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(71) Applicant *(for all designated States except US)*: **CJ CORP.** [KR/KR]; 500 Namdaemunro5-ga, Jung-gu, Seoul 100-095 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEE, Chan-Kyu** [KR/KR]; 992-21 Doksan3-dong, Gumcheon-gu, Seoul 153-827 (KR). **KIM, Deog-Yeor** [KR/KR]; Da-201 Samil Green Villa, 214-3 Samseon-dong, Seongbuk-gu, Seoul 136-041 (KR). **SUH, Jung-Woo** [KR/KR]; 1104-1002 Mokdong Apt., 1279, Shinjeong-dong, Yangcheon-gu, Seoul 158-865 (KR). **CHANG, Jun-Hwan** [KR/KR]; 1-705 Kyungnam Apt., 649, Gaepo1-dong, Kangnam-gu, Seoul 135-806 (KR).

(74) Agent: **LEE, Young-Pil**; The Cheonghwa Bldg., 1571-18 Seocho-dong, Seocho-gu, Seoul 137-874 (KR).

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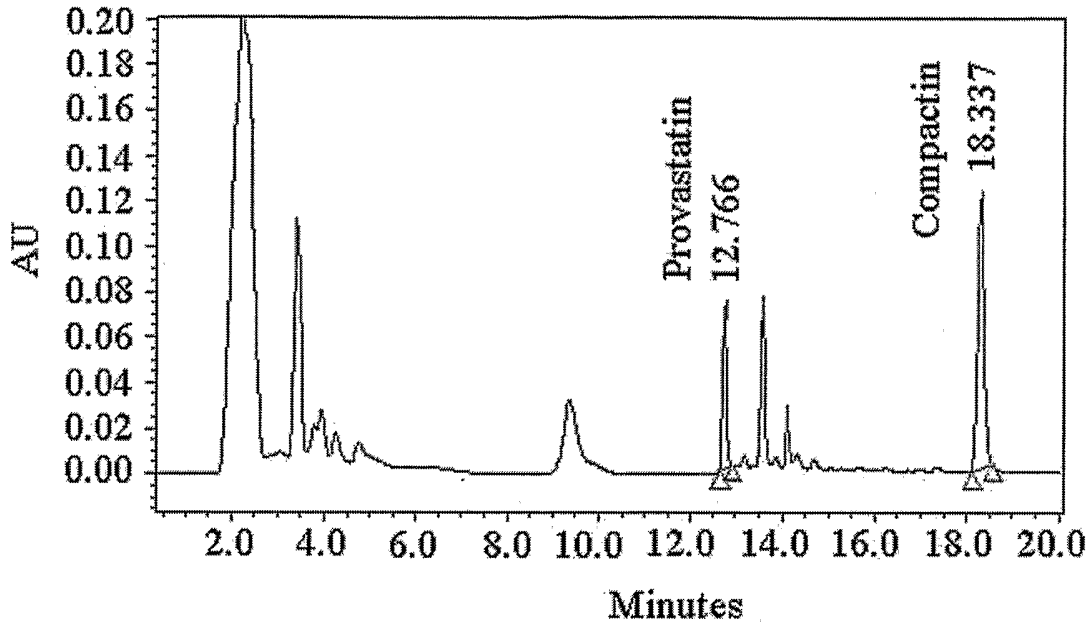
[Continued on next page]

(54) Title: STREPTOMYCES SP. CJPV975652 CAPABLE OF CONVERTING COMPACTIN TO PRAVASTATIN AND METHOD FOR PRODUCING PRAVASTATIN USING THE SAME



(57) Abstract: Provided is a Streptomyces sp. CJPV 975652 (KCCM-10497) capable of converting compactin to pravastatin and a method for producing pravastatin using a strain of Streptomyces sp. CJPV 975652 (KCCM-10497), which includes: culturing the strain of Streptomyces sp. CJPV 975652 (KCCM-10497) in a compactin-containing medium; and recovering pravastatin from the culture.

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

Description

STREPTOMYCES SP. CJPV975652 CAPABLE OF CONVERTING COMPACTIN TO PRAVASTATIN AND METHOD FOR PRODUCING PRAVASTATIN USING THE SAME

Technical Field

[1] The present invention relates to a novel *Streptomyces sp.* capable of converting compactin to pravastatin and a method for producing pravastatin using the same.

Background Art

[2] Pravastatin and compactin are substances that are effective in the treatment of hyperlipidemia. Hyperlipidemia is a major risk factor for atherosclerosis and coronary artery syndrome which are major causes of death by cardiovascular diseases in advanced countries. Hyperlipidemia refers to an excess elevation of lipids, cholesterol, and neutral fats in the bloodstream due to disorder of lipoprotein metabolism . More than 60% of cholesterol in the body is derived from biosynthesis. More than 25 enzymes are involved in cholesterol biosynthesis. Among these enzymes, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase is a major rate-limiting enzyme in cholesterol biosynthesis. Therefore, selective inhibition of HMG-CoA reductase can effectively treat cholesterol associated diseases including hyperlipidemia. Compactin and pravastatin are competitive inhibitors of HMG-CoA reductase. When any one of compactin and pravastatin is present, cholesterol biosynthesis can be blocked. For example, it is known that daily dosage of 5-10 mg of pravastatin for 3 months reduces a total cholesterol level and a LDL-cholesterol (harmful in the body) level by 20-40%. Pravastatin may be prepared by hydroxylation of compactin by microorganism. Pravastatin is more effective than compactin as a HMG-CoA reductase inhibitor.

