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**Señora Jefe
DIVISIÓN DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Bogotá, D.C.**

SUPERINDUSTRIA Y COMERCIO
Radicación : 03055528 00000004 Folios: 2
Fecha (GMD): 2005-05-22 10:17:10
Tramite : 002 PATENTES D 1 REGISTRO/ 530 PETICION
Dependencia: 2920 DIVISION DE NUEVAS CREACIONES

**Re.: Expediente No. 03 55.528
Solicitud de Patente a nombre de
SMITHKLINE BEECHAM p.l.c.
Código 02 - 01 - 538**

En relación con la solicitud contenida en el Expediente de la referencia y, en particular, con la publicación del extracto de la misma en la Gaceta de la Propiedad Industrial No. 548 del 28 de Enero de 2005, por medio del presente escrito muy respetuosamente me permito solicitarle se sirva proceder a efectuar el **EXAMEN DE PATENTABILIDAD** de la solicitud aquí contenida.

Para tal efecto, acompaño el comprobante de pago de la tasa correspondiente,
Recibo No. 05 46375

De Usted Atentamente,

GERMAN CASTILLO GRAU
T.P. No. 2978 del C.S.J.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800174089-2

SUPERINDUSTRIA Y COMERCIO

RECIBO OFICIAL DE CAJA : 05 - 46,375
FECHA : JUNIO 21 DE 2005

Radicacion : 62033526 00000004 Folios: 2
Fecha (AMB): 2005-06-22 10:17:10
Tramite : 002 POTENTES D 1 REGISTRO/ 530 PETICION
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

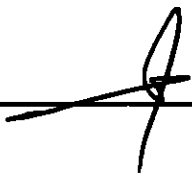
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CASTILLO GRAU Y ASOCIADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	31561222	21/06/2005	900,000.00

***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01	SOLICITUDES	
		05 EXAMEN DE PATENTABILIDAD DE INVEN	371,000.00
		TOTAL :	\$ 371,000.00

SON: TRESCIENTOS SETENTA Y UNO MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

DIVISION DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 03-55528

**EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION
FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 548, DE
FECHA 28 DE ENERO DE 2005, EL TERMINO HABIL SEÑALADO POR LA LEY
EXPIRA EL 27 DE ABRIL DE 2005.**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI ☐ ☐

NO ☐ **X** ☒

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Doctor
GERMAN CASTILLO GRAU
Smithkline Beecham Biologicals
(Apoderado)

ASUNTO	Radicación	03-55528
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	00013440
	Folios	

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

Documentos estudiados:

Los textos estudiados fueron la descripción (Folios: 4 a 15) y las reivindicaciones 1 a 16 (folios: 16 a 17).

La presente solicitud no reivindica prioridad.

Objeto de Invención:

El objeto de la presente solicitud se refiere a un método para preparar ansamitocinas que comprende las etapas de a) cultivar un microorganismo productor de ansamitocina en medio líquido, b) tratar el medio de cultivo para facilitar la extracción del producto, c) extraer las ansamitocinas con un disolvente hidrocarbonado aromático, d) concentrar las ansamitocinas y purificar las ansamitocinas por cristalización. El microorganismo es *Actynosynnema* spp más específicamente ATCC31585 o ATCC 31281, donde el tratamiento consiste en calentamiento a 75°C, la extracción se efectúa con tolueno o xileno a 45°C. también se reclama el método donde la ansamitocinas son aciladas y pueden experimentar escisión reductora para formar maitansinol. También se reclaman las ansatomicinas y complejos de maitansimolde.

El objeto de la solicitud definido anteriormente se clasificó en la IPC A61K 35/86, A61K 48/41

Determinación del estado de la técnica:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "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.

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Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40°.

Considerando que la fecha de presentación de la solicitud es 2 de Julio de 2003 se buscaron documentos del estado de la técnica relacionados, con fecha de publicación anterior a esta.

Se hizo la búsqueda de documentos relevantes en el estado de la técnica, entendiendo como estado de la técnica la definición provista en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina y las excepciones fijadas en el artículo 17 de la misma.

Se encontró como anterioridad (documentos cuya fecha de publicación es anterior a la fecha de presentación de la primera solicitud de patente sobre la cual se reivindica prioridad o de la fecha de presentación de esta solicitud) que puede afectar la patentabilidad del objeto de la presente solicitud:

Nº	Documento	Título	Fecha de Publicación
1	WO2001/077360	Methods for ansamitocin production	2001-10-18

Evaluación de los requisitos de Patentabilidad:

El Artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina el cual establece: "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".

Se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

Evaluación de Novedad:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "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.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40°.

Esta se evaluó por comparación entre lo solicitado y el documento encontrado en el estado de la técnica, que se relaciona con el objeto reivindicado y de acuerdo con la definición de novedad del Artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina.

