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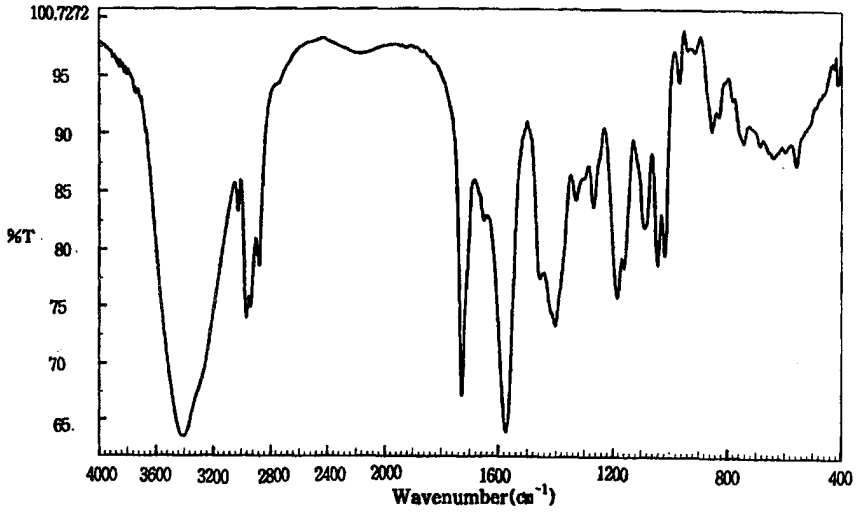
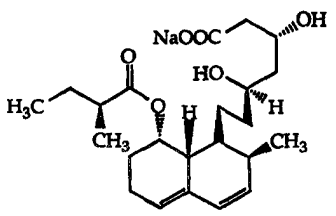
(54) Title: A NEW MICROORGANISM *STREPTOMYCES EXFOLIATUS* YJ–118 AND A METHOD FOR PRODUCING PRAVASTATIN SODIUM BY USING THE STRAIN

(57) Abstract

The present invention is related to a new microorganism *Streptomyces exfoliatus* YJ–118 and a method for producing pravastatin sodium represented in formula (I) by using this microorganism which shows a strong tolerance to ML–236B and a high hydroxylation activity of ML–236B to pravastatin.





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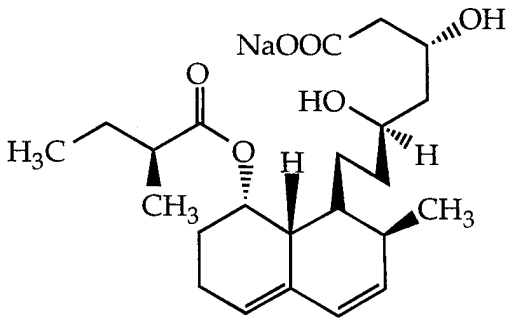
A NEW MICROORGANISM *Streptomyces exfoliatus* YJ-118 AND A METHOD FOR PRODUCING PRAVASTATIN SODIUM BY USING THIS STRAIN.

BACKGROUND OF THE INVENTION

Field of the invention

The present invention is related to a new microorganism *Streptomyces exfoliatus* YJ-118 and a method for producing pravastatin sodium represented in formula I by using this microorganism which shows a strong tolerance to ML-236B and a high hydroxylation activity of ML-236B to pravastatin.



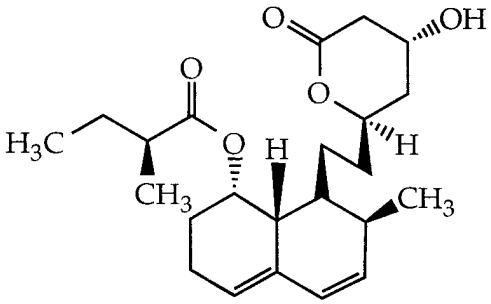
(I)