[3] Among currently known antihyperlipidemic agents, simvastatin and atorvastatin are synthetic drugs that inhibit HMG-CoA reductase. Furthermore, lovastatin and pravastatin are representative drugs that can be produced by microorganism fermentation. Pravastatin is a drug that is biosynthesized by hydroxylation of compactin which is a precursor of pravastatin. Since pravastatin was first developed in early 1980's, efficacy and safety of pravastatin have been demonstrated. Therefore, pravastatin has been actively used in treating patients with hyperlipidemia.

[4] It is reported that compactin can be converted to pravastatin by hydroxylation by

microorganism including bacteria such as the genus *Nocardia* of the *Actinomycetes*; the genus *Actinomudura* of the *Maduromycetes*; and *Streptomyces roseochromogenus* and *Streptomyces carbophilus* of the *Streptomycetes*, and various genera of fungi (U.S. Patent Nos. 5,179,013, 4,448,979, 4,346,227, and 4,537,859, and Japanese Patent No. 58-10573).

Disclosure of Invention

Technical Problem

[5] However, a microorganism capable of producing pravastatin in high yield in a fermentation broth and an improved method for producing pravastatin using the microorganism are still being required.

Technical Solution

[6] The present invention provides a novel *Streptomyces* strain capable of converting compactin to pravastatin in a high efficiency.

[7] The present invention also provides a method for producing pravastatin, which includes converting comactin to pravastatin by the novel *Streptomyces* strain.

[8] According to an aspect of the present invention, there is provided a *Streptomyces* sp. CJPV 975652 (KCCM-10497) capable of converting compactin to pravastatin.

[9] According to another aspect of the present invention, there is provided a method for producing pravastatin using a strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497), which includes: culturing the strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497) in a compactin-containing medium; and recovering pravastatin from the culture.

Advantageous Effects

[10] According to *Streptomyces* sp. CJPV 975652 (KCCM-10497) of the present invention, compactin can be converted to pravastatin in high efficiency.

[11] According to a method for producing provastatin using *Streptomyces* sp. CJPV 975652 (KCCM-10497) of the present invention, pravastatin can be produced in high yield.

Description of Drawings

[12] The above and other features and advantages of the present invention will become more apparent by describing in detail exemplary embodiments thereof with reference to the attached drawings in which:

[13] FIG. 1 is a HPLC chromatogram of pravastatin produced by *Streptomyces* sp. CJPV 975652 (KCCM-10497) of the present invention wherein the retention time of pravastatin is 12.766 minutes (area: 401298) and the retention time of compactin is

18.337 minutes (area: 230258);

[14] FIG. 2 is a LC-MS spectrum of a HPLC fraction of pravastatin measured in negative mode wherein a peak of m/z 423 indicates pravastatin;

[15] FIG. 3 is a MS daughter scan spectrum of a HPLC fraction of pravastatin measured in negative mode wherein a peak of m/z 423 indicates pravastatin and a peak of m/z 321 indicates a specific product obtained by fragmentation of pravastatin;

[16] FIG. 4 is a UV spectrum of purified pravastatin;

[17] FIG. 5 is a H-NMR spectrum of purified pravastatin; and

[18] FIG. 6 is an electron microscopic image of *Streptomyces* sp. CJPV 975652 (KCCM-10497) of the present invention.

Best Mode

[19] Hereinafter, the present invention will be described more specifically by Examples. However, the following Examples are provided only for illustrations and thus the present invention is not limited to or by them.

[20] Example 1: Isolation of microorganism from soil

[21] A soil sample was taken from a ginseng field (a district of Gangwha, Gyeonggi-do, Korea) and dried at 40 ℃ for 24 hours. 1.0 g of the dried soil sample was suspended in 10ml of sterilized distilled water and consecutively twice diluted. 0.1 ml of the diluted solution was plated on 20ml of agar media (see Table 1) for separation of *Actinomyces* strains and cultured at 28 ℃ for 14-21 days. Thereafter, soil microorganism was isolated. Humic acid-vitamin agar media containing 50 ㎍ /ml of cycloheximide as a fungicidal agent and 200 ㎍ /ml of nalidixic acid as an antibacterial agent were used as the agar media for separation of *Actinomyces* strains. The fungicidal agent and the antibacterial agent were separately filtration sterilized and then added to the humic acid-vitamin agar media that had been sterilized at 121 ℃ for 15 minutes. The composition of the humic acid-vitamin agar media is summarized in Table 1 below.

[22] Table 1: Composition of humic acid-vitamin agar media

Component	Content	Remark
Humic acid	1.0g	10 ml of 0.2N NaOH dissolution
Na_2HPO_4	0.5g	
KCl	1.71g	
$MgSO_4 \cdot 7H_2O$	0.05g	

$FeSO_4 \cdot 7H_2O$	0.01g	
Vitamin B group	0.5mg	0.5 mg of each of cyamin-HCl, B_2, niacin, pyridoxine-HCl, inositol, Ca-pentothetate, and p-amino benzoic acid, 0.25 mg of biotin
Cycloheximide	50mg	
Nalidixic acid	200 μg /ml	
Agar	20g	
Distilled water	1L	pH 7.0

[23] *Actinomyces* colonies obtained according to the above method were purely separated. After compactin was added to *Actinomyces*-containing culture media, *Actinomyces* producing pravastatin from compactin were separated and designated as *Streptomyces* sp. CJPV 975652 (KCCM-10497).

