United States Patent [19]

Terahara et al.

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[45] **Date of Patent: * Aug. 27, 1985**

[54] **PROCESS FOR PREPARING 3-HYDROXY-ML-236B DERIVATIVES KNOWN AS M-4 AND M-4'**

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[*] Notice: The portion of the term of this patent subsequent to Oct. 18, 2000 has been disclaimed.

[21] Appl. No.: **442,840**

[22] Filed: **Nov. 18, 1982**

[30] **Foreign Application Priority Data**

Nov. 20, 1981 [JP] Japan 56-186641

[51] **Int. Cl.**[3] **C12P 7/42;** C12P 7/62; C12N 1/20

[52] **U.S. Cl.** **435/146;** 435/135; 435/253; 435/872

[58] **Field of Search** 435/135, 136, 146, 253

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,281,330	10/1966	Fonken et al.	435/136
3,392,171	7/1968	Fonken et al.	435/121
4,410,629	10/1983	Terahara et al.	435/135

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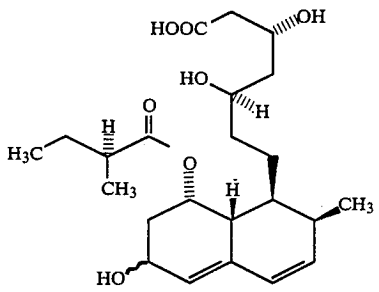
Attorney, Agent, or Firm—Frishauf, Holtz, Goodman & Woodward

[57] **ABSTRACT**

Compounds of formula (I):



(I)

(wherein ~~OH represents ◄OH or ||||OH), that is to say M-4 carboxylic acid and M-4' carboxylic acid, as well as pharmaceutically acceptable salts and esters thereof and the corresponding ring-closed lactones may be prepared by contacting an ML-236B compound with a microorganism of the genus Nocardia or a cell-free, enzyme-containing extract thereof and then, if necessary, subjecting the resulting product to one or more of the following reactions: hydrolysis, salification, esterification and lactonisation. The resulting M-4 and M-4' derivatives have the ability to inhibit the biosynthesis of cholesterol and are therefore of value in the therapy and/or prophylaxis of hyperlipaemia and arteriosclerosis.

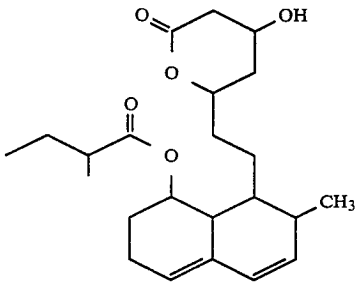
22 Claims, No Drawings

PROCESS FOR PREPARING 3-HYDROXY-ML-236B DERIVATIVES KNOWN AS M-4 AND M-4'

BACKGROUND TO THE INVENTION

The present invention relates to a process for preparing certain 3-hydroxy-ML-236B derivatives known as M-4 and M-4', as well as salts and esters of these compounds.

ML-236B, which can exist in the form of an acid (known as "ML-236B carboxylic acid") or a lactone (known as "ML-236B lactone"), is disclosed in United Kingdom Patent Specification No. 1,453,425 and, in its lactone form, has the formula:



Subsequently, United Kingdom Patent Specification No. 1,555,831 disclosed a variety of salts and esters of ML-236B. ML-236B and its salts and esters were found to inhibit the biosynthesis of cholesterol by competing with 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is the rate-determining enzyme for chloesterol biosynthesis; these compounds were thus found to exhibit a very marked ability to reduce serum cholesterol levels.