Description of the Prior Art

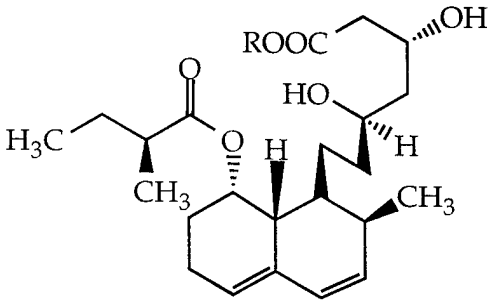
An elevated plasma cholesterol level has long been recognized as a major risk factor for atherosclerotic disease specifically for coronary heart disease. It was expected that plasma cholesterol could be reduced as a result of inhibition of cholesterol biosynthesis because more than 70% of the total input of body cholesterol is derived from *de novo* synthesis in humans. In 1975, ML-236B, a potent

inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A(HMG-Co A) reductase, a rate-limiting enzyme in the biosynthesis of cholesterol, was discovered in the culture broth of *Penicillin citrium*. After thorough screening of hundreds of not only microbial products but also chemically or biologically modified derivatives of ML-236B, pravastatin sodium was chosen as a candidate for development, because of its stronger and more tissue-selective activity than the prototype compound.

Pravastatin has been produced by two-step fermentation: first, biosynthesis of ML-236B and second, bioconversion of this compound to pravastatin sodium. Lactone and carboxylate of ML-236B are represented in formula II-a and formula II-b, respectively.



(II-a)



(II-b)

wherein, R is H or Na.

It was reported that *Streptomyces roseochromogenus* NRRL-1233, *Streptomyces roseochromogenus* IFO-3363, *Streptomyces roseochromogenus* IFO-3411(US. Patent No. 4,346,227) and *Streptomyces carbophilus* SANK-62585(Ferm BP-1145), *Streptomyces halstedii* IFO-3199(JP No. Pyung 4-349034) transformed ML-236B to pravastatin. These fungi, however, are not tolerable to a

relatively high amount of ML-236B in the culture broth because of antifungal activity of the substrate, and show low productivity of pravastatin.

SUMMARY OF THE INVENTION

The present inventors investigated to find out a new microorganism which is tolerable to higher amount of ML-236B and also has strong transformation activity. Finally, they found a new microorganism, *Streptomyces exfoliatus* YJ-118.

Description of the Drawings

The invention will be described in more detail in the drawings.

Fig. 1 is the IR spectrum of pravastatin sodium

Fig. 2 is the ^{13}C-NMR spectrum of pravastatin sodium

Fig. 3 is the ^{1}H-NMR spectrum of pravastatin sodium

Detailed Description of the Invention

The present inventors isolated a new microorganism, *Streptomyces exfoliatus* YJ-118. This microorganism showed strong tolerance to high amount of pravastatin precursor represented in formula II-a and formula II-b. Pravastatin known as plasma cholesterol lowering agent is produced by using this organism.

(A) Isolation of a new microorganism

Soil samples diluted 10^{-3}~10^{-5} fold with sterile distilled water are spread on ISP No.2 agar plate containing 0.05~0.5%(w/v) ML-236B and culture at 27℃ for 7 days.

Among 500 strains isolated from the cultivation, one strain which shows strong tolerance to ML-236B and high hydroxylation activity was selected. This strain was named YJ-118.

(B) Identification of the new microorganism

The properties of the new microorganism YJ-118 obtained from the above are as follows.

(1) Morphological properties

YJ-118 was grown on a medium suggested by ISP (International Streptomyces Project) at 27℃ for 7~14 days. The strain has grown actively on that medium and formed substrate mycelium, aerial mycelium and spores.

Scanning electron micrograph has revealed that the surface of spore was smooth, cylindrical and the appearance of spore chain was linear or rectiflexible.

(2) Chemical components of strain YJ-118

Cell wall type of YJ-118 were analyzed in the way reported in a reference[Yamada et al., Gen. Appl. Microbiol. 16; 103~113, 1970].

L.L-diaminopimelic acid, glutamic acid, alanine were discovered and so it was proved that YJ-118 cell wall could be assigned to Type 1.

Sugar pattern in YJ-118 cell were analyzed in the way suggested in[M. P. Lechevalier et al. Journal of Laboratory and clinical medicine, 71, 934 (1968)] and abnormal patterns were not shown.

The results obtained from the above have proved that the isolated microorganism from this invention belongs to genus *Streptomyces*.