[24] The growth of the microorganism was wholly good. Aerial mycelia exhibited mainly yellow or gray color with a pale yellow underneath. An electron microphotograph of the microorganism revealed straight spore chains, smooth spore surfaces, and fragmented spores (FIG. 6). The microorganism consumed most sugars contained in culture media, including D-glucose, sucrose, D-mannitol, D-fructose, D-xylose, L-arabinose, I-inositol, rhamnose, and raffinose. Gelatin liquefaction and starch digestion were positive. Furthermore, the sugar analysis of cell walls revealed glucose, arabinose, and galactose, and ribose, and the amino acid analysis revealed meso-diaminopimellic acid.

[25] As a result of comparison between the separated microorganism and existing *Actinomyces* in terms of morphological, cultural, and physiological characteristics, the microorganism was judged to be a new strain of the genus *Streptomyces*. Therefore, the microorganism was designated as *Streptomyces* sp. CJPV 975652 and deposited in the Korean Culture Center of Microorganisms (KCCM), which is an international depository authority under the Budapest treaty, on May 12, 2003 (deposition number: KCCM-10497). The cultural characteristics of *Streptomyces* sp. CJPV 975652 (KCCM-10497) are summarized in Table 2 below.

[26] Table 2: Cultural characteristics of *Streptomyces* sp. CJPV 975652 (KCCM-10497)

Medium	Growth	Color of aerial mycelia	Color of underneath	Water-soluble pigment
Tryptone-yeast extract agar medium	Good	Light yellow	Light brown	-
Yeast extract-malt extract agar medium	Excellent	Bright gray	Brown	-
Oatmeal agar medium	Good	White, pale yellow	Pale yellow	-
Inorganic salt-starch agar medium	Excellent	White, pale yellow	Yellow	-
Glycerol-asparagine agar medium	Good	Yellow, white	Light brown	-
Tyrosine agar medium	Excellent	Light yellow	Yellow	Bright yellow
Nutrient agar medium	Normal	White, pale yellow	Yellow	-
Sugar agar medium	Excellent	Pale yellow	Yellow	-
Glucose-asparagine agar medium	Good	Pale yellow	Pale yellow	-

[27] Characteristics of the carbon source utilization of *Streptomyces* sp. CJPV 975652 (KCCM-10497) are summarized in Table 3 below.

[28] Table 3: Characteristics of carbon source utilization of *Streptomyces* sp. CJPV 975652 (KCCM-10497)

Carbon source	Utilization
D-glucose	+

L-arabinose	+
Sucrose	+
D-xylose	+
I-inositol	+
D-mannitol	+
D-fructose	+
Raffinose	+

[29] The physiological characteristics of *Streptomyces* sp. CJPV 975652 (KCCM-104 97) are summarized in Table 4 below.

[30] Table 4: Physiological characteristics of *Streptomyces* sp. CJPV 975652 (KCCM-10497)

Section	Characteristics
Melanin production	Negative
Nitrate reduction	Positive
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Casein digestion	Negative
Casein coagulation	Positive

[31] The cell wall components of *Streptomyces* sp. CJPV 975652 (KCCM-10497) are summarized in Table 5 below.

[32] Table 5: Cell wall components of *Streptomyces* sp. CJPV 975652 (KCCM-10497)

Section	Component
DAP(diaminopimellic acid)	Meso-DAP
Glycine	-
Sugar	Sucrose Galactose Arabinose Ribose

[33] Example 2: Identification of pravastatin produced from comactin by Streptomyces sp. CJPV 975652 (KCCM-10497)

[34] A strain of *Streptomyces* sp. CJPV 975652 was used.

[35] A culture method was as follows: 50 ml of a preculture medium (Table 6) was placed in an 500 ml Erlenmeyer flask and pressure-sterilized at 121 °C for 20 minutes. A culture piece (0.5 cm x 0.5 cm) (containing the strain) that had been cultured in a plate medium for 2 weeks was inoculated on the sterilized preculture medium and cultured in a rotary stirring incubator at 250 rpm at 28 °C for 2 days.

[36] 50 ml of a main medium (Table 7) that had been pressure-sterilized at 121 °C for 20 minutes was placed in a 500 ml Erlenmeyer flask. 10% (5 ml , v/v) of the resultant preculture was inoculated on the main medium and cultured in a rotary stirring incubator at 250 rpm at 28 °C for 5 days. From 2 days after the culture, 0.5 g/L of sterilized compactin (in the form of sodium salt) was added twice a day to induce the conversion of compactin to pravastatin. After 10 ml of a fermentation broth was taken and centrifuged at 450xg for 10 minutes, PMV (Packed Mycelium Volume) was measured to evaluate the growth of the strain.

[37] As a result, pH of the fermentation broth was 7.50 and PMV was 23%. HPLC was performed using waters C18 column (3.9 x 300 mm) and Shimadzu HPLC system (Model LC-10AD). For HPLC analysis, 400 μl of ethanol was added to 600 μl of the fermentation broth and centrifuged for 15-30 minutes. Here, a mixture of methanol: tri-ethylamine: acetic acid: water (500 : 1 : 1: 500) was used as a mobile phase and the flow rate of the mobile phase was 1 ml/min. As a result of the HPLC analysis, pravastatin in the fermentation broth was identified (FIG. 1). LC-MS and UV spectra also revealed pravastatin (FIGS. 2, 3, and 4). Here, PDA2996 (Waters) was used for LC (liquid chromatography) and Ultima-PT (Micro-Mass) was used for MS (mass spectrometry). MS analysis was performed in negative mode under the conditions of 1,970 V of capillary tube voltage, 97 V of cone voltage, and 13 eV of collision energy.