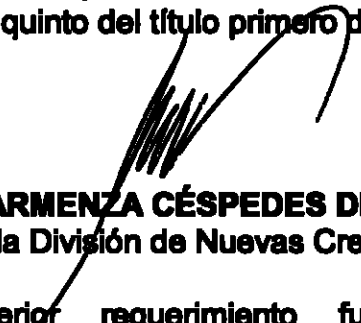
Comparando elemento por elemento el objeto de esta solicitud con lo mencionado

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en la anterioridad se encuentra que la materia de la presente solicitud ya había sido divulgada en su totalidad por el Documento WO2001/077360 y por ende el objeto reclamado no cumple con el requisito de Novedad.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha 3 NOV 2006

CONCEPTO TÉCNICO Nº: 1791	EXAMINADOR TÉCNICO Código Nº: 202063	2006-11-17
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(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 1 272 653 B1

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(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
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28.08.2006 Bulletin 2006/28

(51) Int CL:

C12P 17/18 (2006.01) C07D 498/18 (2006.01)
A61K 47/48 (2006.01) C07D 498/18 (2006.01)
C07D 303/00 (2006.01) C07D 265/00 (2006.01)
C07D 225/00 (2006.01)

(21) Application number: 01986770.8

(22) Date of filing: 11.04.2001

(86) International application number:
PCT/GB2001/001861

(87) International publication number:
WO 2001/077380 (18.10.2001 Gazette 2001/42)

(54) METHODS FOR ANSAMITOCIN PRODUCTION

VERFAHREN ZUR HERSTELLUNG VON ANSAMITOCIN

PROCEDE POUR LA PRODUCTION D'ANSAMITOCINE

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
SI

- STEFANSKA, Anna,
GlaxoSmithKline
Harlow, Essex CM19 5AW (GB)
- THIRKETTLE, Jan,
GlaxoSmithKline
Worthing,
West Sussex BN14 8QH (GB)

(30) Priority: 12.04.2000 US 196361 P

(43) Date of publication of application:
08.01.2003 Bulletin 2003/02

(74) Representative: Connell, Anthony Christopher
GlaxoSmithKline
Corporate Intellectual Property (CN6.25.10)
980 Great West Road
Brentford, Middlesex TW8 9GS (GB)

(73) Proprietor: SMITHKLINE BEECHAM PLC
Brentford,
Middlesex TW8 9GS (GB)

(72) Inventors:
• FULSTON, Mark,
GlaxoSmithKline
Worthing,
West Sussex BN14 8QH (GB)

(56) References cited:
EP-A- 0 019 934 EP-A- 0 026 338
EP-A- 0 031 430 WO-A-91/09134
GB-A- 1 554 396 US-A- 3 929 992
US-A- 4 228 239

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 92(1) European Patent Convention)

P 1 272 653 B1

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38**Description****Field of the invention**

[0001] This invention relates to processes for the preparation of ansamitocins, in particular ansamitocins which can be converted to maytansinol.

Background of the invention

[0002] Highly cytotoxic maytansinoid drugs and their therapeutic use have been described in U.S. Pat. No. 5,208,020. These drugs can be prepared from ansamitocin precursors produced by fermentation of microorganisms such as *Actinosynnema*.

[0003] Under defined culture conditions, *Actinosynnema* spp. such as *Actinosynnema pretiosum* produce a number of related ansamitocins. The major product is ansamitocin P-3 with an isobutyryl moiety at the C-3 position. Other ansamitocins differing only in the C-3 acyl side chain are produced as minor components, P-1 (ethionyl moiety), P-2 (propionyl moiety), P-3' (butyryl moiety), P-4 (isovaleryl moiety) and P-4' (valeryl moiety). All these compounds may undergo reductive cleavage to produce a common product, maytansinol (form P-0). In addition, a number of other ansamitocins are produced at low levels, which are modified at other sites in the molecule (hydroxylated or n-demethylated). These do not produce the desired P-0 on deacylation.

[0004] Processes for ansamitocin P-3 production from *Actinosynnema* spp. have been described in US Pat. Nos. 4,162,940; 4,228,230; 4,356,265; and 4,450,234. In general, these methods require adding a filter aid and a water-miscible solvent to whole fermentation broth, removing solids and extracting the aqueous fraction with a water-immiscible solvent, concentrating and precipitating with petroleum ether, purifying the precipitate using silica chromatography, and crystallizing followed by further chromatography or re-crystallization. Alternative processes utilize Dialon HP-10 adsorption instead of silica chromatography followed by further solvent extraction and crystallization.

[0005] These processes can be used to gain acceptable yields of ansamitocin P-3, but the methods involve a large number of stages which introduce limitations in large-scale production operations, particularly because of the extremely toxic nature of the ansamitocin compounds and the necessity of ensuring the safety of the human operators of the processes. Thus, a need exists to have a safer alternative procedure available, utilizing fewer and more contained stages.