(3) Numerical identification of the new streptomyces strain

50 taxonomic unit characters of the strain for assigning to Major clusters and 34 unit characters for Minor clusters were tested in the way suggested by William et al. and the data were analyzed numerically using TAXON program. TAXON program is devised by Dr. Alan Ward[Department of Microbiology, Newcastle upon Tyne University, UK]. The data are input in the form of + or - and analyzed by CLUSTAN and numerically classified. On the basis of the numeric classification, probabilistic identification matrix is formed. By comparing the characteristics of probabilistic identification matrix with that of unidentified *Streptomyces* strain, the numeric identification was accomplished[Journal of general Microbiology(1983), 129, 1743~1813; Rho.Y.T, Ph. D. Thesis, Seoul National Univ(1993)].

54 unit characters of YJ-118 needed to identify this strain are shown in the following Table 1a and Table 1b.

Table 1a

Unit characters			YJ-118
Morphology and Pigmentation	Spore chain morphology	rectiflexible	+
		spiral	-
	Color of sporemass	red	-
		grey	+
	Mycelial pigment	red/orange	-
	Diffusible pigment	production	+
		yellow/brown	+
	Melanin production on	PYI medium	+
		Tyrosine medium	+
Antimicrobial activity	*Bacillus subtilis*		-
	Micrococcus luteus		-
	Candida albicans		-
	Saccharomyces cerevisiae		-
	Streptomyces murinus		-
	Aspergillus niger		+
Biochemical test	Lecithinase		+
	Lipolysis		+
	Pectin hydrolysis		+
	Nitrate reduction		+
	H_2S production		+
	Hippurate reduction		-
Degradation test	Elastin		+
	Xanthine		+
	Albutin		+

Table 1b

Unit characters		YJ-118
Antibiotic resistance	Neomycin	-
	Rifampicin	-
	Oleandomycin	+
	Penicillin G	+
Growth test	45℃	-
	NaCl	-
	Sodium azide	-
	Phenol	-
	Potassium tellurite	-
	Thallus acetate	-
Compound as sole source of nitrogen	DL-α -amino-n-butyric acid	+
	L-Cysteine	+
	L-Valine	+
	L-phenylalanine	+
	L-histidine	+
	L-Hydroxyproline	+
Organic compound as sole source of carbon	Sucrose	-
	meso-Inositol	-
	Mannitol	-
	L-Rhamnose	-
	Raffinose	-
	D-Melizitose	+
	Adonitol	+
	Dextran	-
	D-Melibiose	+
	Xylitol	-

Identification scores of the isolated *Streptomyces* YJ-118 to the major clusters of *Streptomyces* by TAXON program are represented in Table 2.

Table 2

Taxon major clusters	Taxon distance	90% TAXON radius	Probability of strain further away(%)	Willcox probability
5	0.3838	0.44554	48.4519	0.999999
6	0.4614	0.4126	0.1274	0.000000
33	0.4721	0.3955	0.0050	0.000000
1C	0.4723	0.3883	0.0016	0.000000
1B	0.5252	0.4404	0.0053	0.000000

Consequently, the isolated YJ-118 showed the highest Willcox probability to Major cluster 5 represented by *Streptomyces exfoliatus*. YJ-118 was compared with 18 strains assigned to Major cluster 5 by simple matching coefficient. The isolate YJ-118 shared only 40% similarity with *Streptomyces nashvillensis* among 18 strains.

So, this inventors named the new isolated microorganism as *Streptomyces exfoliatus* YJ-118. This strain was accepted by international depositary authority, Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures and given the accession number KCTC 0318BP on February 7, 1997.

Streptomyces exfoliatus YJ-118 has not yet been used to produce pravastatin. The present inventors used this strain to produce pravatatin sodium. Transformed *Streptomyces exfoliatus*

YJ-118 by mutation and DNA recombination may be also used to produce pravastatin.

A method for producing pravastatin sodium using *Streptomyces exfoliatus* YJ-118 is described below in detail.

