analogues have immunosuppressant property.

US 5,508,398 discloses a process for separating a neutral
20 non-polypeptide macrolide from acidic, basic and non-polar neutral
impurities present in a concentrate of fermentation broth extracts
or mother liquors containing said neutral macrolide which
comprises in any order, extraction step (a) and optionally one or
both of steps (b) and (c), wherein (a) involves extraction with
25 aqueous base, (b) involves extraction with aqueous acid and (c)
involves treatment with non-aromatic hydrocarbon solvent.

US 5,616,595 discloses a process for recovering water
insoluble compounds (including FK506, FK520 and rapamycin) from
a fermentation broth which includes sequential steps of
30 concentrating, solubilizing and diafiltering the compound of

5 interest, all through a single closed recirculation system to recover -
the compound for further downstream purification.

The prior art methods for the recovery of macrolide compounds are tedious, does not result in pure product or require special setup for filtration.

10 The instant invention relates to a novel process for recovery of macrolide compounds.

SUMMARY OF THE INVENTION

The instant invention relates to a novel process for recovery of macrolide compounds.

15 The novel process of the instant invention comprises:

- a) treatment of an impure macrolide with water immiscible solvent and concentration and/or mixing, the impure macrolide or concentrate, with water, water miscible solvent or mixture thereof,
- 20 b) performing hydrophobic interaction chromatography,
- c) extraction into water immiscible solvent and concentration,
- d) addition of a water miscible solvent to effect separation of impurities present with the macrolide compound
- e) performing silica gel chromatography
- 25 f) isolating the macrolide compound in substantially pure form.

The product thus obtained is of pharmaceutically acceptable quality.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned earlier, The instant invention relates to a novel process for the production and recovery of macrolide compounds.

30 The novel process of the instant invention comprises:

- 5 a) treatment of a macrolide with water immiscible solvent and concentration and/or mixing, the impure macrolide or concentrate with water, water miscible solvent or mixture thereof,
- b) performing hydrophobic interaction chromatography,
- 10 c) extraction into water immiscible solvent and concentration,
- d) addition of a water miscible solvent to effect separation of impurities present with the macrolide compound,
- e) performing silica gel chromatography
- f) isolating the macrolide compound in substantially pure form.

15 The macrolide of the present invention can be produced by fermentation, chemical synthesis or combination of both. The broth obtained by fermentation can be directly extracted by water immiscible solvent. Any crude material in solid, semisolid or liquid form obtained either from broth, synthetic process or combination

20 thereof can be treated with water immiscible solvent to effect solubilization of the macrolide into the water immiscible solvent. The water immiscible solvent containing the macrolide can be concentrated. The concentration can be affected by methods known per se. The concentration can be affected by vaporization of

25 the solvent. The vaporization of the solvent can be carried out by heating without or with reduced pressure. The concentrate thus obtained can be diluted with water, water miscible solvent or mixture thereof. The water miscible solvent can be selected from alcohol, ketone or dielectric aprotic solvent. Preferably, the water

30 miscible solvent can be selected from one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile. The

5 mixture can be then subjected to hydrophobic interaction chromatography. The hydrophobic interaction chromatography can be carried out on matrix selected from a group comprising polystyrene divinyl benzene, methacrylates, polystyrene, resin linked or coated with hydrocarbons or any other suitable

10 hydrophobic matrix. The fraction containing the macrolide can be collected and extracted with water immiscible solvent. The water immiscible solvent can be selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters. Preferably, the water immiscible solvents can be selected from

15 benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate, n-butyl acetate or t-butyl acetate. The solution can be subjected to charcoalization. The solution can be concentrated by method discussed earlier. The concentrate can be treated with a water miscible solvent to effect separation of

20 impurities present with the macrolide. The impurities may be present in form of solids or liquid, immiscible with the solvent or both. The impurities can be separated out by filtration, phase separation or both. Preferably, the water miscible solvent can be selected from acetone, methanol, or acetonitrile. The filtrate or

25 product containing phase can be concentrated and the concentrate can be subjected to silica gel chromatography. The elute from the chromatography can be mixed with water immiscible solvent. The water immiscible solvent can be selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters. Preferably

30 the solvent can be selected from hydrocarbon, ether or ester. The solvent used can be a mixture of two or more solvents. Pure

5 macrolide can be isolated from the water immiscible solvent or mixture thereof.

In particular, the novel process of the instant invention comprises:

- 10 a) treatment of an impure macrolide in broth with water immiscible solvent, preferably toluene or ethyl acetate and concentration,
- b) mixing with water, water miscible solvent or mixture thereof, preferably a mixture of acetone and water,
- c) performing hydrophobic interaction chromatography,
- 15 d) extraction into water immiscible solvent, preferably ethyl acetate and concentration,
- e) addition of a water miscible solvent, preferably acetonitrile, to effect separation of impurities from the macrolide compound,
- f) performing silica gel chromatography,
- 20 g) crystallization from one or more solvents selected from ether, ethyl acetate and hexane to afford the macrolide compound in substantially pure form.

In particular, the novel process of the instant invention also comprises:

- 25 a) mixing the impure macrolide with water, water miscible solvent or mixture thereof, preferably a mixture of acetone and water,
- b) performing hydrophobic interaction chromatography,
- c) extraction into water immiscible solvent and concentration,
- 30 d) addition of a water miscible solvent to effect separation of impurities from the macrolide compound,

- 5 e) performing silica gel chromatography,
f) crystallization from one or more solvents selected from ether, ethyl acetate and hexane to afford the macrolide compound in substantially pure form.

The following examples further illustrate the invention, it being
10 understood that the invention is not intended to be limited by the details disclosed therein.

EXAMPLES

Example 1: RECOVERY OF RAPAMYCIN

The fermentation broth (31Kg) containing rapamycin was
15 twice extracted with 31 L of toluene. The toluene extract was concentrated to obtain 52 g of residue. The residue was dissolved in 5 L of acetone. 15 L of water was added to this. The solution was passed through a 1.2L column packed with Sepabeads SP 825 resin. The column was washed with 3-column volume of water and
20 3-column volume of 25% acetone in water. The product was eluted with 75% acetone in water. The product containing fractions were pooled and diluted with water to make 50% acetone in the solution. The diluted solution was extracted with ethyl acetate. 4 g of activated charcoal was added to the ethyl acetate extract and
25 mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 15 g of oily residue. 100 ml of acetonitrile was added to the residue. The precipitate obtained after acetonitrile addition was filtered. The filtrate was concentrated to obtain 14 g of oily residue. The residue was
30 applied to a silica gel column. The column was washed with 2 column volumes of 15% acetone in hexane and 2 column volumes

5 of 30% acetone in hexane. The product was eluted with 40% acetone in hexane. The product containing fractions were pooled and concentrated to obtain 5.5 g oily residue. To the residue 10 ml of diethyl ether was added and the product was crystallized at 4 °C. The product was filtered and dried. The product yield was 54%.
10 The chromatographic purity was 99.8%

EXAMPLE 2: RECOVERY OF TACROLIMUS

The fermentation broth (5Kg) containing tacrolimus was twice extracted with 5 L of ethyl acetate. The ethyl acetate extract was concentrated to obtain 22 g of residue. The residue was
15 dissolved in 1 L of acetone. 4 L of water was added to this. The solution was passed through a 0.6L column packed with Sepabeads SP 825 resin. The column was washed with 3-column volumes of water and 3-column volumes of 25% acetone in water. The product was eluted with 75% acetone in water. The product
20 containing fractions were pooled and concentrated. The concentrated solution was extracted with ethyl acetate. 1.5 g of activated charcoal was added to the ethyl acetate extract and mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 5.8 g of oily residue. The
25 residue was twice extracted with acetonitrile. The acetonitrile extract was concentrated to obtain 3.9 g of oily residue. The residue was applied to a silica gel column. The column was washed with 3 column volumes of 25% ethyl acetate in hexane and 3 column volumes of 50% ethyl acetate in hexane. The product was
30 eluted with 75% ethyl acetate in hexane. The product containing fractions were pooled and concentrated to obtain 1 g oily residue.