Summary of the invention

[0006] One aspect of the present invention is a method for preparing purified ansamitocins comprising the steps of:

- a. culturing an ansamitocin-producing microorganism in a liquid culture medium;
- b. treating the culture medium to facilitate solvent extraction of ansamitocins;
- c. extracting ansamitocins from the culture medium with an aromatic hydrocarbon solvent;
- d. concentrating the extracted ansamitocins; and
- e. purifying the ansamitocins by crystallization.

[0007] Another aspect of the present invention is a method for preparing purified ansamitocins comprising the steps of:

- a. culturing an ansamitocin-producing microorganism in a liquid culture medium;
- b. extracting ansamitocins from the culture medium with an aromatic hydrocarbon solvent;
- c. concentrating the extracted ansamitocins; and
- d. purifying the ansamitocins by crystallization.

Detailed Description of the invention

[0008] Methods are provided for preparing purified ansamitocins without a filtration step. The methods comprise the steps of culturing an ansamitocin-producing microorganism in a liquid culture medium, treating the culture medium to release ansamitocins from the microorganism into the culture medium thereby facilitating solvent extraction, extracting ansamitocins from the treated culture medium with an aromatic hydrocarbon solvent, concentrating the extracted ansamitocins, and purifying the ansamitocins by crystallization. Alternatively, the treatment step can be omitted.

[0009] The purified ansamitocins may be reduced to maytansinol and include ansamitocin P-3, P-1, P-2, P-3', P-4 and P-4'. The purified ansamitocins contain only very low levels of undesirable ansamitocins with modifications at other sites in the molecule. Preferably, the ansamitocin-producing microorganism is *Actinosynnema* spp. Particularly preferred is *Actinosynnema pretiosum* ATCC 31585. Also particularly preferred is *Actinosynnema pretiosum* ATCC 31281. The microorganisms can be grown by fermentation culture techniques well-known to those skilled in the art such as those disclosed in U.S. Pat. No. 4,450,234.

[0010] One embodiment of the method of the invention uses treatment of the microorganism to aid release of intracellular ansamitocins and render cell-associated ansamitocins more amenable to solvent extraction. Exemplary treatment methods include sonication, increased pressure or increased temperature. Preferably, the treatment is a heat treatment. The heat treatment can be conducted at about 60°C to about 80°C. Preferably, the heat treatment step is conducted at about 75°C which kills the microorganism and facilitates solvent extraction of ansamitocins. In an alternative embodiment, no treatment step is used prior to extraction since approximately 60% of total ansamitocins can be found in the culture

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medium.

[0011] Ansamitocins can be extracted from culture medium with the use of aromatic hydrocarbon solvents. The aromatic hydrocarbons have a particular selectivity for the ansamitocins over other broth constituents, which ensures only simple processes are required downstream to isolate pure product. Preferably, the aromatic hydrocarbon solvent is toluene or xylene. Particularly preferred is toluene as this is more amenable to low temperature evaporation. The properties of toluene and xylene are such that the solvent and aqueous layers are readily separated under gravity without the need for mechanical separation and thus greater containment of the process and increased operator safety are achieved. The extraction can be conducted at about 20°C to about 80°C. Preferably, the extraction is conducted at about 45°C. The pH of the aqueous solution prior to extraction should be in the range 3 to 9. Preferably, the pH is near neutrality, i.e., pH 6 to 8.

[0012] In general, one equivalent volume of solvent to whole broth (1:1) is preferred for the extraction. Alternative ratio ranges of 2:1 to 1:4 can also be used. The solutions are generally mixed slowly with stirring and the mixture stirred until about >80% of the ansamitocins have been extracted into the organic layer. Preferably the mixture is left to settle under gravity at a temperature between 15°C and 50°C. The preferred temperature for settling is about 45°C.

[0013] The organic layer is removed, and concentration of the extract by volume reduction of the solvent may be carried out *in vacuo* or by other methods well-known to those skilled in the art. After volume reduction, the concentrated extract can be optionally dissolved in a polar solvent such as methanol, and clarified using a membrane filter such as PTFE, or a depth filter such as silica or alumina.

[0014] Crystallization is used to purify the desired ansamitocins, particularly P-3, by reducing the levels of unwanted ansamitocins. A preferred solvent mixture for crystallization is ethyl acetate and heptane. Such crystallization is performed in a conventional manner. To aid dissolution of the solid for crystallization, a small volume of methanol or similar polar solvent may be added prior to addition of ethyl acetate and then treatment with larger volumes of heptane, optionally with stirring and cooling to afford the crystallized product. The product may be recrystallized following the same procedure.