For the seed culture, a medium(I) containing glucose 0.5~3%, yeast extract 1.0~3.0%, beef extract 0.1~1.0%, Casein hydrolyte (N-Z amine A) 0.5~2.0% is prepared. To medium(I) is added 0.01~0.05%(w/v) ML-236B represented in formula II-a and formula II-b which is sterilized by filtration. *Streptomyces exfoliatus* YJ-118 is inoculated in medium(I) and cultured at 27℃ for 2~3 days. ML-236B added to the seed culture medium(I) is thought to play a role as an activator of enzymatic hydroxylating reaction. More than 0.05%(w/v) of the concentration of ML-236B in broth, however, the preculture time increased and cell growth is not good.

Ten percent of seed culture is inoculated in a production medium(II) containing glucose 1.0~3.0%, yeast extract 0.5~2.0%, polypeptone 0.1~2.0%, K_2HPO_4 0.01~0.5%, $MgSO_4 \cdot 7H_2O$ 0.01~0.1%, NaCl 0.01~0.1% and cultures at 27℃ on a rotary shaker.

0.1~5.0%(w/v) of ML-236B, preferentially 0.1~3.0%(w/v), is added to the culture broth and the cultivation was continued at 27℃. Two or three days later, 0.3~0.5%(w/v) glucose solution was fed. It supposed that additive glucose might be used in hydroxylation of ML-236B as an energy source and a catalyst. During production culture, ML-236B concentration might be maintained in a range of 0.1~5.0%. Lower than 0.1%, pravastatin productivity decreases and higher than 5.0%, cell growes very slowly and pravastatin productivity also decreases.

It has been reported that microorganisms producing pravastatin could be tolerant to the range of 0.05~0.1%(w/v) ML-236B concentration in culture broth. However, *Streptomyces exfoliatus* YJ-118 isolated from this invention showes tolerance to 5%(w/v) ML-236B concentration.

For the purification of pravastatin sodium, the culture broth was centrifuged and cell mass was discarded. The supernatant was adsorbed to a column of polymeric adsorbent resin and washed with water. Pravastatin sodium is eluted with 20~50% acetone solution or methanol solution. Pravastatin sodium fraction is concentrated *in vacuo*. The residue is applied to HPLC of ODS reverse phase and active fraction of pravastatin sodium is dried *in vacuo*. Pravastatin sodium is obtained as white crystal in ethanol and ethyl acetate.

The present invention is represented in detail by the examples below, which aren't intended to be exemplary only.

MANUFACTURING EXAMPLE

Soil samples collected from all around South Korea were diluted 10^{-3}~10^{-5} fold with sterile distilled water and spread on ISP No.2 agar plates containing 0.05~0.5%(w/v) pravastatin precursor, ML-236B, represented in formula II-b.

Cultivation was done at 27℃ for 5~10 days. About 500 pure isolates were obtained and inoculated in 125*ml* Erlenmeyer flask containing 20*ml* Bennett's medium and cultured at 27℃ for 5 days on a rotary shaker. Two days later, 0.05% ML-236B was added and the cultivation was continued for 2 more days. The amount of

pravastatin sodium produced from culture broth was analyzed by HPLC. Among 10 strains showing hydroxylation activity of ML-236B, one strain which had strong tolerance to ML-236B was selected and named *Streptomyces exfoliatus* YJ-118.

EXAMPLE 1

To 125*ml* Erlenmeyer flask containing 20*ml* seed culture medium(I) that comprises glucose 1%, yeast extract 0.2%, skim milk 0.2%, casein hydrolyte (N-Z amine) 0.5%, pH 7.0, 0.02%(w/v) ML-236B was added and *Streptomyces exfoliatus* YJ-118 isolated from manufacturing Example was inoculated. The cultivation was done at 27℃, 200 rpm, for 2 days on a rotary shaker. 20*ml* of seed culture above was inoculated in 2 *l* Erlenmeyer flask containing 400*ml* production medium(II) that comprises glucose 1.0%, yeast extract 1.0%, polypeptone 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.01~0.1%, pH 7.2 and the flask was cultured at 27℃, 150 rpm. One day after cultivation, 0.05%(w/v) ML-236B(formula II -a) was added every day till the final concentration of ML-236B in culture broth became 0.2%(w/v). The cultivation was continued at 27℃, 150 rpm for 6 days and 0.3% glucose was fed once every two days 2 times in total. After then, the culture broth was adjusted to pH 9.0 and stirred for 3hr. After centrifugation cell mass was removed and the supernatant was applied to a column of HP-20 500*ml*. After washed with water, pravastatin sodium was eluted with 25% acetone solution. Pravastatin sodium fraction was concentrated *in vacuo* and the residue was applied to semi preparative HPLC(Kromasil C_{18} resin). Pravastatin sodium was

eluted with 35% acetonitrile solution and was obtained as white crystal 1,254mg(627 mg/ *l*).