- 5 To the residue 2.5 ml of ethyl acetate was added. The product was crystallized at 4 °C by slow addition of hexane. The product was filtered and dried. The product yield was 60%. The chromatographic purity was greater than 99.5%

EXAMPLE 3: RECOVERY OF IMMUNOMYCIN

- 10 The fermentation broth (16Kg) containing immunomycin was extracted with 16 L of ethyl acetate. The ethyl acetate extract was concentrated to obtain 36 g of residue. The residue was dissolved in 2 L of acetone. 8 L of water was added to this. The solution was passed through a 1.2L column packed with Sepabeads SP 825
- 15 resin. The column was washed with 3-column volumes of water and 3-column volumes of 25% acetone in water. The product was eluted with 75% acetone in water. The product containing fractions were pooled and concentrated. The concentrated solution was extracted with ethyl acetate. 15 g of activated charcoal was added
- 20 to the ethyl acetate extract and mixture was stirred for 20 minutes. The mixture was filtered. The filtrate was concentrated to obtain 62 g of oily residue. The residue was twice extracted with acetonitrile. The acetonitrile extract was concentrated to obtain 43 g of oily residue. The residue was applied to a silica gel column. The column
- 25 was washed with 3 column volumes of 25% ethyl acetate in hexane and 3 column volumes of 50% ethyl acetate in hexane. The product was eluted with 75% ethyl acetate in hexane. The product containing fractions were pooled and concentrated to obtain 12 g oily residue. To the residue 12.5 ml of ether was added. The
- 30 product was crystallized at 4 °C by slow addition of hexane. The

5 product was filtered and dried. The product yield was 56%. The chromatographic purity was greater than 99.6%

5 We claim:

1. A novel process for the recovery of a macrolide in substantially pure form comprising:
 - a) treating the macrolide with water immiscible solvent followed by concentration,
 - 10 b) mixing with water, water miscible solvent or mixture thereof,
 - c) performing hydrophobic interaction chromatography and collecting the fractions,
 - d) extracting the fraction containing macrolide with water immiscible solvent followed by concentration;
 - 15 e) adding water miscible solvent to effect separation of impurities from the macrolide compound,
 - f) performing silica gel chromatography and collecting the fractions,
 - 20 g) isolating the macrolide compound in substantially pure form.
2. A process as in claim 1, wherein the macrolide is selected from rapamycin, tacrolimus or immunomycin.
3. A process as in claim 1, wherein the water immiscible solvent is selected from a group comprising hydrocarbons,
25 heterocyclic compounds, ethers, esters or a mixture of two or more of these.
4. A process as in claim 3, wherein the water immiscible solvents is selected from benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate,
30

- 5 n-butyl acetate, t-butyl acetate or a mixture of two or more of these.
5. A process as in claim 1, wherein the water miscible solvent is selected from one or more among alcohol, ketone or dielectric aprotic solvent.
- 10 6. A process as in claim 5, wherein the water miscible solvent is selected one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile.
7. A process as in claim 1, wherein the hydrophobic interaction chromatography is performed using matrix selected from
- 15 polystyrene divinyl benzene, methacrylates, polystyrene or matrix linked or coated with hydrophobic groups.
8. A process as in claim 1 step (e), wherein the solvent is selected from acetone, methanol or acetonitrile.
9. A process as in claim 1, wherein the macrolide compound is
- 20 isolated by crystallization.
10. A process as in claim 9, wherein the crystallization is carried out in one or more solvents selected from ethyl acetate, diethyl ether or hexane.
11. A process as in claim 1, wherein the macrolide is obtained by
- 25 fermentation.
12. A process as in claim 1, wherein the macrolide is obtained by synthetic process.
13. A novel process for the recovery of the macrolide in substantially pure form as in claim 1 from crude macrolide
- 30 comprising:

- 5 a) mixing with water, water miscible solvent or mixture thereof,
- b) performing hydrophobic interaction chromatography and collecting the fractions,
- c) extracting the fraction containing macrolide with water
10 immiscible solvent followed by concentration,
- d) adding water miscible solvent to effect separation of impurities from the macrolide compound,
- e) performing silica gel chromatography and collecting the fractions,
- 15 f) isolating the macrolide compound in substantially pure form.
14. A process as in claim 13, wherein the macrolide is selected from rapamycin, tacrolimus or immunomycin.
15. A process as in claim 13, wherein the water immiscible
20 solvent is selected from a group comprising hydrocarbons, heterocyclic compounds, ethers or esters.
16. A process as in claim 15, wherein the water immiscible solvents is selected from benzene, toluene, hexane, butanol, dichloromethane, chloroform, ethyl acetate, isobutylacetate
25 or butyl acetate.
17. A process as in claim 13, wherein the water miscible solvent is selected from one or more among alcohol, ketone or dielectric aprotic solvent.
18. A process as in claim 17, wherein the water miscible solvent
30 is selected one or more among methanol, ethanol, isopropyl alcohol, acetone or acetonitrile.

- 5 19. A process as in claim 13, wherein the hydrophobic interaction-
chromatography is performed using matrix selected from
polystyrene divinyl benzene, methacrylates, polystyrene or
matrix linked or coated with hydrophobic groups.
- 20 A process as in claim 13 step (d), wherein the solvent is
10 selected from acetone, methanol or acetonitrile.
21. A process as in claim 13, wherein the macrolide compound is
isolated by crystallization.
22. A process as in claim 21, wherein the crystallization is carried
out in one or more solvents selected from ethyl acetate,
15 diethyl ether or hexane.
23. A process as in claim 13, wherein the macrolide is obtained
by fermentation.
24. A process as in claim 13, wherein the macrolide is obtained
by synthetic process.
- 20

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(54) Title: PURIFICATION OF RAPAMYCIN

(57) Abstract: Purified rapamycin and a chemical process for obtaining the purified rapamycin are described.

20

PURIFICATION OF RAPAMYCIN

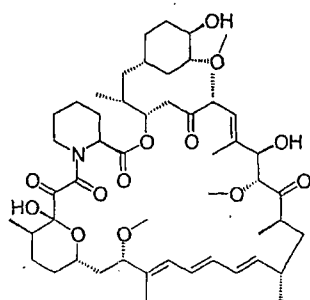
BACKGROUND OF THE INVENTION

5 This invention relates to a chemical process for the purification of rapamycin.

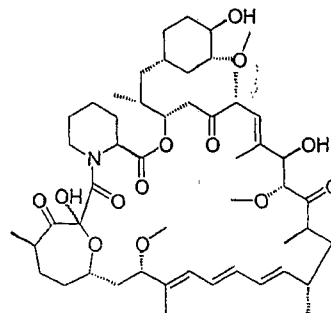
Rapamycin is a macrocyclic triene antibiotic produced naturally by *Streptomyces hygroscopicus*. It has been found useful in an array of applications based on its antitumoral and immunosuppressive effects. Uses include preventing or treating systemic lupus erythematosus, pulmonary inflammation, insulin dependent diabetes mellitus, smooth muscle cell proliferation and intimal thickening following vascular surgery, adult T-cell leukemia/lymphoma, and ocular inflammation. Rapamycin and rapamycin derivatives continue to be studied for treatment of these and other conditions.

Isomers of rapamycin are known which have structures below, referred to herein as Isomer B and Isomer C:

15



Isomer B



Isomer C

Conventional production of rapamycin is by way of fermentation. The fermentation process yields a low grade rapamycin product containing impurities, which is often colored (including troublesome yellow color), as opposed to a pure white product. Current purification methods require isopropanol recrystallization and/or charcoal treatment methods. The product obtained by these methods has a purity of approximately 94% (based on the sum of the individual purities of isomers B and C), and a yellow index of about 2. Repeated recrystallizations are often necessary to increase rapamycin purity, reduce the yellow index and increase the low B:C isomeric ratio to

20

25

meet a minimum of approximately 23 : 1, resulting in low yields upon crystallization. As a result, production costs remain high.

What is needed is a chemical purification process to increase yield of rapamycin having sufficient purity and yield to meet quality and regulatory standards for pre-clinical and commercial use.