[0015] Alternatively, prior to crystallization, the impure ansamitocins extracted from the fermentation broth may be purified using silica gel, e.g., by passing a solution of the solvent extract through a bed of silica. Solvents used for the chromatography may be toluene and toluene-methanol mixes. Other solvents known to those skilled in the art can also be used. The fractions containing ansamitocin P-3 are pooled and concentrated under reduced pressure.

[0016] Methods are also provided for analysis of ansamitocins by HPLC. Quantitation of ansamitocin P-3

and analysis of ansamitocin ratios are achieved by the methods. These methods were employed in the Examples set forth below.

[0017] Quantitation of ansamitocin P-3 in broth and extraction samples can be determined on a C18 Waters Q Spherisorb 95 ODS2 column, 4.6 x 250 mm, with a 10 mm guard column. UV detection is at 252 nm and 205 nm. An isocratic mobile phase of 1 ml/min 80% MeCN (0.05% TFA) in water (0.05% TFA) and a 20 µl injection volume are used.

[0018] Analysis of ansamitocin ratios in downstream process samples can be determined on a C8 Waters Symmetry Shield column, 3.9 x 150 mm, with no guard column. UV detection is at 252 nm and 205 nm. A gradient mobile phase of 1 ml/min 35-45% MeCN (0.05% TFA) in water (0.05% TFA) over 30 minutes with a 10 minute re-equilibration at 40°C and a 10 µl injection volume are used.

[0019] Analysis of ansamitocin ratios in chromatography fractions and final product can be determined on a C8 Waters Symmetry Shield column, 3.9 x 150 mm, with no guard column with an LC-MS detection system, atmospheric pressure electrospray ionisation, +ve ion mode. 30V cone voltage. Mass detection to positively identify the peaks is achieved by full scan MS (scan from 600-700 amu with quad 1). MS-MS fragmentation to determine class of ansamitocin is carried out using the same gradient system. A gradient mobile phase of 1 ml/min 35-45% MeCN (0.05% TFA) in water (0.05% TFA) over 30 minutes with a 10 minute re-equilibration at 40°C and a 10 µl injection volume are used.

[0020] Quantitation of ansamitocin P-3 in waste streams and other low-level samples requiring high sensitivity can be determined on a C18 Waters Spherisorb S5 ODS2 column, 4.6 x 250 mm, with a 10 mm guard column and LC-MS-MS atmospheric pressure electrospray ionisation detection system, +ve ion mode, 30V cone voltage. For determination of structural type, the molecular ions of the major species were selected and the fragmentation patterns observed were a predominant 547 ion indicating N-methylated and a 533 indicating N-demethylated. An isocratic mobile phase of 1 ml/min 80% MeCN (0.05% TFA) in water (0.05% TFA) and a 20 µl injection volume are used.

[0021] The process of the invention can be used to make cell-binding agent/maytansinoid complexes which are useful as tumor-activated pro-drugs. Ansamitocins prepared by the process of the invention can undergo reductive cleavage to maytansinol which can be used as described in U.S. Patent No. 5,208,020 to produce N-methyl-L-alanine containing maytansinoid derivatives. These derivatives are then conjugated to cell-binding agents, preferably antibodies, via various linkers such as a disulfide link.

[0022] An exemplary cell-binding agent/maytansinoid complex can be prepared by a process comprising the following steps:

- (1) reducing ansamitocins prepared by the process of the invention to maytansinol;
- (2) esterifying maytansinol with N-methyl-L-alanine derivatives to form a disulfide-containing maytansinoid ester;
- (3) reducing the disulfide-containing maytansinoid ester prepared by step (2) to a thiol-containing maytansinoid;
- (4) introducing dithiopyridyl groups into a cell-binding agent; and
- (5) linking the thiol-containing maytansinoid produced by step (3) to the dithiopyridyl cell-binding agent of step (4) by a disulfide link.

[0023] The present invention will now be described with reference to the following specific, non-limiting examples.

Example 1

Extraction of *Actinosynnema pretiosum* Culture Broth and Ansamitocin Purification

[0024] 37L of whole broth containing the producing strain *Actinosynnema pretiosum* ATCC 31565 (ansamitocin P-3 titer 86.3 mg/L) were heat-treated *in situ* at 75°C for 60mins to kill the micro-organism and facilitate solvent extraction of the ansamitocins. 40L of toluene were added and the mixture warmed to 45°C. Phases were agitated such that a vortex of upper toluene phase was drawn into the lower broth phase but without emulsification or complete homogenization of the phases. Extraction was completed within 18 hours, and separation under gravity within 2 hours.