EXAMPLE 2

Except for that 12hr after the cultivation, 0.04%(w/v) ML-236B represented in formula II-b was added to culture broth once every 12hr, pravastatin sodium was produced from the same method as described in above Example 1. The cultivation was done at 27 ℃, 150 rpm for 7 days. As a result, pravastatin sodium was obtained 2.68g (1,340 mg/ *l*).

EXAMPLE 3

Except for that ML-236B was not added in seed culture, pravastatin sodium was produced from the same method as described above in Example 1. The obtained pravastatin sodium was 778mg (389 mg/ *l*). This amount of pravastatin sodium was 60~65% higher than that of other microorganisms.

COMPARATIVE EXAMPLE : US Patent No. 4, 346, 227

Streptomyces roseochromogenus NRRL 1233 was inoculated in twenty 500*ml* flask containing 100*ml* medium that comprises glucose 2.0%, corn steep liquor 0.2%, K_2HPO_4 0.15%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.15%, $ZnSO_4 \cdot 7H_2O$ 0.001%, NH4NO3 0.1%, peptone 0.1%, pH 7.0 and cultured at 26℃, 120 rpm for 2 days on a rotary shaker. 0.05%(w/v) ML-236B represented in formula II-b was added to the culture broth, 2 days after cultivation. After

cultivation was done for 5 days, 120mg(60 mg/ l) pravastatin sodium was obtained from the method described above in Example 1.

Considering ML-236B concentration in culture broth and pravastatin productivity, the present invention is more excellent than Comparative Example described above.

EXPERIMENTAL EXAMPLE

The physical properties of pravastatin sodium obtained from Example 1 and Comparative Example are described in Table 3.

Table 3

Article	EXAMPLE 1	COMPARATIVE EXAMPLE
Formula	$C_{23}H_{35}O_7Na$	$C_{23}H_{35}O_7Na$
Molecular weight	446.52	446.52
UV spectrum (MeOH)	230, 237, 245nm	230, 237, 245nm
IR spectrum (cm^{-1}, KBr)	3415, 2964, 2935, 2875, 1727, 1578, 1402, 1265, 1184, 1157, 1085, 1043, 1015, 854, 557, 421	2960, 2930, 2875, 1725, 1400, 1330, 1300,1 265, 1180, 1160, 1080, 1045, 1015, 965
Specific rotation $[\alpha]^{26}$	+150~160°	+150~160°

IR spectrum, ^{13}C-NMR spectrum, ^{1}H-NMR spectrum of pravastatin sodium obtained from this invention are represented in Fig. 1, Fig. 2 and Fig. 3, respectively.

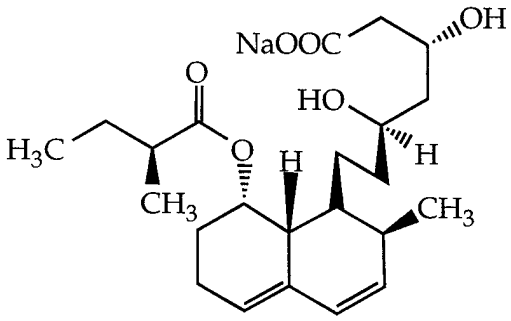
By using a new microorganism *Streptomyces exfoliatus* YJ-118 isolated from this invention, ML-236B concentration in culture broth could be raised to 0.5%(w/v) and pravastatin sodium productivity was increased up to 600~1,340 mg/ *l* much higher than that of other microorganisms(60 mg/ *l*).