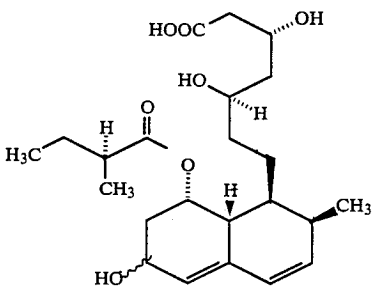
Subsequently, certain 3-hydroxy-ML-236B derivatives were isolated as products of the animal metabolism of ML-236B lactone and similar derivatives were found to be produced by the enzymatic hydroxylation of ML-236B lactone or carboxylic acid or salts or esters thereof, effected by means of various microorganisms of the genera Absidia, Cunninghamella, Syncephalastrum, Streptomyces, Mucor, Rhizopus, Zygorinchus, Circinella, Actinomucor, Gongronella, Phycomyces, Mortierella, Pycnoporus and Rhizoctonia. These processes are disclosed in U.S. Pat. No. 4,346,227, filed 5th June 1981, by A. Terahara and M. Tanaka and the compounds thus produced are described in that patent application as M-4, M-4', IsoM-4 and IsoM-4'. These compounds were found to have an ability to inhibit the biosynthesis of cholesterol which is at least comparable with and, in some instances, substantially exceeds that of ML-236B itself.

ML-236B and its derivatives, including the M-4 and M-4' compounds, are thus of therapeutic value for the treatment of hyperlipaemia and the prophylaxis of arteriosclerosis.

BRIEF SUMMARY OF INVENTION

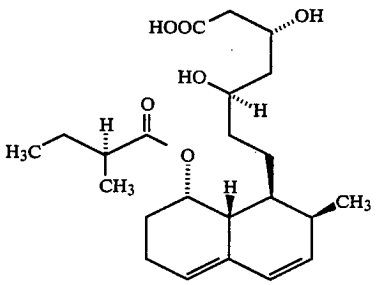
We have now discovered that M-4 and M-4' can also be produced from ML-236B and various derivatives thereof by treatment with a microorganism of the genus Nocardia or a cell-free, enzyme-containing extract thereof. The use of microorganisms of the genus Nocardia has the advantage over the use of those microorganisms disclosed in U.S. Pat. No. 4,346,227 that the Ml-236B compound employed as substrate can be present in the reaction medium to a much higher concentration than when the prior art microorganisms were used. This is most surprising as the ML-236B compounds have been found to possess antifungal and antibiotic properties.

Accordingly, the present invention provides a process for preparing a compound of formula (I):



(I)

(wherein ~~~ OH represents ▬ OH or ····· OH), pharmaceutically acceptable salts and esters thereof and the corresponding ring-closed lactones, which process comprises contacting an ML-236B compound selected from the group consisting of ML-236B carboxylic acid, having the formula (II):



(II)

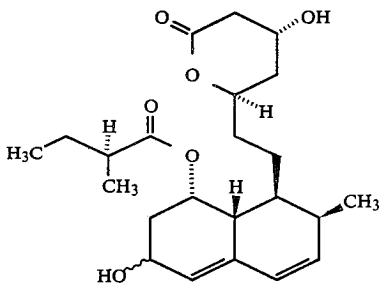
salts and esters thereof and the corresponding ML-236B lactone with a hydroxylation enzyme produced by a microorganism of the genus Nocardia; if necessary, subjecting the resulting product to one or more reactions selected from the group consisting of hydrolysis, salification, esterification and lactonisation; and isolating the product from the reaction mixture.

DETAILED DESCRIPTION OF INVENTION

The compound of formula (I) in which ~~~ OH represents ▬ OH is called M-4 carboxylic acid and the corresponding salts and esters are known as M-4 carboxylates. The compound of formula (I) in which ~~~ OH represents ····· OH is referred to as M-4' carboxylic acid and the corresponding salts and esters are referred to as M-4' carboxylates.

The ring-closed lactones corresponding to the compounds of formula (I) may be represented by the formula (Ia):

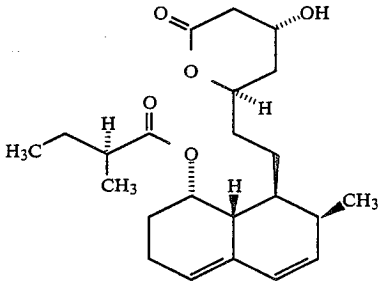
(Ia)



wherein ~~ OH represents ▬ OH or ····· OH and are known as M-4 lactone and M-4' lactone respectively.