[0025] 39L of toluene containing 80mg/L P-3 was recovered by siphon, and evaporated using a 20L rotary evaporator (bath temperature 40-45°C, rate ~8L/hr). 11.2g of mobile oil, containing 3.1g of P-3; 27.6% w/w) was generated after evaporation. The resulting extract was transferred to a flask by dissolution in toluene and re-evaporation. (Extraction stage yield = 97%)

[0026] The extract was taken up in 120ml toluene and loaded onto a silica column (Kieselgel 60, 125ml bed volume packed in toluene, 4cm diam. x 10cm) in 375ml toluene (3bv). The column was washed with 2bv toluene and then eluted with 4 x 2bv of 2% MeOH in toluene, followed by 12 x 1bv of 4% MeOH in toluene. The column was eluted at 40ml/min and produced tight bands of color. Fractions 7-10 containing ansamitocin P-3 were bulked and evaporated to produce 3.2g of oily solid containing 2.5g of P-3. This material was analyzed by LC-MS and MS-MS.

[0027] At this stage the product contained 85.1% ansamitocin P-3, and a total of 93.9% of the desired ansamitocins. (Column stage yield = 80.6%)

[0028] The product from the silica column was taken up in 200mL EtOAc warmed to 40°C. Heptane (200mL) was added and the solution allowed to cool. The solution

was seeded with 1mg pure P-3 crystals (crystallization also spontaneously occurred at other sites in the flask). After 4 hours at ambient temperature the supernatant was analyzed by HPLC and 0.8g P-3 (30%) was determined to still be in solution. Further heptane was added (150ml) and the flask left for a further 3 hours and re-analyzed. This indicated that 0.4g of P-3 (16%) remained in solution. The flask was left overnight at 4°C. Subsequent analysis indicated that only 70mg of P-3 (3%) remained in solution. The mother liquors were removed by aspiration, using a sintered filter line assembly. The white needle crystals were washed with 2 x 15mL 1:3 EtOAc:heptane. The crystals were dried *in situ* under vacuum on a rotary evaporator at 30°C for 10hours. 2.5g of crystals were obtained. (Crystallization yield = 86%)

[0029] The final product contained 86% ansamitocin P-3, and a total of 98.4% of the desired acylated ansamitocins (P-1, P-2, P-3, P-3', P-4, P-4').

20 Example 2

Extraction of *Actinosynnema pretiosum* Culture Broth and Ansamitocin Purification

25 [0030] 1,100L of whole broth containing the producing strain *Actinosynnema pretiosum* ATCC 31565 (ansamitocin P-3 titer 76.1 mg/L, 82.6g P-3) were heat-treated *in situ* at 75°C for 60 mins to kill the microorganism and facilitate solvent extraction of the ansamitocins. 77.6g of P-3 remained after the heat kill process. An equal volume of toluene pre-warmed to 45°C was added and the mixture was maintained at 45°C. Phases were agitated such that a vortex of upper toluene phase was drawn into the lower broth phase but without emulsification or complete homogenization of the phases. Extraction was carried out for 46 hours, followed by separation under gravity which occurred within 30 min. (Extraction stage yield 90.3%.)

30 [0031] 1,127L of the toluene extract, containing the ansamitocins, were concentrated to 22L using a falling film evaporator (FFE). The concentrate was transferred to a 50L rotary evaporator and evaporated to low volume (evaporation rate 14.6L/hr). The FFE was rinsed with 2 x 20L toluene and the rinsings passed to the evaporator to ensure complete transfer of product. The concentrate was evaporated to dryness.

35 [0032] The dry extract was taken up in 7.2L of 4% methanol in toluene and loaded onto a silica column (Kieselgel 60, 4.8L bed volume packed in 4% methanol in toluene, 15.0cm diam. x 27.0cm, loading rate 120 mL/min). The column was eluted isocratically using 4% methanol in toluene at a flow rate of 227-384 mL/min and produced tight bands of color. Initial fractions were one bed volume; fractions 3 to 6 were collected as half bed volumes. Fractions were monitored by TLC (Kieselgel 60 F₂₅₄ plates, run in 5% methanol in dichloromethane, visualized by UV at 254nm) and those containing ansamitocins were monitored by HPLC and LC-MS. Fraction

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selection for crystallization was based on HPLC and LC-MS analysis of the fractions to optimize ansamitocin P-3 recovery and minimize undesired ansamitocins. Fractions 5 to 10 containing ansamitocin P-3 were bulked and evaporated to produce a solid containing 54.8g of P-3. At this stage the product contained 77.1% ansamitocin P-3, and a total of 98.1% of the desired ansamitocins. (Column stage yield = 82.6%)

[0033] The product from the silica column was taken up in 91 mL methanol previously warmed to 48°C, followed by 548 mL ethyl acetate warmed to the same temperature. Further aliquots of methanol were added to aid dissolution of the solid. A total of 188 mL of methanol was added to the mixture. Heptane (100mL) warmed to 50°C was added to the first sign of cloudiness and then the solution was allowed to cool to ambient as crystallization commenced. After 4 hours, a further 1,324 mL of heptane (at ambient temperature) was added. The supernatant was analyzed by HPLC and 6.8g P-3 was determined to still be in solution. The mixture was cooled on ice and further aliquots of heptane (200 and 400 mL) were added until 3.9g of P-3 (6.8%) remained in the mother liquor.