CLAIMS

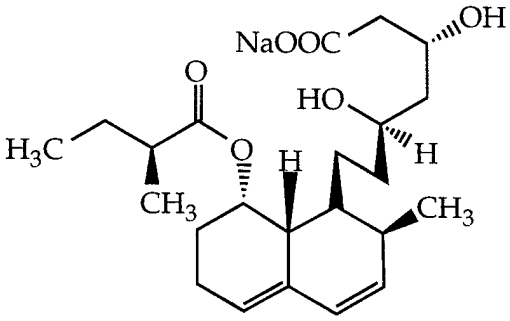
What is claimed is:

1. A new strain *Streptomyces exfoliatus* YJ-118.

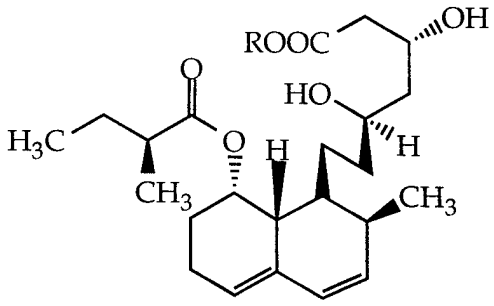
2. A method for producing pravastatin sodium represented in formula I from hydroxylation of ML-236B represented in formula II-a and formula II-b using *Streptomyces exfoliatus* YJ-118.



(I)



(II-a)

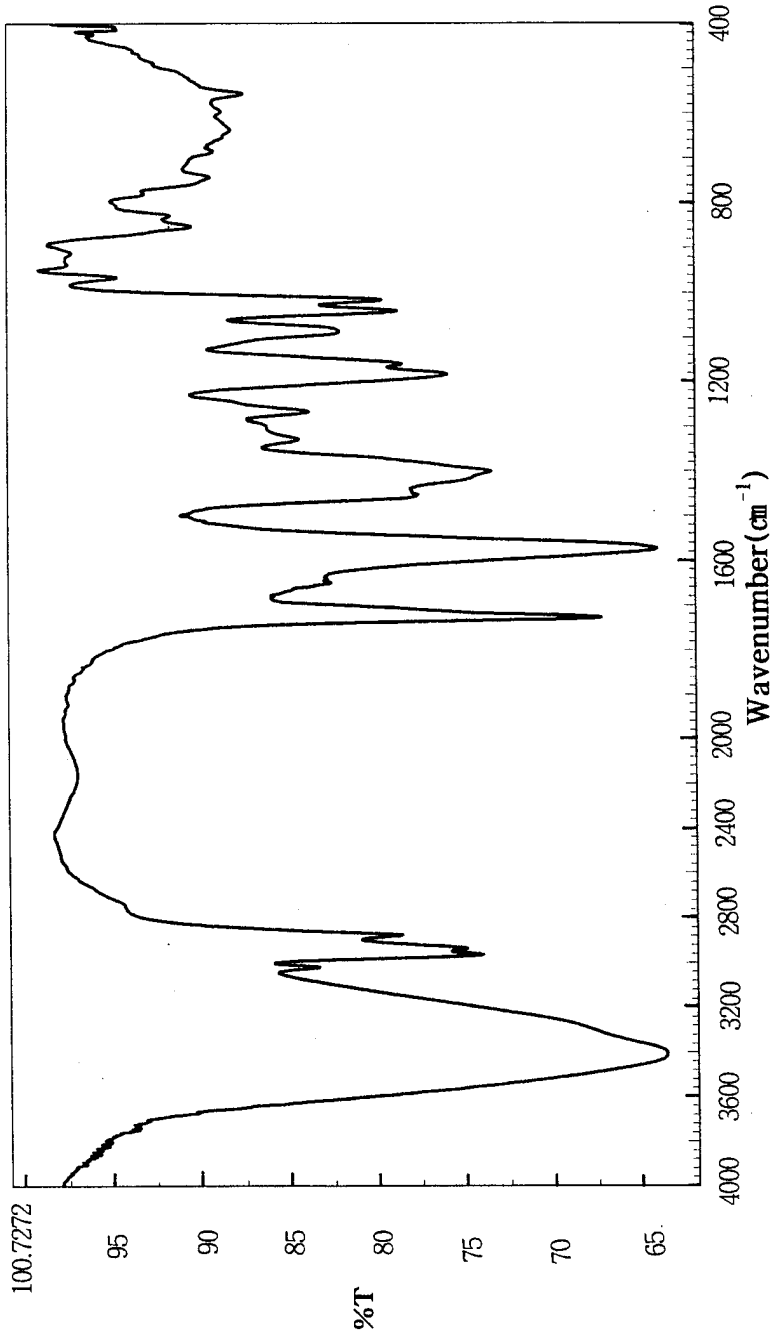


(II-b)

3. The method for producing pravastatin sodium according to claim 2, by using a seed culture medium(I) containing ML-236B lower than 0.05%(w/v).