The ring-closed lactone corresponding to ML-236B carboxylic acid of formula (II) may be represented by the formula (IIa):

(IIa)



and is known as ML-236B lactone.

Preferred species of the genus Nocardia for use in the process of the present invention are *Nocardia autotrophica, Nocardia asteroides, Nocardia farcinica* and *Norcardia coeliaca,* particularly *Nocardia autotrophica.* Of these species, the following strains are preferred:

Nocardia autotrophica FERM P-6181 (SANK 62781);

Nocardia autotrophica subsp. *canberrica* subsp. nov. FERM P-6182 (SANK 62881);

Nocardia autotrophica subsp. *amethystina* subsp. nov. FERM P-6183 (SANK 62981);

Nocardia autotrophica IFO 12743 (SANK 91279);

Nocardia asteroids IFO 3424 (SANK 62065);

Nocardia farcinica ATCC 3318 (SANK 64265) and

Nocardia coeliaca ATCC 17040 (SANK 63665).

Of the above preferred strains, *Nocardia autotrophica* IFO 12743, *Nocardia asteroides* IFO 3424, *Nocardia farcinica* ATCC 3318 and *Nocardia coeliaca* ATCC 17040 are all known strains which are freely and publicly available from the appropriate culture collection, i.e. the Institute for Fermentation, Osaka, Japan (IFO) or the American Type Culture Collection, U.S.A. (ATCC), under the accession numbers given.

The strains of *Nocardia autotrophica* identified by the accession numbers FERM P-6181, FERM P-6182 and FERM P-6183 are all new strains of the microorganism, newly isolated from soil and deposited on the sixteenth day of October 1981 at the Fermentation Research Institute, Ibaraki-Ken, Japan (FERM).

The morphological and physiological properties of the newly isolated microorganisms were determined using conventional media and the methods described by Shirling and Gottlieb [International Journal of Systematic Bacteriology 16, 313–340 (1966)], together with several supplementary tests. Observations of the culture were made after incubation at 28° C. for 2 weeks. The colour names used were assigned according to the "Guide to Colour Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan). The characteristics of the cultures were compared with those of various known species of actinomycetes described in "The Actinomycetes, Vol. 2" by Waksman, "The ISP Report" by Shirling and Gottlieb, "Bergey's Manual of Determinative Bacteriology", 8th edition and other recent literature concerning the taxonomy of the family Nocardiaceae. The new microorganisms are identified by their FERM accession numbers.

Morphological characteristics

TABLE 1

	FERM P-6181	FERM P-6182	FERM P-6183
Spore chain morphology	RF	RF	RF
Branching	simple	simple	simple
Fragmentation	yes	yes	yes
Surface structure of segmented hyphae (spores)	smooth	smooth	smooth
Other organs	knots, nest-like tangles	knots	none

RF = rectus-flexibilus

Growth on taxonomic media

All of the new strains showed good growth on a variety of media.

Strain FERM P-6181 had white aerial mycelia on a yellowish-grey to pale yellow-orange growth. In certain media a pale yellow-brown soluble pigment was observed, but only to a small extent.

Strain FERM P-6182 had brown-white to pale yellow-orange aerial mycelia on a greyish-yellow-brown growth. No soluble pigment was observed.

Strain FERM P-6183 had a brown-white to pale yellow-orange growth and brown-violet spots were observed as the cultivation proceeded. Brownish-grey aerial mycelia were present on all media, except for the yeast-malt medium.

The culture properties on the 14th day of cultivation at 28° C. in a variety of media are shown in Table 2. The abbreviations used in the Table are as follows:

G=growth;

AM=aerial mycelium;

R=reverse;

SP=soluble pigment.