[0034] The mother liquors were removed by aspiration, using a sintered filter line assembly. The crystals were washed with 2 x 150 mL 1:3 ethyl acetate:heptane. The crystals were dried *in situ* on a rotary evaporator, initially under low vacuum, followed by high vacuum (1.0-1.3 mbar) at 30°C for 88.5 hours. 78.4g of crystals were obtained.

[0035] The final product contained 74.5% ansamitocin P-3 (56.9g) and a total of 97.8% of the desired acylated ansamitocins. (Overall yield = 89%)

Example 3

Silica Chromatography and Crystallization of the Ansamitocins

[0036] 1,008L of *Actinosynnema pretiosum* whole broth with a titer of 64.8mg/L ansamitocin P-3 were heat treated, extracted with toluene and evaporated essentially as described in Example 1.

[0037] The concentrate containing 34.5 g ansamitocin P-3 was taken up in 3 L toluene and loaded onto a silica column (Kieselgel 60, 3.0 L bed volume, packed in toluene, 13.8 cm diam. x 18.6cm). The column was topped with a 4 cm bed of sand. The column was washed with 5L of toluene, followed by 20L of 2% MeOH in toluene, which was collected as 5 L fractions. The column was then eluted with 20 L of 4% MeOH in toluene, collected as 2.5 L fractions. The ansamitocins were eluted in fractions 9 through to 15. Fraction selection for crystallization was based on HPLC and LC-MS analysis of the fractions to optimize ansamitocin P-3 recovery and minimize undesired ansamitocins. Fractions 10 to 12 were bulked and evaporated to dryness to yield an oil containing 32.5 g of ansamitocin P-3.

[0038] At this stage the product contained 91.8% ansamitocin P-3, and a total of 95.3% of the desired acylated ansamitocins. (Column stage yield = 94.2%)

[0039] The concentrate from the bulked silica fractions, was warmed in a water bath at 50°C and dissolved in a minimum volume of warm methanol/ethyl acetate (50°C). 80mL of methanol was added initially, followed by slow addition of 300mL of ethyl acetate. A further 20mL of warm methanol was added at which point the concentrate was completely dissolved. 200mL of warm heptane (50°C) was added and the crystallization solution removed from the water bath. Crystallization commenced, and further heptane was added in 200mL aliquots until a total volume of 800mL had been added. The mixture was cooled in an ice bath for 18 hours. The crystallization was monitored by HPLC analysis of the mother liquors. At 18 hours 6.3% ansamitocin P-3 remained in the mother liquors. A further 200mL of heptane was added and the mixture cooled for a further five hours. HPLC analysis indicated 4.0% ansamitocins remained in the mother liquors.

[0040] The crystals were recovered by aspiration of the dark brown mother liquors. The crystals were washed three times with 50mL of heptane:ethyl acetate, 3:1 and were dried under vacuum (0.8mBar) overnight. The crystals were uniform, fine, off-white needles containing 28.2g of ansamitocin P-3. (Crystallization stage recovery = 88.9%)

[0041] The final product contained 83.1% ansamitocin P-3 and a total of 98.4% desired acylated ansamitocins.

Example 4

Purification and Crystallization of Ansamitocins from Toluene Extract

[0042] 1,001L of *Actinosynnema pretiosum* whole broth with a titer of 74.5 mg/L ansamitocin P-3 were heat treated, extracted with toluene and evaporated essentially as described in Example 1 to give 9.5 L of concentrated extract containing 54.7 g of ansamitocin P-3.

[0043] The concentrate was heated to 45°C and 14.5 L of heptane was added over 33 min to precipitate the ansamitocins. The mixture was cooled on ice. A further 5 L of heptane was added and the mixture allowed to settle overnight. The mother liquor was removed by aspiration and the precipitate was washed with 10 L of toluene:heptane, 1:1. HPLC analysis indicated the mother liquor contained 6.5% of ansamitocin P-3, and the wash contained 1.8% P-3. The precipitate was dissolved in 2 L of methanol and filtered through a 0.2 micron filter.

[0044] The methanol solution was evaporated to dryness and re-dissolved in 85 mL of methanol at 50°C. 510 mL of ethyl acetate was added to the solution followed by 200 mL of heptane. The mixture was cooled to ambient and a further 800 mL of heptane was slowly added as crystallisation commenced. 4.14 g of ansamitocin P-3 remained in the mother liquor. A further 400 mL of heptane

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tane was added and the mixture cooled on ice. 2.7 g of ansemitocin P-3 remained in the mother liquor.