[38] In FIG. 1, the retention time of pravastatin was 12.766 minutes (area: 401298) and the retention time of compactin was 18.337 minutes (area: 230258). According to the HPLC chromatogram of FIG. 1, the ratio of the area of pravastatin to the sum of the areas of compactin and pravastatin was about 64%. This demonstrated that the conversion ratio of compactin to pravastatin was very high by about 64% or more.

[39] In FIGS. 2 and 3, a peak of m/z 423 indicates pravastatin. Since pravastatin was measured in negative mode, it is considered that the peak is for a sodium-free form of pravastatin. A peak of m/z 321 is a specific peak of pravastatin that indicates a daughter type substance obtained by fragmentation of pravastatin.

[40] Table 6: Preculture medium of *Streptomyces* sp. CJPV 975652

Component	Content
Yeast extract	0.1%
Soybean powder	1.5%
$NaNO_3$	0.5%
Glucose	1.5%
Glycerol	0.25%
$CaCO_3$	0.4%

[41] Table 7: Main culture medium of *Streptomyces* sp. CJPV 975652

Component	Content
Yeast extract	0.1%
Soybean powder	2.0%
Peptone	0.5%
$NaNO_3$	0.5%
Glucose	3.0%
Glycerol	0.25%
$CaCO_3$	0.4%
NaCl	0.5%

[42] Example 3: Purification of pravastatin

[43] After the culture was completed, 3 L of the microorganism culture was diluted with 2x distilled water and diatomite (3% volume) was added thereto with stirring. The resultant solution was filtered with a filter funnel packed with diatomite. Then, the filtrate was adsorbed on a column packed with 500ml of HP-20 resin, washed with a sufficient amount of water, and eluted with a 50% ethanol solution. The eluted solution was discolored using activated carbon and alumina resin and concentrated under a reduced pressure.

[44] The crude concentrate was adsorbed on a column packed with 200 ml of XAD-1600 (Amberlite) resin and washed with 10% ethanol. Thereafter, pravastatin was separated with a 40% ethanol solution. The separated solution was concentrated under a reduced pressure, followed by crystallization with ethanol and ethylacetate, filtration, and drying under a reduced pressure, to give 1.3 g of pravastatin as a white crystal.

Pravastatin thus obtained was subjected to H-NMR analysis. Here, Brucker ARX400 FT-NMR was used as a NMR machine and MeOH-d4 was used as a solvent.

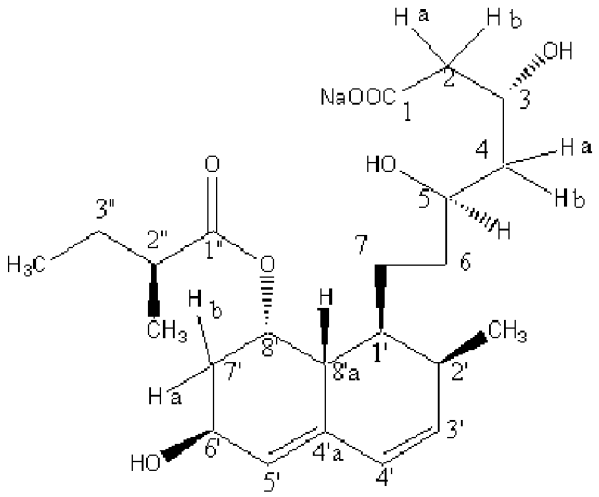
[45] The H-NMR analysis result is shown in FIG. 5. The assignment of each chemical shift of FIG. 5 is presented in Table 8 below and the resultant compound is represented by the following Formula 7.

[46] Table 8: Assignments of chemical shifts

Chemical shift (ppm)	Assignment	Remark	Number of protons
0.91	3'-CH3	t	
0.92	2'-CH3	d	
1.12	2'-CH3	d	
1.19	H6b	m	
1.36	H7a	m	
1.36	H7b	m	
1.46	H3'b	m	
1.53	H4a	m	10
1.54	M6a	m	
1.57	H4b	m	
1.58	H7'a	m	
1.61	H3'a	m	
1.67	H1'	m	
2.24	H2a	dd	
2.34	H2b	dd	
2.35	H2'	m	
2.36	H8'a	m	
2.41	H2'	m	4
2.47	H7'b	m	
3.69	H5	m	
4.06	H3	m	
4.29	H6'	m	

5.36	H8'	brs	
5.50	H5'	brs	
5.88	H3'	dd	
5.98	H4'	dd	

[47] Formula 7



[48] As shown in FIG. 5, pravastatin was identified

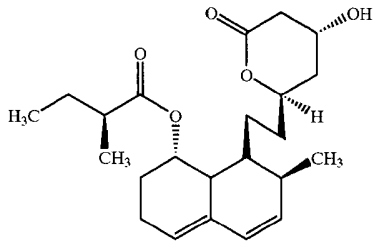
Mode for Invention

[49] The present invention provides a strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497) capable of converting compactin to pravastatin. The strain can convert compactin in a culture medium to pravastatin in high yield.