SUMMARY OF THE INVENTION

The invention provides a chemical process as an alternative method for the purification of rapamycin. This method is useful for crude rapamycin or second crop recovered from mother liquors during the isopropanol recrystallization. This material in general is dark brown in color with low purity and low B:C isomeric ratio. The purification process includes treatment of rapamycin with chlorotrimethylsilane to yield 31,42-bis-trimethylsilyl ether, heptane extraction, and de-protection of the 31,42-bis-trimethylsilyl ether to yield a purified rapamycin product.

In another aspect, a rapamycin generated is buffered in order to increase the ratio of B isomer to C isomer in the drug substance.

In another embodiment, purified rapamycin is precipitated, collected via filtration, washed, and then dried under vacuum in order to yield a final product.

Other aspects and advantages of the invention will be apparent from the following detailed description of the invention.

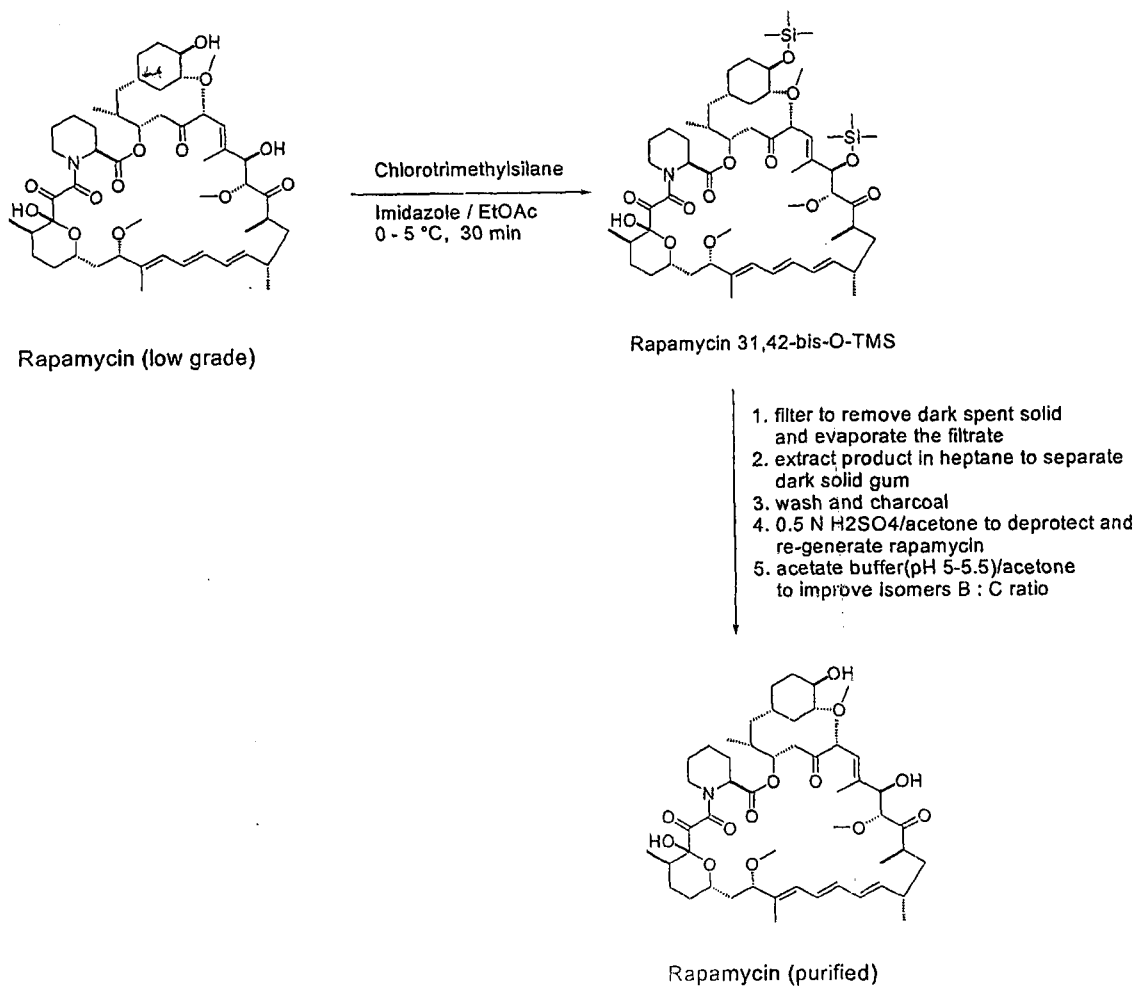
DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel chemical process for the purification of a rapamycin.

As used herein, the terms **low grade** or **crude** are intended to mean rapamycin having a purity of less than 90%, a yellow index greater than 2, and a B:C isomeric ratio (also referred to herein as an isomeric B:C ratio) less than 20:1. In one embodiment, the B:C isomeric ratio is in the range of 13:1 to 11:1.

Scheme 1 on the following page provides an overview of the purification process. In the first step, low grade rapamycin is silylated in order to form rapamycin 31,42-bis-O-trimethylsilyl ether (also known as rapamycin 31,42-bis-O-TMS). This silylation reaction is described in detail in US Patent No. 6,277,983 (Shaw, *et al.*).

Scheme 1



Briefly, silylation is accomplished by treating the crude rapamycin at about 0°C to about 5°C with chlorotrimethylsilane in an inert solvent in the presence of a suitable base. In one embodiment, the solvent is ethyl acetate (EtOAc). In another embodiment, the silylation agent is chlorotriethylsilane. In one embodiment, the base is an imidazole, such as imidazole or 1-methylimidazole. However, in other embodiments, triethylamine and N,N-diisopropylethylamine may be used as the base. Following silylation, the mixture is filtered by conventional means to remove spent organic salts and other dark solids.

In one embodiment, the rapamycin 31,42-bis-O-trimethylsilyl ether is extracted in heptane at room temperature and then separated from the dark insoluble impurities by filtration, using conventional means. Following filtration, the filtrate is mixed with charcoal. In one embodiment, Darco® KB charcoal is used. However, other charcoal
5 may be selected by one of skill in the art, such as Nuchar® SA charcoal or Darco® G-60 charcoal. The charcoal contributes to the removal of color. Additionally, it prevents formation of emulsions during sodium bicarbonate washing.

The solution is then filtered and the filtrate is subjected to one or more water and basic washes. In one embodiment, the basic wash is sodium bicarbonate. In another
10 embodiment, the filtrate is first washed with water, then with saturated sodium bicarbonate solution, and then again with water.

The organic layer is then removed and concentrated under reduced pressure at about 25°C to about 30°C to obtain a pale yellow foam. The foam is then dissolved in a suitable solvent and cooled to about 0°C to about 5°C. In one embodiment the solvent is
15 acetone. In other embodiments, the solvent is tetrahydrofuran, acetonitrile, or acetic acid. Other suitable solvents may be selected by one of ordinary skill in the art.

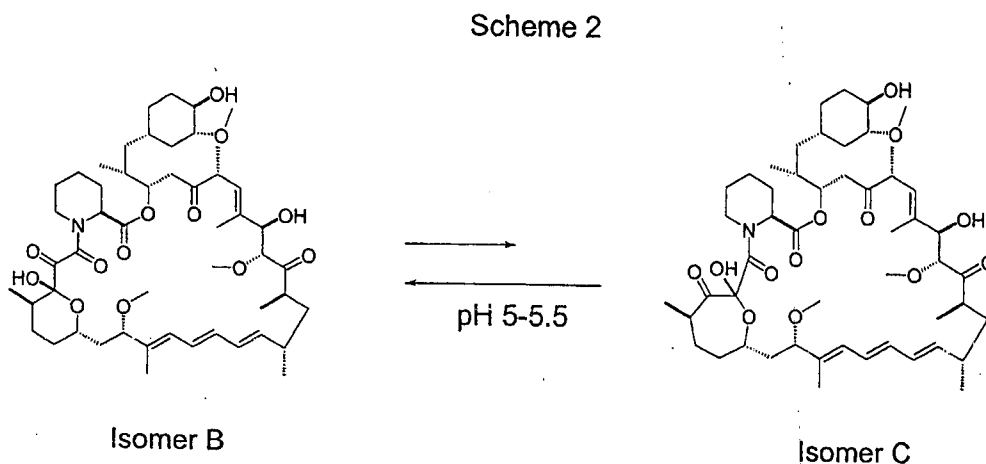
The silyl groups of the extracted rapamycin 31,42-bis-O-TMS are then removed, *i.e.*, the rapamycin 31,42-bis-O-TMS is de-protected, using a suitable acid in order to generate rapamycin. In one embodiment, the acid used is sulfuric acid. In another
20 embodiment, the sulfuric acid is 0.5 N sulfuric acid.