[0045] The mother liquor was removed by aspiration and the crystals were dissolved in 110 mL of methanol and 520 mL ethyl acetate at 50°C. Heptane was added in two 75 mL aliquots, the mixture was cooled to ambient and another 900 mL added. A further 400 mL of heptane was added and the mixture cooled on ice and left overnight to crystallize. HPLC analysis indicated 2.3 g of ansemitocin P-3 remained in the mother liquor. The mother liquor was removed by aspiration and the crystals were washed with heptane:ethyl acetate, 3:1 and were dried under vacuum (0.8 mBar) overnight. The 47.8 g of crystals contained 84.3% ansemitocin P-3, and a total of 97.0% desired acylated ansemitocins.

Example 5

Extraction of *Actinosynnema pretiosum* Culture Broth with Xylene and Ansemitocin Purification

[0046] 220 mL of heat treated *Actinosynnema pretiosum*, with a titre of 44 ug/ml ansemitocin P-3, were placed in a 45°C water bath and an equal volume of xylene was added. The phases were mixed such that a vortex of xylene was drawn in to the lower broth phase but without forming an emulsion. After 20 hours, 84% of the ansemitocin P-3 had extracted into the xylene phase.

[0047] The mixture was separated under gravity and 170 mL of xylene were removed. The xylene extract was evaporated to dryness. The xylene extract was purified using silica chromatography. The extract was dissolved in 2 mL of 4% methanol in toluene and loaded and eluted using the same solvent mix. Fractions (0.5 mL) were collected and assayed by TLC (Kieselgel 60 F₂₅₄ plates run in dichloromethane:methanol 20:1). Fractions containing ansemitocin P-3 were crystallized using the procedure described in previous examples. The crystalline product was of comparable quality in terms of colour and purity with that produced by toluene extraction of fermentation broth.

[0048] The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof, and, accordingly, reference should be made to the appended claims, in the light of the foregoing specification, as indicating the scope of the invention.

Claims

1. A method for preparing purified ansemitocins comprising the steps of:

- culturing an ansemitocin-producing microorganism in a liquid culture medium;
- treating the culture medium to facilitate solvent extraction of ansemitocins;

- extracting ansemitocins from the culture medium with toluene or xylene;
- concentrating the extracted ansemitocins; and
- purifying the ansemitocins by crystallization wherein said method does not include a filtration step.

2. A method for preparing purified ansemitocins comprising the steps of:

- culturing an ansemitocin-producing microorganism in a liquid culture medium;
- extracting ansemitocins from the culture medium with toluene or xylene;
- concentrating the extracted ansemitocins; and
- purifying the ansemitocins by crystallization wherein said method does not include a filtration step.

3. The method of claim 1 or 2 wherein the ansemitocin producing microorganism is *Actinosynnema* spp.

4. The method of claim 3 wherein the *Actinosynnema* spp. is *Actinosynnema pretiosum* ATCC 31585.

5. The method of claim 3 wherein the *Actinosynnema* spp. is *Actinosynnema pretiosum* ATCC 31281.

6. The method of claim 1 wherein the treatment is heating at about 75°C.

7. The method of claim 1 or 2 wherein the solvent is toluene.

8. The method of claim 1 or 2 wherein the solvent is xylene.

9. The method of claim 7 or 8 wherein the ratio of toluene or xylene to heat-treated culture medium is about 1:1.

10. The method of claim 9 wherein the extraction is at about 45°C.

11. The method of claim 1 or 2 wherein the ansemitocins comprise acylated ansemitocins that can undergo reductive cleavage to form maytanol.

Patentansprüche

1. Verfahren zur Herstellung gereinigter Ansemitocine, das die folgenden Schritte umfaßt:

- Kultivieren eines Ansemitocin-erzeugenden Mikroorganismus in einem flüssigen Kulturme-