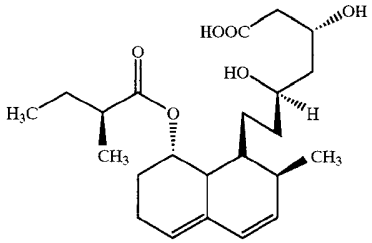
[50] The present invention also provides a method for producing pravastatin using a strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497), which includes: culturing the strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497) in a compactin-containing medium and recovering pravastatin from the culture.

[51] As used herein, the term 'compactin' encompasses a lactone structure known as mevastatin, and its free acid and salt, as represented by the following Formulae 1, 2, and 3, respectively. In particular, the compound of Formula 3 is a sodium salt of compactin.

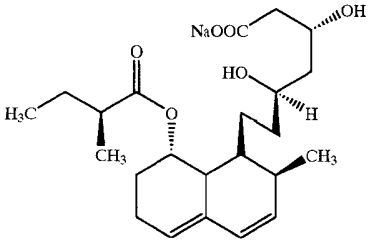
[52] Formula 1



[53] Formula 2

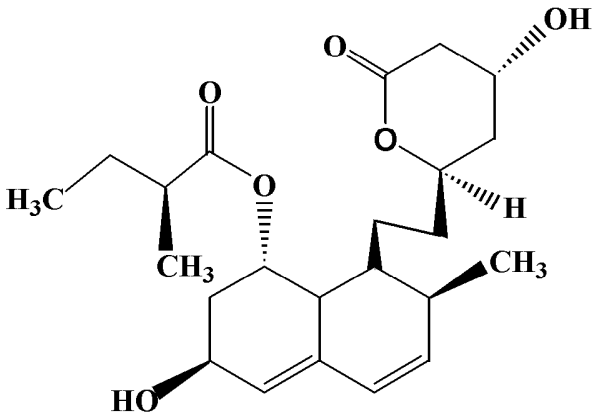


[54] Formula 3

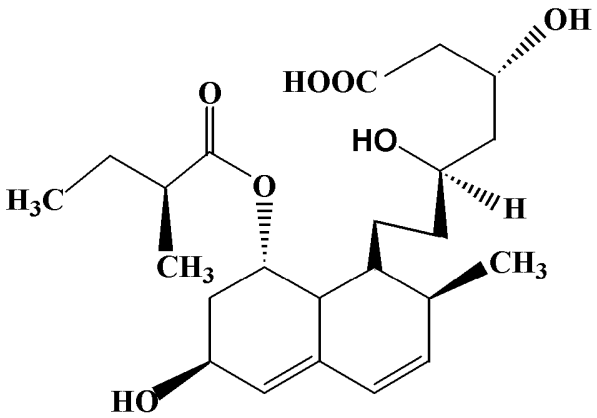


[55] As used herein, the term 'pravastatin' refers to a compound obtained by hydroxylation at the 6 β -position of compactin and encompasses a lactone structure, and its acid and salt, as represented by the following Formulae 4, 5, and 6, respectively. The compound of Formula 6 is a sodium salt of pravastatin.

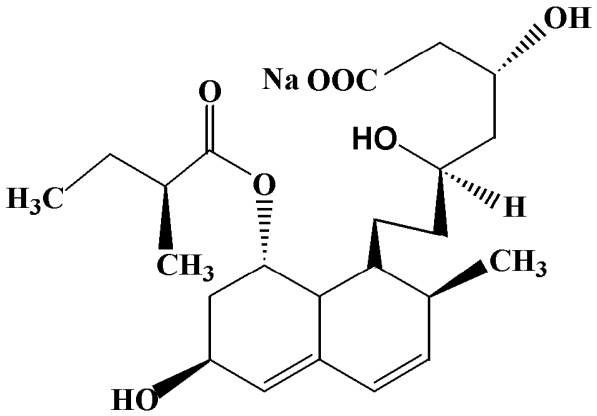
[56] Formula 4



[57] Formula 5



[58] Formula 6



[59] There are no particular limitations on the compactin-containing medium provided that it can be used in culturing common strains of the genus *Streptomyces*. The recovering pravastatin from the culture may be carried out using common separation and purification technique. For example, extraction and HPLC may be used.

[60] While the present invention has been particularly shown and described with reference to exemplary embodiments thereof, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope of the present invention as defined by the following claims.

Applicant's or agent's file reference	International application No.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 2, line 17.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

KCCM (Korean Culture Center of Microorganisms)

Address of depositary institution *(including postal code and country)*

361-221, Yurim B/D, Honje 1, Sudaemun, Seoul, 120-091, Republic of Korea

Date of deposit	Accession Number
12 May 2003	KCCM-10497

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
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Form PCT/RO/134 (July1998)

Claims

[1] A *Streptomyces* sp. CJPV 975652 (KCCM-10497) capable of converting compactin to pravastatin.

[2] A method for producing pravastatin using a strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497), which comprises:
culturing the strain of *Streptomyces* sp. CJPV 975652 (KCCM-10497) in a compactin-containing medium; and
recovering pravastatin from the culture.