In one embodiment, the rapamycin produced according to the present invention has a purity greater than about 95% (by HPLC area %). In another embodiment, the rapamycin has a purity of greater than about 98%. In one embodiment, the rapamycin has a yellow index (also referred to herein as a yellow color index) of 1.0 or lower. In
25 yet another embodiment, the rapamycin has a purity of greater than 95% and a yellow color index less than 1. In still another embodiment, the rapamycin has a purity of greater than 98% and a yellow color index less than 1.

Optionally, in order to increase the B:C isomeric ratio of the product, the pH of the reaction mixture is adjusted to a pH of about 5 to about 6. In one embodiment,
30 aqueous sodium bicarbonate and acetic acid are added to an acetone solution of rapamycin. In one embodiment, the volume ratio of acetone and buffer is then adjusted via addition of sodium acetate-acetic acid buffer solution. In another embodiment,

potassium acetate or zinc acetate is used. In another embodiment, the buffer solution has a pH from about 5 to about 5.5. Other buffer systems, acids, *etc.*, to adjust the pH would be known to one of skill in the art and are contemplated by the process of the invention.

Scheme 2 on the following page provides a representation of the change in isomer balance generated through this pH adjustment.



In one embodiment, the isomeric B:C ratio obtained through buffering is greater than about 30:1. In another embodiment, the isomeric B:C ratio is greater than about 35:1.

The purified rapamycin solution is stirred at room temperature (about 20°C to about 25°C) to yield precipitated product. In one embodiment, the reaction mixture is stirred for 60 hours. The product is then collected via conventional filtration means. The collected product is then washed. In one embodiment, the product is washed with a 1:1 (v/v) mixture of acetone and water. In another embodiment, the washing is performed twice. One of skill in the art would be aware of other schemes to wash the product, which are contemplated by the process of the invention.

In one embodiment, the washed product is dried under vacuum at approximately 35 to 40°C, although the drying conditions are not a limitation of the invention.

The purified rapamycin obtained by the processes of the invention is useful in pharmaceutical compositions. Thus, the rapamycin obtained can be formulated by any suitable method described in the art for rapamycin. In one embodiment, a composition

contains the purified rapamycin obtained and a physiologically compatible carrier. As used herein the term **carrier** is intended to mean a physiologically compatible carrier. Suitable carriers for use in compositions of the invention are described below.

Oral formulations containing the active compounds of this invention may
5 comprise any conventionally used oral forms, including tablets, capsules, buccal forms, troches, lozenges and oral liquids, suspensions or solutions. Capsules may contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (*e.g.*, corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and
10 microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium lauryl sulfate,
15 microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. Preferred surface modifying agents include nonionic and anionic surface modifying
20 agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the
25 absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

In some cases it may be desirable to administer the compounds directly to the airways in the form of an aerosol.

30 The compounds may also be administered parenterally or intraperitoneally. Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as

hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

5 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of
10 microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

 The parenteral formulations useful in this invention can be used to produce a dosage form that is suitable for administration by either direct injection or by addition to
15 sterile infusion fluids for intravenous infusion.

 Transdermal administrations are understood to include all administrations across the surface of the body and the inner linings of bodily passages including epithelial and mucosal tissues. Such administrations may be carried out using the present compounds, or pharmaceutically acceptable salts thereof, in lotions, creams, foams, patches,
20 suspensions, solutions, and suppositories (rectal and vaginal).

 Transdermal administration may be accomplished through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non-toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms
25 such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such
30 as a semi-permeable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

5 The present invention further provides packaging and kits containing the purified rapamycin produced according to the present invention and formulated for administration by a suitable delivery method. In one embodiment, the purified rapamycin is present in unit dosage form. A variety of suitable containers, including bottles, vials, blister packs, and the like are known to those of skill in the art. Such
10 packaging and kits may further contain other components, including, *e.g.*, instructions for use, syringes, applicators, and the like.

The following example is illustrative of the present invention, but is not a limitation thereof.

15

EXAMPLE - Purification of rapamycin via rapamycin 31, 42-bis-O-trimethylsilyl ether

A solution of crude rapamycin (20.0 g, 19.5 mmol, yellow index =24.23, purity=89% and B:C isomeric ratio=11:1) in 300 ml ethyl acetate was cooled to 0 - 5 °C. Imidazole (6.0 g, 88.1 mmol) was added and the mixture stirred to form a solution. To this cold solution was added dropwise 8.73 g (80.4 mmol) of chlorotrimethylsilane over 30 min and stirred for a further 30 min at 0 - 5°C to complete the formation of rapamycin 31,42-bis-O-trimethylsilyl ether. The reaction mixture was filtered and the filtrate
20 concentrated under reduced pressure to a dark foam, which was stirred with 400 ml of heptane at room temperature for 20-30 minutes. The mixture was filtered to separate the insoluble material. The filtrate was stirred with 3.0 g of Darco® KB charcoal at room temperature for 20-30 minutes then filtered and the filtrate was further washed with water (160 ml), saturated sodium bicarbonate solution (80 ml) then with water (2 x 80
25 ml) to pH 6-7. The light yellow organic layer was concentrated under reduced pressure at 25 - 30°C to obtain 24.0 g of product as a pale yellow foam.
30

The foam (24.0 g) was dissolved in 100 ml of acetone, stirred and cooled to 0 - 5°C. To this cold solution was added, dropwise, 20 ml of 0.5 N sulfuric acid over 10 minutes. The mixture was stirred at 0 - 5°C until reaction completion. A solution of sodium bicarbonate (1.68g/20 ml water) was added over 2-5 minutes. The pot
5 temperature was allowed to rise to 10 - 15°C. Acetic acid (2 ml) was added and the mixture stirred for 15-20 minutes. To the reaction mixture was added, portionwise, 50 ml of sodium acetate buffer solution (pH 5-5.5) over 10 minutes and the mixture stirred at ambient temperature for 60 hours. The reaction mixture was filtered and washed with acetone-water (1:1;v/v) mixture (2 x 60 ml). The product was dried in vacuum oven at 35
10 - 40°C to constant weight to obtain 15.58 g of product as a white solid. The proton NMR of the product was identical with an authentic sample.

The recovery yield was 87.6%, the purity of the product was 98.7 %, the B:C isomeric ratio was 35:1, and the yellow index was 0.73.

All patents, publications, and other documents identified herein are incorporated by reference. One of skill in the art will recognize that minor modifications to the conditions and techniques described in the specific embodiments described herein can be varied without departing from the present invention. Such minor modifications and variants are within the scope of the invention as defined by the following claims.

CLAIMS:

1. Rapamycin having a purity of greater than 98% and a yellow color index less than 1.
2. Rapamycin according to claim 1 having an isomeric B:C ratio greater than 30:1.
3. Rapamycin according to claim 1 having an isomeric B:C ratio greater than 35:1.
4. A composition comprising rapamycin according to claim 1 and a physiologically compatible carrier.
5. A composition comprising rapamycin having an isomeric B:C ratio greater than 30:1.
6. The composition according to claim 5, wherein the isomeric ratio is greater than 35:1.
7. A process for purifying rapamycin, which comprises:
 - (a) treating rapamycin with chlorotrimethylsilane in an inert solvent in the presence of a suitable base to provide rapamycin 31,42-bis-O-trimethylsilyl ether;
 - (b) filtering the rapamycin 31,42-bis-O-trimethylsilyl ether;
 - (c) extracting the rapamycin 31,42-bis-O-trimethylsilyl ether in heptane;
 - (d) washing the extracted rapamycin 31,42-bis-O-trimethylsilyl ether; and
 - (e) deprotecting the extracted rapamycin 31,42-bis-O-trimethylsilyl ether with acid to generate rapamycin.