4. The method for producing pravastatin sodium according to claim 2, by using a production medium(II) containing 0.1~5.0%(w/v) ML-236B.

FIG. 1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95
90
85
80
75
70
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4000
3600
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2800
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2000
1600
1200
800
400
Wavenumber(cm^{-1})

FIG. 2

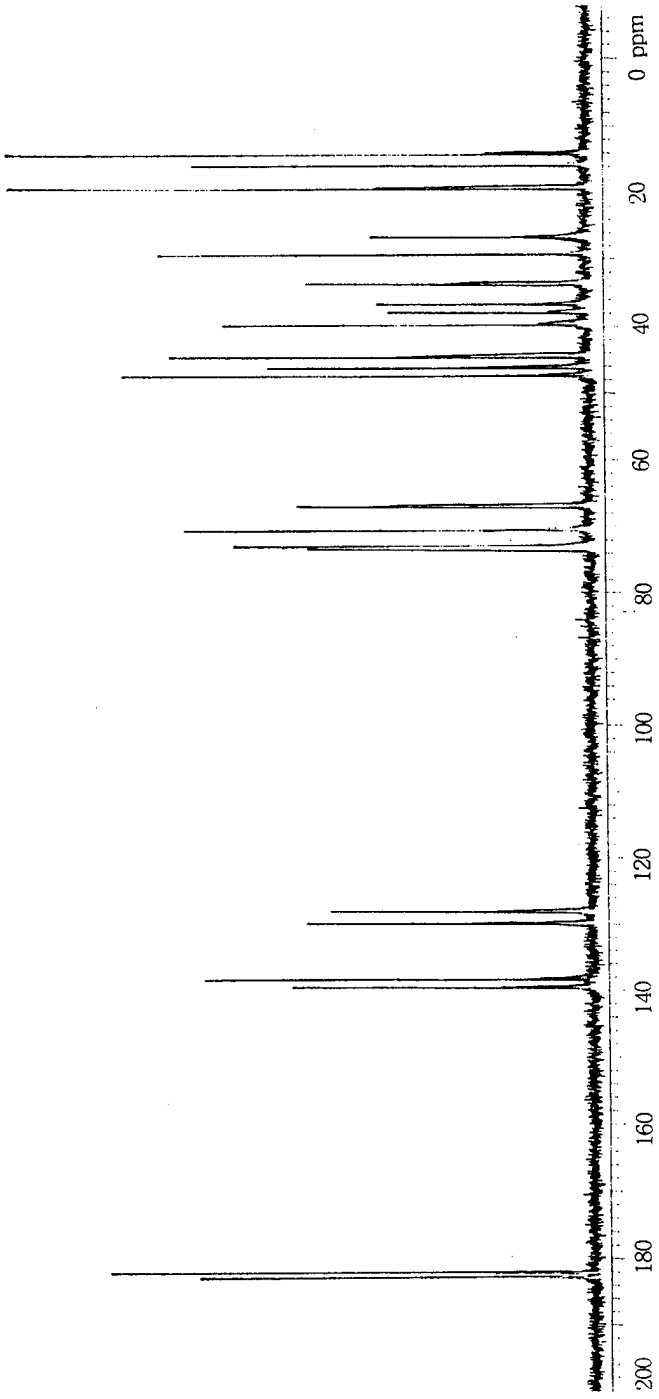
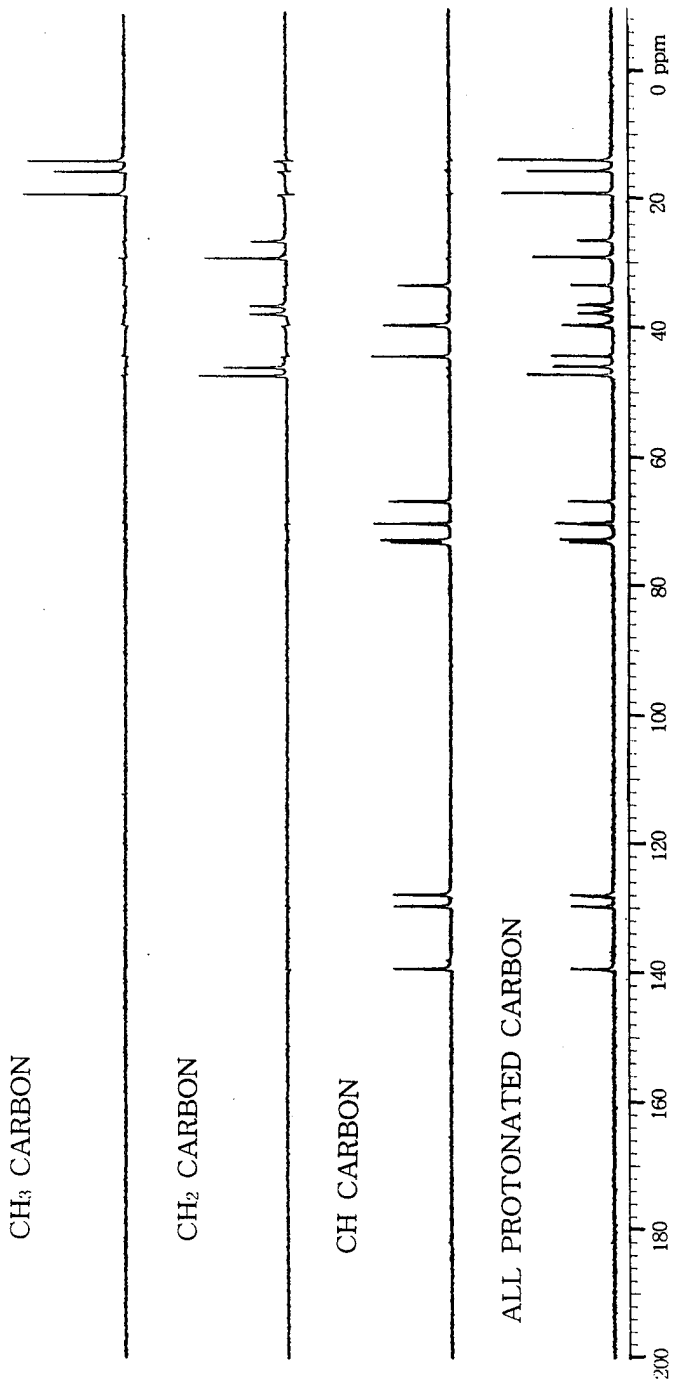