TABLE 2

Media		FERM P-6181	FERM P-6182	FERM P-6183
Yeast-malt agar (ISP 2)	G	Very good, pale yellow-brown (6-7-9)	Very good, brown (6-4-1)	Very good, brown-white (2-9-8) to greyish-red-brown (4-3-5)
	AM	Abundant, white	Abundant brownish-white (2-9-7)	Trace, white
	R	Dull, yellow-orange (8-8-8)	Brown (4-4-7)	Brown-white (2-9-8) to greyish-red-

TABLE 2-continued

Media		FERM P-6181	FERM P-6182	FERM P-6183
				brown (4-3-5)
	SP	Yellow-brown (8-7-9)	None	None
Oatmeal agar (ISP 3)	G	Good, pale brown (2-8-9)	Very good, pale yellow-orange (2-9-9)	Very good, dark red-brown (4-3-4)
	AM	Fair, white	Abundant, pale yellow-brown (2-9-9)	Fair, pale pink (2-8-4)
	R	Yellowish-brown (4-7-9)	Pale yellow-brown (4-8-9)	Brown-violet (3-3-2)
	SP	Pale yellow-brown (4-8-9)	None	None
Starch/ inorganic salt agar (ISP 4)	G	Good, yellowish-grey (2-9-10)	Poor, yellowish-grey (1-9-10)	Very good, brown-violet (3-3-2)
	AM	Fair, white	Abundant, pale yellow-orange (2-9-9)	Good, bright brown-grey (2-8-2)
	R	Pale yellow (3-9-10)	Pale yellow-orange (2-9-9)	Dark red-brown (4-3-4)
	SP	None	None	None
Glycerin/ asparagine agar (ISP 5)	G	Good, pale brown (2-8-9)	Good, greyish-yellow-brown (4-5-7)	Very good, pale brown (2-9-9) to brown-violet (3-3-2)
	AM	Abundant, white	Abundant, brown-white (1-8-6)	Abundant, white
	R	Pale yellow-brown (6-8-9)	Brown (4-4-6)	Pale yellow-orange (2-9-9) to greyish-red-brown (4-3-6)
	SP	Pale yellow-brown (6-9-11)	None	None
Tyrosine agar (ISP 7)	G	Very good, pale-yellow-orange (3-8-8)	Very good, greyish-yellow-brown (4-5-7)	Good, greyish-brown (4-6-6)
	AM	Abundant, white	Abundant, brown-white (2-9-7)	Trace, white
	R	Yellowish-grey (1-9-10)	Bright brown (6-5-7)	Pale yellow-orange (2-9-9) to brown-violet (3-3-2)
	SP	Pale yellow-brown (6-7-9)	None	None
Sucrose nitrate agar	G	Good, pale yellow-brown (2-9-9)	Poor, pale yellow-brown (2-9-9)	Poor, pale yellow-orange (2-9-9)
	AM	Fair, white	Abundant, brown-white (2-9-7)	Fair, white
	R	Yellowish-grey (1-9-10)	Brown-white (1-9-6)	Pale yellow-orange (2-9-9)
	SP	None	None	None
Glucose/ asparagine agar	G	Very good, pale yellow-orange (2-9-9)	Good, greyish yellow-brown (4-5-7)	Very good, pale yellow-orange (2-9-9) to brown-violet (3-3-2)
	AM	Fair, white	Abundant, bright brown-white (1-7-6)	Fair, white
	R	Pale yellow-brown (4-8-9)	Greyish-red-brown (4-3-6)	Pale yellow-orange (2-9-9) to greyish-red-brown (4-3-6)
	SP	Pale yellow-brown (4-8-9)	None	None
Nutrient agar	G	Good, yellowish-grey (2-9-10)	Very good, pale yellow-brown (6-8-9)	Good, pale yellow-orange (2-9-9)
	AM	Fair, white	Pale yellow-orange (2-9-9)	Trace, white
	R	Yellowish-grey (4-9-10)	Pale yellow-brown (6-8-9)	Pale yellow-orange (2-9-9)
	SP	None	None	None
Water agar	G	Poor, yellowish-grey (1-9-10)	Poor, colourless	Poor, pale yellow-orange (2-9-9)
	AM	Fair, white	Abundant, white	Fair, white