FIG. 1

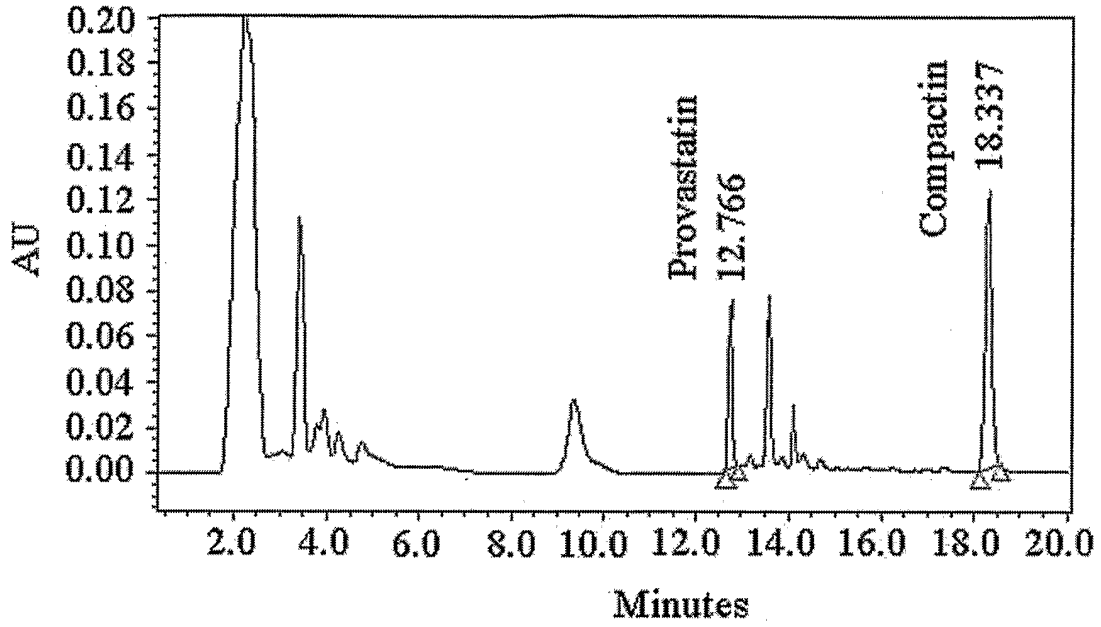

0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AU
Provastatin
12.766
Compactin
18.337
2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0
Minutes

FIG. 2

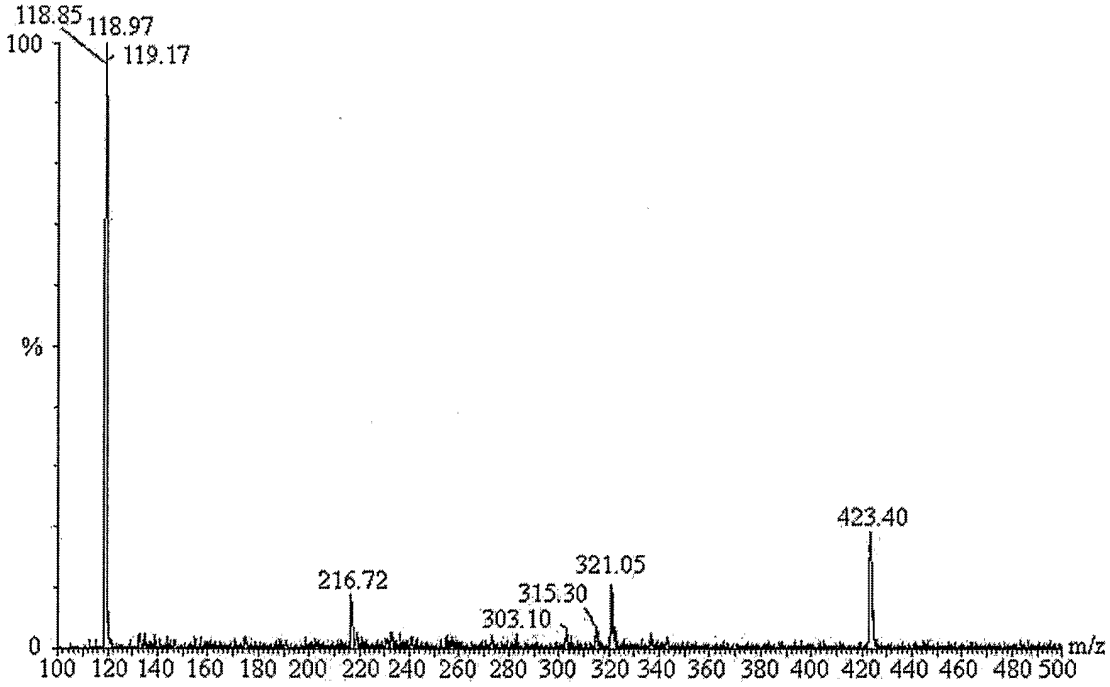

118.85
118.97
119.17
100
%
0
423.40
321.05
216.72
315.30
303.10
m/z
100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500