- dium;
 (b) Behandeln des Kulturmediums zur Erleichterung der Lösungsmittelextraktion von Ansamitocinen;
 (c) Extrahieren von Ansamitocinen aus dem Kulturmedium mit Toluol oder Xylol;
 (d) Aufkonzentrieren der extrahierten Ansamitocine; und
 (e) Reinigen der Ansamitocine durch Kristallisation, worin das Verfahren keinen Filtrations-schritt einschließt.
2. Verfahren zur Herstellung gereinigter Ansamitocine, das die folgenden Schritte umfaßt:
- (a) Kultivieren eines Ansamitocin-erzeugenden Mikroorganismus in einem flüssigen Kulturmedium;
 (b) Extrahieren von Ansamitocinen aus dem Kulturmedium mit Toluol oder Xylol;
 (c) Aufkonzentrieren der extrahierten Ansamitocine; und
 (d) Reinigen der Ansamitocine durch Kristallisation, worin das Verfahren keinen Filtrations-schritt einschließt.
3. Verfahren gemäß Anspruch 1 oder 2, worin der Ansamitocin-erzeugende Mikroorganismus *Actinosynema* spp. ist.
4. Verfahren gemäß Anspruch 3, worin die *Actinosynema* spp. *Actinosynema pretiosum* ATCC 31585 ist.
5. Verfahren gemäß Anspruch 3, worin die *Actinosynema* spp. *Actinosynema pretiosum* ATCC 31281 ist.
6. Verfahren gemäß Anspruch 1, worin die Behandlung ein Erwärmen auf ca. 75°C ist.
7. Verfahren gemäß Anspruch 1 oder 2, worin das Lösungsmittel Toluol ist.
8. Verfahren gemäß Anspruch 1 oder 2, worin das Lösungsmittel Xylol ist.
9. Verfahren gemäß Anspruch 7 oder 8, worin das Verhältnis von Toluol oder Xylol zu wärmebehandeltem Kulturmedium ca. 1:1 ist.
10. Verfahren gemäß Anspruch 9, worin die Extraktion bei ca. 45°C erfolgt.
11. Verfahren gemäß Anspruch 1 oder 2, worin die Ansamitocine acylierte Ansamitocine umfassen, die eine reduktive Spaltung unter Bildung von Maytansinol erfahren können.

Revendications

1. Procédé de préparation d'ansamitocines purifiées comprenant les étapes de :
- a. culture d'un microorganisme produisant des ansamitocines dans un milieu de culture liquide ;
 b. traitement du milieu de culture pour faciliter l'extraction par solvant des ansamitocines ;
 c. extraction des ansamitocines à partir du milieu de culture avec du toluène ou du xylène ;
 d. concentration des ansamitocines extraites ;
 et
 e. purification des ansamitocines par cristallisation,
- où ledit procédé ne comprend pas d'étape de filtration.
2. Procédé de préparation d'ansamitocines purifiées comprenant les étapes de :
- a. culture d'un microorganisme produisant des ansamitocines dans un milieu de culture liquide ;
 b. extraction des ansamitocines à partir du milieu de culture avec du toluène ou du xylène ;
 c. concentration des ansamitocines extraites ; et
 d. purification des ansamitocines par cristallisation,
- où ledit procédé ne comprend pas d'étape de filtration.
3. Procédé selon la revendication 1 ou 2, dans lequel le microorganisme produisant des ansamitocines est *Actinosynema* spp.
4. Procédé selon la revendication 3, dans lequel l'*Actinosynema* spp est *Actinosynema pretiosum* ATCC 31585.
5. Procédé selon la revendication 3, dans lequel l'*Actinosynema* spp est *Actinosynema pretiosum* ATCC 31281.
6. Procédé selon la revendication 1, dans lequel le traitement est un chauffage à environ 75°C.
7. Procédé selon la revendication 1 ou 2, dans lequel le solvant est le toluène.
8. Procédé selon la revendication 1 ou 2, dans lequel le solvant est le xylène.
9. Procédé selon la revendication 7 ou 8, dans lequel le rapport de toluène ou du xylène au milieu de cul-

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ture traité à chaud est d'environ 1 :1.

10. Procédé selon la revendication 8, dans lequel l'extraction est réalisée à environ 45°C.

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11. Procédé selon la revendication 1 ou 2, dans lequel les ansemitocines comprennent des ansemitocines acylées qui peuvent subir un clivage réducteur pour former du maytansinol.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RADICACIÓN: 3-55528

EDICTO No. 8133

**LA SECRETARÍA GENERAL AD-HOC
HACE SABER:**

Que esta Superintendencia profirió Resolución No. 7880 de 23-MAR-2007. Que el contenido de la parte resolutive es como sigue: El Superintendente de Industria y Comercio.

RESUELVE

ARTICULO PRIMERO: Negar el privilegio de patente de invención para la solicitud correspondiente a "METODOS PARA LA PRODUCCION DE ANSAMITOCINA".

ARTICULO SEGUNDO: Notificar personalmente el contenido de la presente resolución al doctor Germán Castillo Grau, o a quien haga sus veces, en su calidad de apoderado de Smithkline Beecham Pte., entregándole copia de la misma, advirtiéndole que contra ella procede el recurso de reposición interpuesto ante el Superintendente de Industria y Comercio, al momento de la notificación o dentro de los 5 días hábiles siguientes a ella.

NOTIFÍQUESE Y CUMPLASE

Dada en Bogotá, D.C., a los **23 MAR 2007**

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,

JAMES PABLO ESCOBAR

Para notificar a los interesados, se fija el presente EDICTO en lugar visible al público en la SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO, por el término de (10) diez días hábiles contados a partir de las 8:00 a.m. de hoy.

Secretaría General Ad-Hoc 09-ABR-2007 Este edicto se desfija hoy 20-ABR-2007 a las 4:30 p.m.
Secretaría General Ad-Hoc
EDICTO No. 8133