TABLE 2-continued

Media		FERM P-6181	FERM P-6182	FERM P-6183
	R	Yellowish-grey (1-9-10)	Pale yellow-orange (2-9-9)	Pale yellow-orange (2-9-9)
	SP	None	None	None
Potato/carrot extract agar	G	Poor, yellowish-grey (1-9-10)	Poor, colourless	Poor, pale yellow-orange (2-9-9)
	AM	Fair, white	Fair, white	Fair, white
	R	Yellowish-grey (1-9-10)	Pale yellow-orange (2-9-9)	Pale yellow-orange (2-9-9)
	SP	None	None	None

Physiological properties

The physiological properties of the new strains are shown in Table 3. The test for melanoid pigment formation was carried out in three media, as follows:

Medium 1: Tryptone-yeast extract broth (ISP 1);

Medium 2: Peptone-yeast extract-iron agar (ISP 6);

Medium 3: Tyrosine gear (ISP 7).

TABLE 3

	FERM P-6181	FERM P-6182	FERM P-6183
Nitrate reduction	−	−	−
Starch hydrolysis	−	−	−
Urea decomposition	+	−	+
Lysozyme resistance	−	+	−
Melanoid pigment formation			
Medium 1	−	−	−
Medium 2	−	−	−
Medium 3	−	−	−
Acid production from			
arabinose	+	−	+
xylose	+	−	+
raffinose	+	−	NG

− = negative;
+ = positive;
NG = no growth.

Utilisation of carbohydrates

The utilisation of carbohydrates by the new strains is shown in Table 4. The medium used was Pridham-Gottlieb agar (ISP 9) and determination was made after cultivation at 28° C. for 14 days.

TABLE 4

	FERM P-6181	FERM P-6182	FERM P-6183
D-Glucose	+	+	+
D-Arabinose	+	−	+
D-Xylose	+	−	+
D-Fructose	+	+	+
L-Rhamnose	+	−	±
Inositol	+	+	+
Sucrose	+	−	−
Raffinose	+	−	−
D-Mannitol	+	+	+
Control	−	−	−

+ = utilised;
± = slightly utilised;
− = not utilised.

Cell wall analysis

Paper chromatographic analyses were performed on acid hydrolyzates of each of the three new strains, following the method of B. Becker et al. [Applied Microbiology, 13, 236 (1965)] and that of M. P. Lechevalier et al. ["The Actinomycetales" by H. Prauser, 311 (1970)]. Meso-2,6-diaminopimelic acid was found in the cell walls and arabinose and galactose were found as saccharide components of the whole cell, thus confirming that each of the strains had cellular components of the type IV-A.

The results of these taxonomic studies demonstrate that all strains belong to the genus Nocardia. Of the known species of Nocardia, the characteristics of the new strains are most closely related to those of *Nocardia autotrophica* [International Journal of Systematic Bacteriology, 30, 337 (1980)[, except only for the differences shown in Table 5. In the Table, symbols and abbreviations are as given in the corresponding Tables 1–4.

TABLE 5

TEST		FERM P-6181	FERM P-6182	FERM P-6183	*Nocardia autotropnica*
Growth colours	AM	white	white to pale yellow-orange	white to brownish-grey	white to pale yellow
	G	yellowish-grey to pale yellow-orange	greyish-yellow-brown	brown-violet	pale yellow to yellowish-grey
Decomposition of urea		+	−	+	+
Resistance to lysozyme		−	+	−	−
Acid production from:					
arabinose		+	−	+	+
xylose		+	−	+	+
raffinose		+	−	NG	−
Utilization of:					
arabinose		+	−	+	+
xylose		+	−	+	+
rhamnose		+	−	±	+

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