FIG. 3


CH_3 CARBON
CH_2 CARBON
CH CARBON
ALL PROTONATED CARBON
200
180
160
140
120
100
80
60
40
20
0 ppm

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Lee, Joo-Kyung
#277-58, Seongsu-dong 2Ga, Seongdong-ku, Seoul, 133-121,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: *Streptomyces exfoliatus* YJ-118	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0318BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **February 26 1997.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures** Address: KCTC, KRIBB #52, Oun-dong, Yusóng-ku, Taejón 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Kyung Sook Bae, Curator Date: **February 7 1997**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00130

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 1/20; C 12 P 17/06 // (C 12 N 1/20; C 12 R 1:465)

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)

IPC[6]: C 12 N 1/20; C 12 P 17/06

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)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 332 574 A (SUGAWARA et al.) 26 July 1994 (26.07.94), abstract.	1
A	EP 0 582 976 A2 (BOEHRINGER MANNHEIM GMBH) 16 February 1994 (16.02.94), claims 1-3,5,6.	1
A	DE 31 22 499 A1 (SANKYO CO., LTD.) 24 December 1981 (24.12.81), abstract.	2
A	US 5 153 124 A (FURUYA et al.) 06 October 1992 (06.10.92), claims.	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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 December 1997 (05.12.97)	05 January 1998 (05.01.98)
Name and mailing address of the ISA/ AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 97/00130

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