8. The process according to claim 7, wherein step (d) comprises:
 - (i) filtering the solution of step (c);
 - (ii) mixing the filtrate with charcoal;
 - (iii) washing the filtrate with water and sodium bicarbonate;
 - (iv) concentrating the filtrate; and
 - (v) dissolving the filtrate in a suitable solvent.
9. The process according to claim 8, wherein the suitable solvent is selected from the group consisting of acetone, tetrahydrofuran, acetonitrile and acetic acid.
10. The process according to claim 8, wherein the suitable solvent is acetone.
11. The process according to any one of claims 7 to 10, further comprising:
 - (f) adjusting the pH of the rapamycin solution to a pH of about 5 to about 6.
12. The process according to claim 11, wherein the rapamycin produced thereby has an isomeric B:C ratio of greater than 30:1.
13. The process according to claim 11, wherein the rapamycin produced thereby has an isomeric B:C ratio of greater than 35:1.
14. The process according to any one of claims 11 to 13, wherein the pH is adjusted with a buffer solution of sodium acetate, potassium acetate or zinc acetate.
15. The process according to claim 14, wherein the buffer solution is sodium acetate.

16. The process according to any one of claims 11 to 15, further comprising the steps of:

- (g) precipitating the rapamycin;
- (h) collecting the rapamycin by filtration;
- (i) washing the rapamycin with a mixture of acetone and water; and
- (j) drying the rapamycin under vacuum.

17. The process according to any one of claims 7 to 16, wherein the inert solvent in step (a) is ethyl acetate.

18. The process according to any one of claims 7 to 17, wherein the suitable base in step (a) is imidazole.

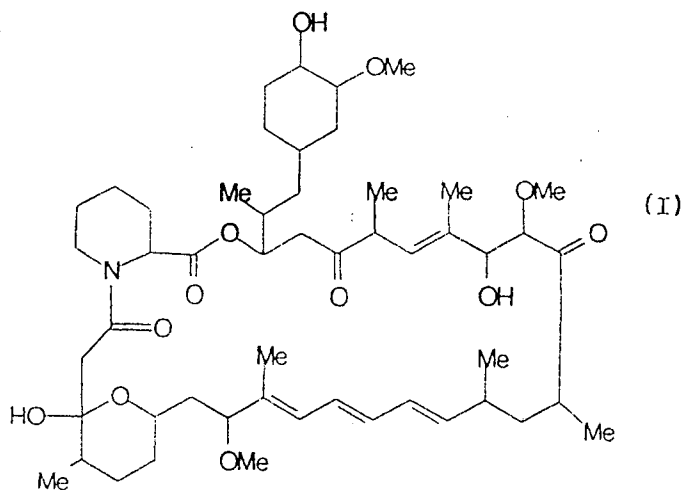
19. The process according to any one of claims 7 to 18, wherein the acid in step (e) is sulfuric acid.

20. The process according to any one of claims 7 to 19, wherein the rapamycin produced thereby has a purity greater than 98%.



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(54) Title: RAPAMYCIN DERIVATIVE AND ITS MEDICINAL USE**(57) Abstract**

A compound of formula (I), processes for their production, pharmaceutical formulations containing them, their use as antifungal agents, immunosuppressants and anticancer agents.

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sterilised by exposure to ethylene oxide before being suspended in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in such suspensions in order to facilitate uniform distribution of the compound.

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It will be recognised by the person skilled in the art that the optimal quantity and spacing of individual dosages of the compound of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration and the particular animal to be treated and that such optima can be determined by conventional techniques.

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The following examples serve to illustrate the present invention.

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Example 1

Preparation of 14-methylene rapamycin from a culture of Streptomyces by fermentation

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A culture producing 14-methylene rapamycin has been classified as Streptomyces sp. and has been deposited in the national Collection of Industrial and Marine Bacteria, 23, St. Machar Drive, Aberdeen AB2 1RY, Scotland, UK. under the accession number NCIB 40319.

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The culture was isolated from a termite hill at Abuke, Gambia.

1.0 Fermentation

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1.1 Inoculum Preparation

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Sporulating cultures grown on starch/casein agar slopes [soluble starch (BDH Poole, Dorset), 10g/l; casein (white soluble), 1g/l; K_2HPO_4 , 0.5g/l; $MgSO_4 \cdot 7H_2O$, 0.5g/l; agar technical (oxid No.3, Oxoid Basingstoke) 18g/l] in McCartney bottles were treated with 5ml 0.02% Tween 80 to produce a spore suspension. Each of two 500ml flasks containing 100ml RS1 medium [Oxoid neutralised Soy peptone, 10g/l; glucose monohydrate, 20g/l; bakers yeast, 5g/l; NaCl, 2g/l; $ZnSO_4 \cdot 7H_2O$, 0.05g/l; $MgSO_4 \cdot 7H_2O$,

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0.125g/l; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02g/l adjusted to pH 7.0 with 5N sodium hydroxide] were inoculated with 2ml spore suspension. Flasks were incubated at 25°C, 240rpm (50mm throw) for 72 hours.

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After 72 hours the contents of the 2 flasks were pooled and 16ml aliquots of the pooled culture used to inoculate each of ten x 2 litre flasks each containing 400ml RS1 medium. Flasks were incubated at 25°C, 240rpm (50mm throw).

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After 48 hours the contents were pooled and used to inoculate a further (tertiary) seed stage which comprised 100L RS1 medium +0.5g/l NOPCO foamaster antifoam sterilised in situ in a 150 litre fermenter for 30 minutes at 121°C. The fermenter was run at 25°C, 180rpm with an airflow of 50l/min at 0.5 bar overpressure. Foaming was controlled by the addition of Pluriol PE8100 antifoam obtained from BASF, Cheshire; (20% solution in soya bean oil).

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1.2 Final Stage Fermentation

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3000 litres of RP2 medium [Arkasoy 50, 20g/l obtained from Arkady Soy Mills, Manchester; glucose 20g/l; L-lysine monohydrochloride, 6g/l; bakers yeast, 6g/l; NaCl, 5g/l; K_2HPO_4 , 2.5g/l; KH_2PO_4 , 2.5g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125g/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g/l; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02g/l; glycerol, 30g/l; soya bean oil, 20g/l] together with 0.5g/l NOPCO Foamaster antifoam were sterilised in situ in a 4500 litre fermenter at 121°C for 45 minutes. After sterilisation the pH was adjusted to pH 6.4 with 9M NH_4OH and then inoculated with 100 litres of 56hr tertiary seed culture.

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The fermenter was run at 25°C, 67rpm (0-11h); 75rpm (11-84h) and 90rpm (84-202h) with an airflow of 1500 l/min at 0.5 bar overpressure. Foaming was controlled by the addition of Pluriol PE8100 antifoam (20% solution in soya bean oil). The pH was allowed to fall naturally and no pH control was used. The fermenter was run for 158h when 1000 litres of broth was removed from the vessel and the airflow was reduced to 1000 l/min to

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maintain a constant 0.5 vvm. The fermenter was run on for a further 44h when the remaining 2100 litres of broth was harvested.

2.0 Isolation Procedure

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2.1 Solvent Extraction

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After 158hr fermentation 1000 litres of whole broth were removed from the fermenter and adjusted to pH4 with sulphuric acid. 500 litres of MIBK was added and the mixture stirred for 2 hours.

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The solvent phase was recovered using a Westfalia SA7-03-076 liquid/solid centrifugal separator (Westfalia Separator Ltd; Oelde Germany). After concentration in vacuo to 20L, the concentrate was stored at 5°C.

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After 202hrs fermentation the remaining 2100 litres of whole broth was removed from the fermenter and adjusted to pH4 with sulphuric acid. 1050l of MIBK were added and the mixture stirred for 1 hour.

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The solvent, phase was recovered as above and combined with the stored concentrate from above. The combined, rich MIBK extracts were concentrated to give a final volume of 68L.