FIG. 3

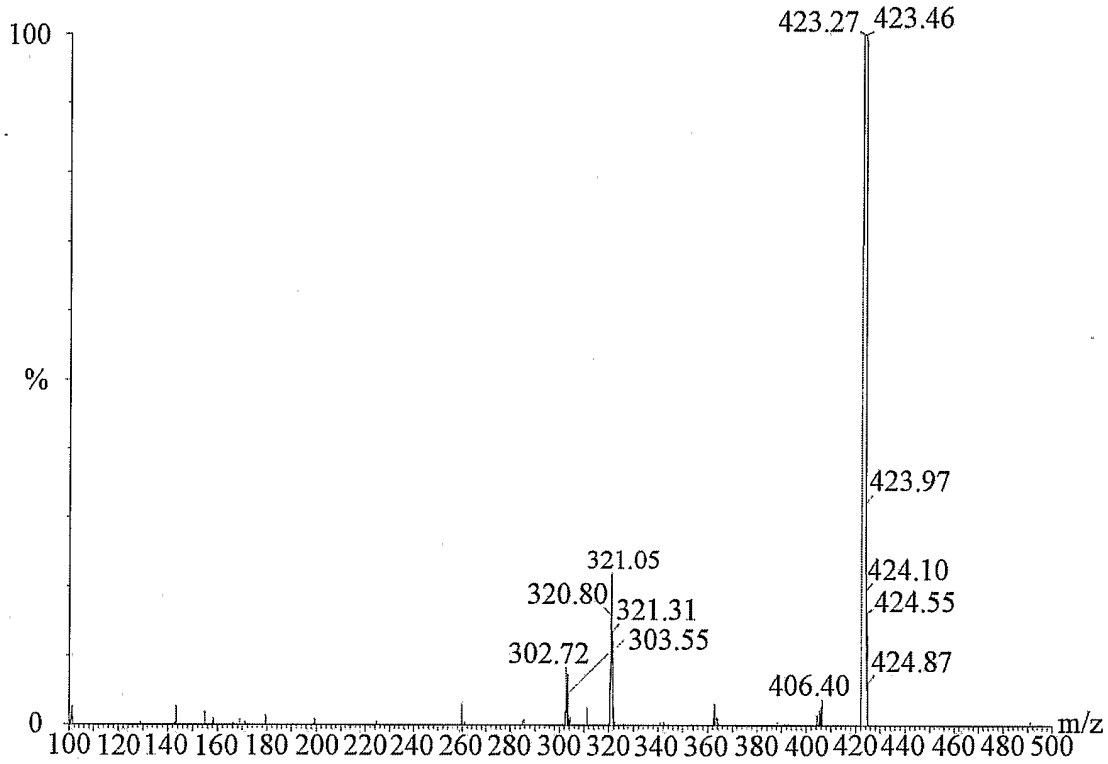

100
%
0
423.27
423.46
423.97
424.10
424.55
424.87
321.05
320.80
321.31
303.55
302.72
406.40
m/z
100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500

FIG. 4

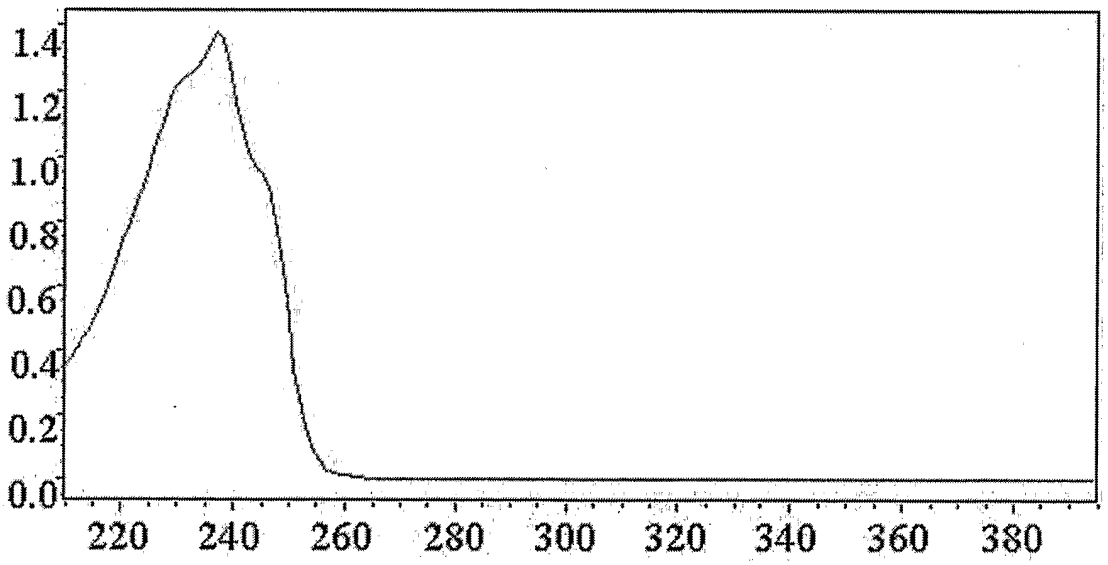

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
220 240 260 280 300 320 340 360 380