2.2 Solvent Partition

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125L of methanol and 125L of hexane were added to the concentrate and the mixture stirred for 1/2 hour. Solvent phases were allowed to separate by gravity overnight. The upper phase was then recovered (190L) and concentrated in vacuo to give 7kg of oil.

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2.3 Initial Chromatographic Purification

The oil was loaded on a Diaion (Mitsubishi Chemical Industries Ltd Tokyo Japan) HP20 column (58 x 30cm) packed in 10:90 acetone - H₂O.

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After loading the column was eluted with 60:40 acetone-H₂O. Fractions were then taken and those containing rapamycin and 14-methylene rapamycin combined and then concentrated in vacuo to give an oil (500g). The oil was dissolved in 2 litres of acetone and stored before silica chromatography.

2.4 Silica chromatography

2.5L hexane was added to the stored acetone solution and the result loaded on a silica (Sorbsil C60 silica 40-60µm, Rhone-Poulenc) column 33 x 30cm packed in 85:15, hexane-acetone. Elution was with a step gradient of acetone in hexane. Fractions eluted with up to 70:30 hexane-acetone contained 14-methylene rapamycin, these were combined and concentrated in vacuo. 34g of oil was obtained.

2.5 Amberchrom CG71 chromatography

2L of Amberchrom CG71 50µm-100µm (Tosohaas, Stuttgart, Germany) were packed in a medium pressure liquid chromatograph (Jobin Yvon, France, 8cm diameter) and equilibrated in hexane. After dissolving 34g oil in toluene, elution continued at up to 8 bar pressure with 4L of 80:20 hexane-ethyl acetate followed by 7.2L 75:25 hexane-ethyl acetate. Fractions containing 14-methylene rapamycin were combined and evaporated in vacuo to give 1.0g of solid.

2.6 Preparative hplc

800mg of the above solid was dissolved in 76:24 methanol-H₂O at up to 75mg/ml and 2ml portions injected separately onto a reverse phase 5µm C₁₈ column and pre column (21.4mm x 25cm and 21.4mm x 5cm) (Rainin Instruments USA). After injection elution continued with 76:24 methanol-H₂O, at 15ml/minute and was monitored for UV absorbance at 275nm. Fractions containing the object compound from a total of six injections were pooled and concentrated in vacuo to yield 83mg of white solid.

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- Fractions containing the object compound were analysed by reverse phase hplc using a Microsorb 5 μ m C₁₈ column 4.6 x 250mm (Rainin Instruments USA) and an Upchurch pre column (2.0 x 20mm). This column was operated at 30°C and monitored by ultraviolet absorbance at 275nm. The column was eluted with methanol-water 74:26 at 1ml/minute. Under these conditions the object compound designated 14-methylene rapamycin had a retention time of 31.4 minutes differing from that of rapamycin.
- 14-methylene rapamycin was characterised by ultra violet absorbance UV max 268,277,289nm by mass spectroscopy FAB (M+Na)⁺ =922 and by proton and ¹³C nuclear magnetic resonance spectroscopy.

3.0 Structural Analysis

Introduction

- All NMR experiments were carried out on 18.5mg/0.5ml solution in CDCL₃/TMS. All measurements were carried out using a Bruker AM400 spectrometer at 300K. FAB MS data was recorded on a VG ZAB mass spectrometer using a matrix of 3-nitrobenzylalcohol and sodium acetate (NOBA/Na). Accurate mass measurements were carried out in duplicate using MNa⁺ ions from rapamycin and demethoxyrapamycin as reference ions.

Results

- Molecular weight determinations after accurate mass measurements were 899.5739 and 899.5746. This is 14 mass units less than rapamycin (molecular formula C₅₁ H₇₉ NO₁₃) and is consistent with a formula of C₅₁ H₈₁ NO₁₂ and with the presence of a methylene in place of a carbonyl moiety in the parent rapamycin.
- Evidence for the structure of 14-methylene rapamycin can be found in the results for ¹H and ¹³C NMR spectra given in Table 3. In particular there is a loss of a carbonyl resonance in the ¹³C NMR spectra and the addition of an isolated methylene in the ¹H and the ¹³C NMR spectra. A COLOC



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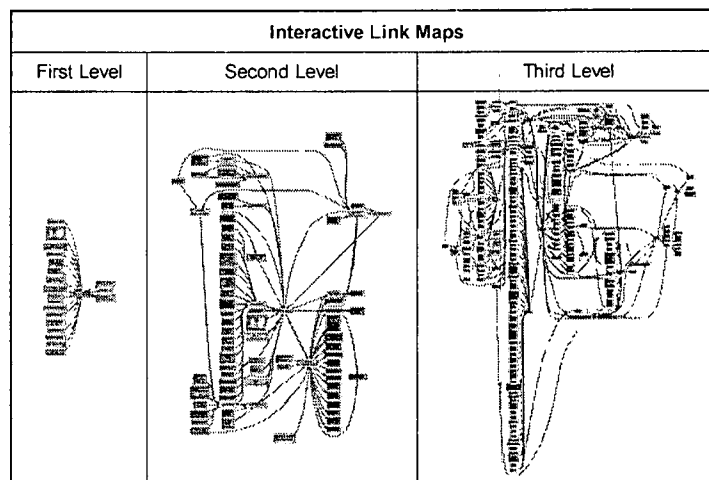
PREVIOUS
isomerism

isomerization

A chemical reaction, the principal product of which is isomeric with the principal reactant. An intramolecular isomerization that in breaking or making of bonds is a special case of a molecular rearrangement. Isomerization does not necessarily imply molecular rearrangement (e.g. in the case of the interconversion of conformational isomers).

Source:

PAC, 1994, 66, 1077 (*Glossary of terms used in physical organic chemistry (IUPAC Recommendations 1994)*) on page 1129



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Isomer

From Wikipedia, the free encyclopedia

In chemistry, **isomers** (from Greek ισομερής, isomerès; isos = "equal", méros = "part") are compounds with the same molecular formula but different structural formulas. Isomers do not necessarily share similar properties, unless they also have the same functional groups. There are many different classes of isomers, like stereoisomers, enantiomers, geometrical isomers, etc. (see chart below). There are two^[*citation needed*] main forms of isomerism: structural isomerism and stereoisomerism (spatial isomerism).

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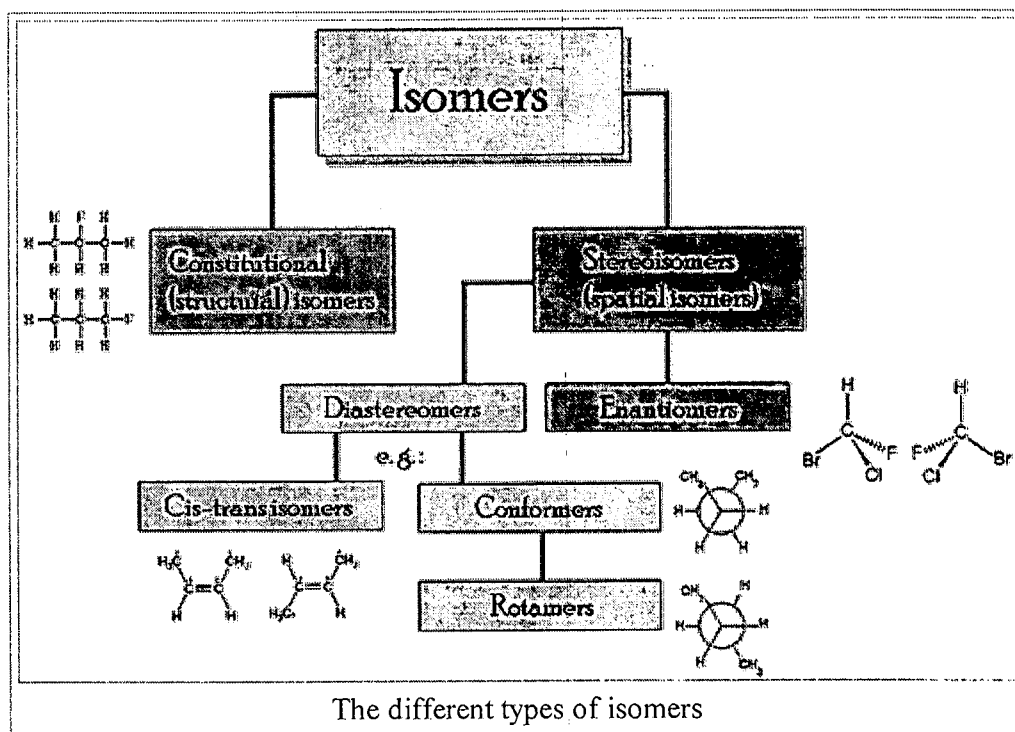
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Forms

Structural isomers

Main article: Structural isomer

In **structural isomers**, sometimes referred to as *constitutional isomers*, the atoms and functional groups are joined together in different ways. Structural isomers have different IUPAC names and may or may not belong to the same functional group.^[1] This group includes **chain isomerism** whereby hydrocarbon chains have variable amounts of branching; **position isomerism** which deals with the position of a functional group on a chain; and **functional group isomerism** in which one functional group is split up into different ones.



In **skeletal isomers** the main carbon chain is different between the two isomers. This type of isomerism is most identifiable in secondary and tertiary alcohol isomers.

Tautomers are structural isomers of the same chemical substance that spontaneously interconvert with each other, even when pure. They have different chemical properties, and consequently, distinct reactions characteristic to each form are observed. If the interconversion reaction is fast enough, tautomers cannot be isolated from each other. An example is when they differ by the position of a proton, such as in keto/enol tautomerism, where the proton is alternately on the carbon or oxygen.

Stereoisomers

Main article: Stereoisomerism

In stereoisomers the bond structure is the same, but the geometrical positioning of atoms and functional groups in space differs. This class includes enantiomers where different isomers are non-superimposable mirror-images of each other, and diastereomers when they are not.

Diastereomerism is again subdivided into "cis-trans isomers", which have restricted rotation within the molecule (typically isomers containing a double bond) and "conformational isomers" (conformers), which can rotate about one or more single bonds within the molecule.

An obsolete term for "cis-trans isomerism" is "geometric isomers".^[2]

For compounds with more than two substituents E-Z notation is used instead of cis and trans. If possible, *E* and *Z* (written in italic type) is also preferred to compounds with two substituents.^[3]

In octahedral coordination compounds *fac*- (with facial ligands) and *mer*- (with meridional ligands) isomers occur.

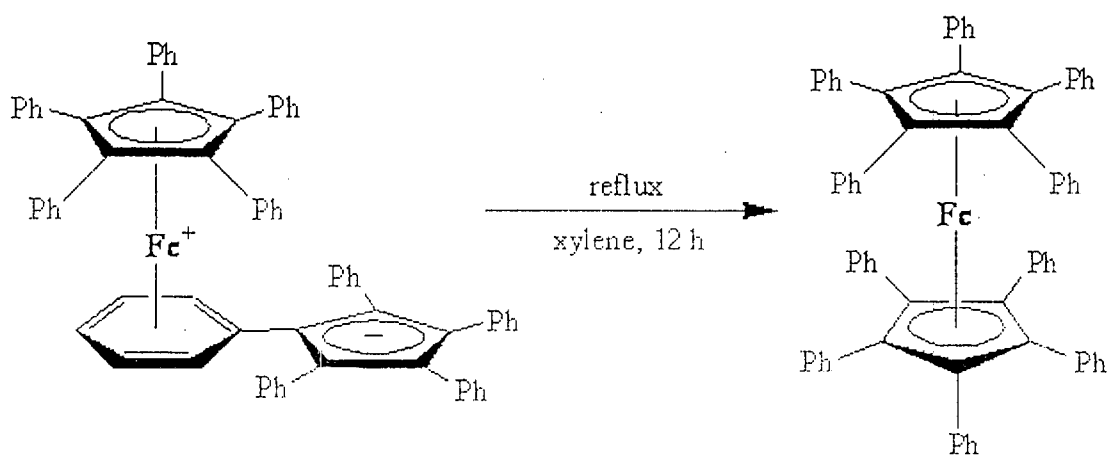
Note that although conformers can be referred to as stereoisomers, they are not **stable** isomers, since bonds in conformers can easily rotate thus converting one conformer to another which can be either diastereomeric or enantiomeric to the original one.

While structural isomers typically have different chemical properties, stereoisomers behave identically in most chemical reactions, except in their reaction with other stereoisomers. Enzymes however can distinguish between different enantiomers of a compound, and organisms often prefer one isomer over the other. Some stereoisomers also differ in the way they rotate polarized light.

Isomerisation

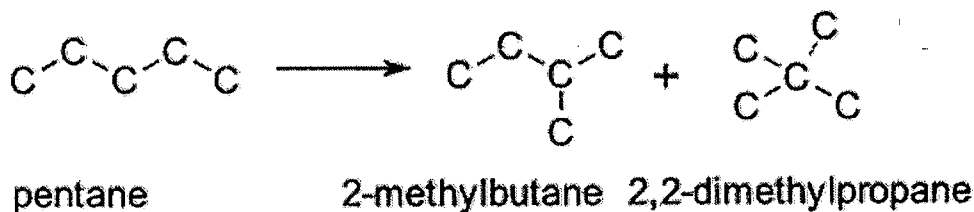
Isomerisation is the process by which one molecule is transformed into another molecule which has exactly the same atoms, but the atoms are rearranged^[4]. In some molecules and under some conditions, isomerisation occurs spontaneously. Many isomers are equal or roughly equal in bond energy, and so exist in roughly equal amounts, provided that they can interconvert relatively freely, that is the energy barrier between the two isomers is not too high. When the isomerisation occurs intramolecularly it is considered a rearrangement reaction.

An example of an organometallic isomerisation is the production of decaphenylferrocene, $[(\eta^5\text{-C}_5\text{Ph}_5)_2\text{Fe}]$ from its linkage isomer.^{[5][6]}

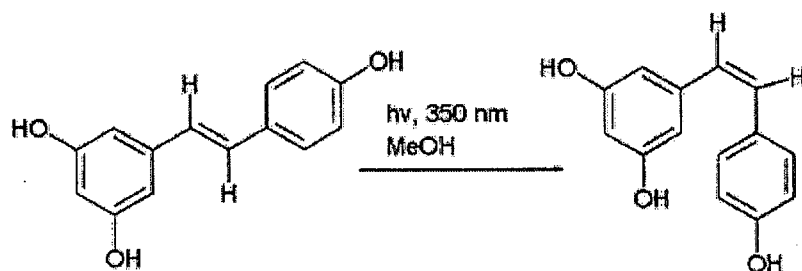


Instances of Isomerization

- Isomerizations in hydrocarbon cracking. This is usually employed in organic chemistry, where fuels, such as pentane, a straight-chain isomer, are heated in the presence of a platinum catalyst. The resulting mixture of straight- and branched-chain isomers then have to be separated. An industrial process is also the isomerisation of n-butane into isobutane.



- **Trans-cis isomerism.** In certain compounds an interconversion of cis and trans isomers can be observed, for instance, with maleic acid and with azobenzene often by photoisomerization. An example is the photochemical conversion of the trans isomer to the cis isomer of resveratrol ^[7]:



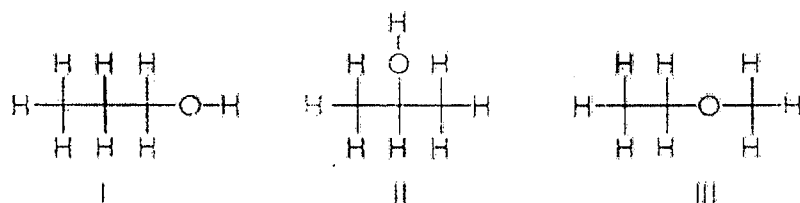
- Aldose-ketose isomerism in biochemistry.
- Isomerisations between conformational isomers. These take place without an actual rearrangement for instance inconversion of two cyclohexane conformations
- Fluxional molecules display rapid interconversion of isomers e.g. Bullvalene.
- **valence isomerisation:** the isomerisation of molecules which involve structural changes resulting only from a relocation of single and double bonds. If a dynamic equilibrium is established between the two isomers it is also referred to as valence tautomerism ^[8]

The energy difference between two isomers is called **isomerisation energy**. Isomerisations with low energy difference both experimental and computational (in parentheses) are endothermic trans-cis isomerisation of 2-butene with 2.6 (1.2) kcal/mol, cracking of isopentane to n-pentane with 3.6 (4.0) kcal/mol or conversion of trans-2-butene to 1-butene with 2.6 (2.4) kcal/mol. ^[9]

Examples

Propanol

A simple example of isomerism is given by propanol: it has the formula C₃H₈O (or C₃H₇OH) and occurs as two isomers: propan-1-ol (n-propyl alcohol; I) and propan-2-ol (isopropyl alcohol; II)



Note that the position of the oxygen atom differs between the two: it is attached to an end carbon in the

first isomer, and to the center carbon in the second.