FIG.5

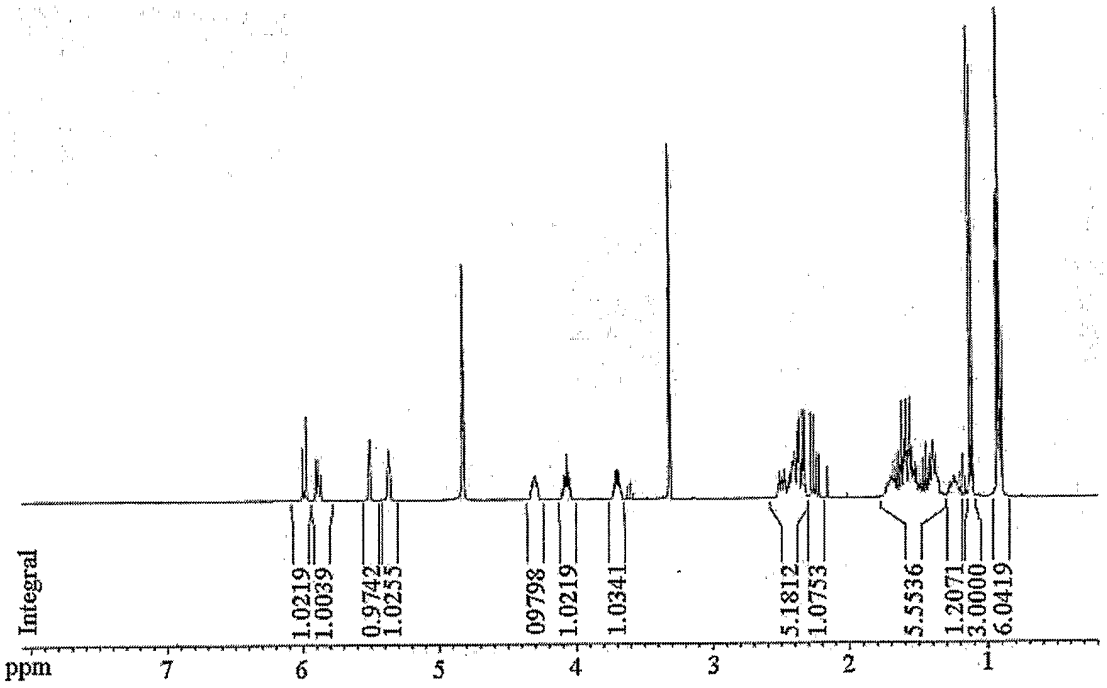
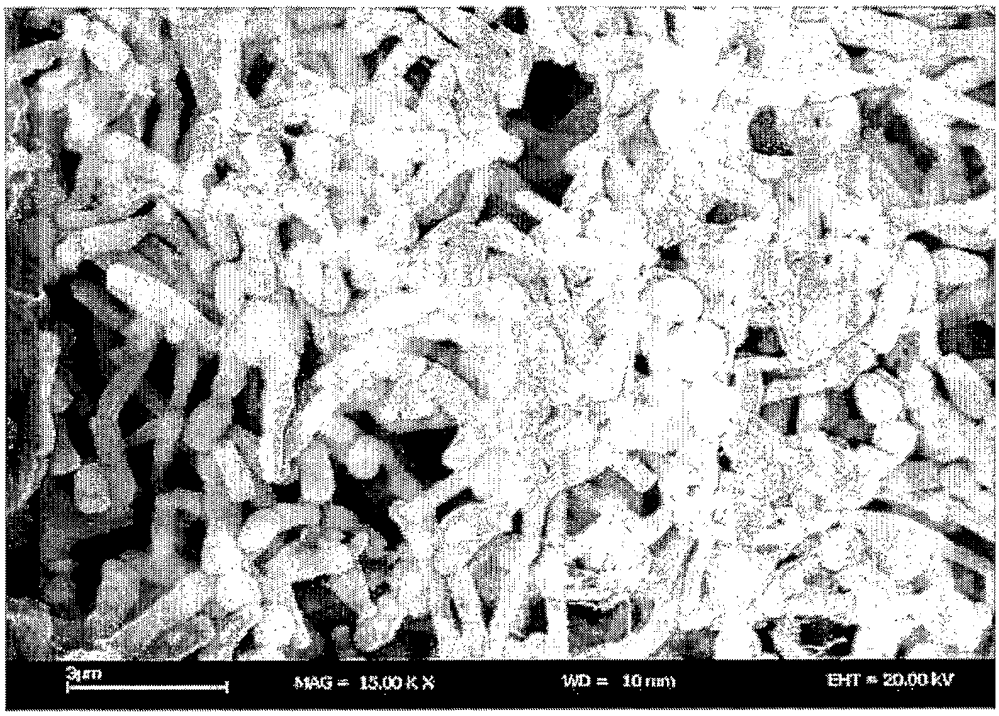

Integral
1.0219
1.0039
0.9742
1.0255
09798
1.0219
1.0341
5.1812
1.0753
5.5536
1.2071
3.0000
6.0419
ppm
7
6
5
4
3
2
1

FIG. 6


3μm
MAG = 15.00 K X
WD = 10 mm
EHT = 20.00 kV

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2004/001364

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 1/20

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)

IPC7 C12N 1/20, A61K31/22 //(C12N 1/20, C12R 1:465)

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

Electronic data base consulted during the internationl search (name of data base and, where practicable, search terms used)

Delphion, PubMed, CA, eKIpass, 'Streptomyces', 'compactin', 'pravastatin'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2003/027302 A1 (BIOCON INDIA LTD.), 03 April 2003. See the whole documents.	1-2
A	WO 1998/045410 A1 (YUNGJIN PHARMACEUTICAL IND. CO., LTD.), 15 October 1998. See the whole documents.	1-2
A	US 4,346,227 A (TERAHARA AKIRA ET AL.), 24 August 1982. See the whole documents.	1-2
A	EP 0 582 976 A2 (BOEHRINGER MANNHEIM GMBH), 16 February 1994. See the whole documents.	1-2

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 AUGUST 2004 (11.08.2004)	27 AUGUST 2004 (27.08.2004)

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
LEE, CHUNG HO
Telephone No. 82-42-481-8160

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
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