There is, however, another isomer of C_3H_8O which has significantly different properties: methoxyethane (methyl-ethyl-ether; **III**). Unlike the isomers of propanol, methoxyethane has an oxygen connected to two carbons rather than to one carbon and one hydrogen. This makes it an ether, not an alcohol, as it lacks a hydroxyl group, and has chemical properties more similar to other ethers than to either of the above alcohol isomers.

Examples of isomers having different medical properties can be easily found. For example, in the placement of methyl groups. In substituted xanthines, Theobromine, found in chocolate, is a vasodilator with some effects in common with caffeine, but if one of the two methyl groups is moved to a different position on the two-ring core, the isomer is theophylline, which has a variety of effects, including bronchodilation and anti-inflammatory action. Another example of this occurs in the phenethylamine-based stimulant drugs. Phentermine is a non-chiral compound with a weaker effect than amphetamine. It is used as an appetite reducing medication and has mild or no stimulant properties. However, a different atomic arrangement gives dextromethamphetamine which is a stronger stimulant than amphetamine.

Allene and propyne are examples of isomers containing different bond types. Allene contains two double bonds, whereas propyne contains one triple bond.

Synthesis of fumaric acid

Industrial synthesis of fumaric acid proceeds via the cis-trans isomerization of maleic acid:



In medicinal chemistry and biochemistry, enantiomers are a special concern because they may possess quite different biological activity. The infamous case of thalidomide arose from the effects of the unwanted enantiomer. Many preparative procedure afford a mixture of equal amounts of both enantiomeric forms. In some cases, the enantiomers are separated by chromatography using chiral stationary phases. In other cases, enantioselective syntheses have been developed.

History

Isomerism was first noticed in 1827, when Friedrich Woehler prepared cyanic acid and noted that although its elemental composition was identical to fulminic acid (prepared by Justus von Liebig the previous year), its properties were quite different. This finding challenged the prevailing chemical understanding of the time, which held that chemical compounds could be different only when they had different elemental compositions. After additional discoveries of the same sort were made, such as Woehler's 1828 discovery that urea had the same atomic composition as the chemically distinct ammonium cyanate, Jöns Jakob Berzelius introduced the term *isomerism* to describe the phenomenon.^[10]

In 1849, Louis Pasteur separated tiny crystals of tartaric acid into their two mirror-image forms. The individual molecules of each were the left and right optical stereoisomers, solutions of which rotate the plane of polarized light to the same degree but in opposite directions.

Other types of isomerism

Other types of isomerism exist outside this scope. Topological isomers called topoisomers are generally large molecules that wind about and form different shaped knots or loops. Molecules with topoisomers include catenanes and DNA. Topoisomerase enzymes can knot DNA and thus change its topology. There are also isotopomers or isotopic isomers that have the same numbers of each type of isotopic substitution but in chemically different positions. In nuclear physics, nuclear isomers are excited states of atomic nuclei. Spin isomers have differing distributions of spin among their constituent atoms.

See also

- Chirality (chemistry)
- Structural isomerism
- Cis-trans isomerism
- Cyclohexane conformation
- Electromerism

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Jim Waters: The Development of GPC and the First HPLC Instruments

Aug 1, 2005
By: Leslie S. Ettre
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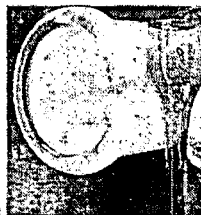
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Leslie S. Ettre

In the May issue of our magazine, I discussed the activities of Csaba Horváth in the 1960s, leading to the first modern, high-pressure liquid chromatograph (1). Naturally, however, his achievements do not imply that nothing had been done previously in converting liquid chromatography (LC) from a purely empirical, manual laboratory technique into a more controlled, automated instrumental method. According to Greek mythology, the Goddess Pallas Athena was born by jumping out of the head of Zeus, grown up and fully armed with spear and shield. However, this only happens in mythology or fairy tales. In science and technology, evolution is more or less gradual, and each major development has its antecedents.

Csaba Horváth's instrument was without question the first modern, high-pressure liquid chromatograph and as such, it essentially represented the start of the present-day instruments one can find today worldwide in almost every laboratory. However, by the middle of the 1960s, instrumentation already existed in two other branches of LC: in amino acid analysis by ion-exchange chromatography and in the investigation of molecular-weight distribution of polymers by the technique of size-exclusion (gel-permeation or GPC) chromatography. In fact, these instruments can be considered the first automated liquid chromatographs.



The development of the first gel-permeation chromatograph is closely connected with Jim Waters, the founder and for 20-years the leader of Waters Associates. However, his merit is not restricted to this single product line. By the end of the 1960s, he succeeded in becoming a major player in the meteorically growing field of LC, transforming his company into "the Liquid Chromatography People." Both achievements can be characterized as true milestones in the evolution of chromatography.

In October, Jim Waters is celebrating his 80th birthday. As a tribute to his many contributions to chromatography instrumentation, the present "Milestones" column will discuss his early career, his involvement in creating GPC standards, his contribution to the development of HPLC, and his

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Reversed-phase chromatography

From Wikipedia, the free encyclopedia

Reversed-phase chromatography (RPC) includes any chromatographic method that uses a non-polar stationary phase.^[1] The name "reversed phase" has a historical background. In the 1970s most liquid chromatography was done on non-modified silica or alumina with a hydrophilic surface chemistry and a stronger affinity for polar compounds - hence it was considered "normal". The introduction of alkyl chains bonded covalently to the support surface reversed the elution order^[2]. Now in RPC, polar compounds are eluted first while non-polar compounds are retained - hence "reversed phase". All of the mathematical and experimental considerations used in other chromatographic methods apply (ie separation resolution proportional to the column length). Today, reversed-phase column chromatography accounts for the vast majority of analysis performed in liquid chromatography.

Contents

- 1 Stationary Phases
 - 1.1 Silica Based Stationary Phases
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Stationary Phases

Silica Based Stationary Phases

Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. The most popular column is a octadecyl carbon chain (C18) bonded silica (USP classification L1) with 297 columns commercially available^[3] This is followed by C8 bonded silica (L7 - 166 columns), pure silica (L3 - 88 columns), cyano bonded silica (L10 - 73 columns) and phenyl bonded silica (L11 - 72 columns). Note that C18, C8 and phenyl are dedicated reversed phase packings while cyano columns can be used in a reversed phase mode depending on analyte and mobile phase conditions. It should be noted at this point that not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping). While the overall retention mechanism remains the same subtle differences in the surface chemistries of different stationary phases will lead to changes in selectivity.

Mobile Phase Considerations

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column. The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF). Other solvents can be used such as ethanol, 2-propanol

(iso-propyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

References

- ↑ IUPAC, *the Gold Book*. Online edition: "reversed-phase chromatography (<http://goldbook.iupac.org/R05376.html>)".
- ↑ I. Molnar and C. Horvarth, Clin.Chem., 22 (1976) 1497.
- ↑ Chromatographic Reagents Used in USP - NF and Pharamcopeial Forum, (2007 edition).

External links

- Reversed Phase Chromatography (<http://www.proteinchemist.com/tutorial/rpc.html>)
- Tables (http://www.materialharvest.com/welcome/silica_products/functional_and_reverse.html) summarizing different types of reverse phases, and information on the functionalization process

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