VOL. 57 NO. 4, APR. 2004 THE JOURNAL OF ANTIBIOTICS pp. 264 — 270

FR901512, a Novel HMG-CoA Reductase Inhibitor Produced by an Agonomycetous Fungus No. 14919

I. Taxonomy of the Producing Organism, Fermentation, Isolation and Physico-chemical Properties

Hidetaka Hatori[a,*], Bunji Sato[a], Ikuko Sato[a], Toshihiro Shibata[b], Yasuhisa Tsurumi[a], Kazutoshi Sakamoto[a], Shigehiro, Takase[a], Hirotsugu Ueda[a], Motohiro Hino[a] and Takashi Fujii[a]

[a] Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan
[b] Research Planning, Research Division, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa, Osaka, Japan

(Received for publication December 24, 2003)

Novel compounds FR901512 and FR901516 were isolated from the fermentation broth of agonomycete strain No. 14919. FR901512 and FR901516 possess unique tetralin ring in their structure. These compounds were potent inhibitors of the cholesterol synthesis in human hepatoma cell line Hep G2. FR901512 shows strong 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitory activity with an IC_{50} value of 0.95 nM.

High levels of blood cholesterol and blood lipids are conditions involved in the onset of arteriosclerosis. It is well known that inhibitors of cholesterol biosynthesis such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors are effective in lowering the level of blood plasma cholesterol, especially low density lipoprotein cholesterol (LDL-C). It has been established that lowering LDL-C levels affords protection from arteriosclerosis and coronary artery disease[1~6].

While screening microbial fermentation products for an inhibitor of sterol synthesis, we identified FR901512 (**1**) and FR901516 (**2**) (Fig. 1.) from culture broth of an agonomycetous fungus No. 14919. In this paper, we describe the cultural characteristics of the producing organism, production, purification, physico-chemical properties and *in vitro* biological activities of compounds **1** and **2**.

Materials and Methods

Cultural Characterizations

The fungus strain No. 14949 was originally isolated from a soil sample, collected at Mt. Kiyosumi, Chiba Prefecture, Japan. Cultural characteristics of strain No. 14919 were determined using various agar media as follows; malt extract agar, potato dextrose agar (Difco 0013), Czapek's solution agar, Sabouraud dextrose agar (Difco 0190), Emerson Yp Ss agar (Difco 0739), corn meal agar (Difco 0386), oatmeal agar (Difco 0552) and MY20 agar. The composition of malt extract agar, Czapek's solution agar and MY20 agar are based on JCM Catalogue of Strains[7].

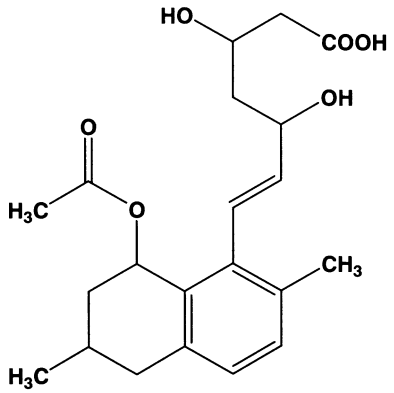
The morphological characteristics were determined based on cultures grown on Miura's LCA agar[8]. These characteristics were observed after 14 days of incubation at 25°C. The color description was based on the Methuen Handbook of Color[9]. The temperature range of growth was determined on potato dextrose agar.

Fermentation

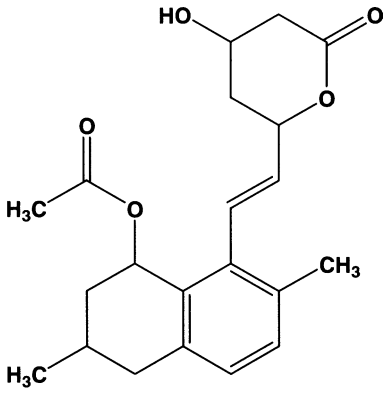
An aqueous seed medium (60 ml) containing sucrose 4%, cotton seed flour 2%, molatein 1%, peptone 1%, KH_2PO_4 1%, Tween 80 0.1% and $CaCO_3$ 0.2% was added to a 250-ml Erlenmeyer flask and sterilized at 120°C for 30 minutes. A loopful of a strain No. 14919 from a slant culture was

* Corresponding author: hidetaka_hatori@po.fujisawa.co.jp

Fig. 1. Structure of FR901512 (**1**) and FR901516 (**2**).



FR901512 (**1**)



FR901516 (**2**)

inoculated into the flask. The flask was shaken on a rotary shaker (220 rpm, 5.1 cm-throw) at 25°C for 4 days. The seed culture was transferred at the rate of 2% to 160 ml of the same sterile seed medium in each of twenty 500-ml Erlenmeyer flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) at 25°C for 3 days. The resultant seed culture was inoculated to 150 liters of production medium (pH 3.8) consisting of glucose 0.5%, glycerin 3.5%, soluble starch 6%, corn steep liquor 1.5%, potato protein 1.5%, β-cycrodextrin 1%, Adekanol LG-109 (defoaming agent, Asahi Denka Co., Ltd.) 0.05% and Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% in a 200-liter jar fermenter. The fermentation was carried out at 25°C for 4 days under aeration of 150 liters/minute and agitation of 200 rpm.

Isolation

The cultured broth (230 liters) was extracted with equal volume of acetone by intermittent mixing. The acetone extract was filtered with an aid of diatomaceous earth and water was added to the filtrate to yield 25% acetone solution. The mixture was passed through a column (25 liters) of Diaion HP-20 (Mitsubishi Chemical Ind. Co., Ltd.). The column was washed with 30% and 40% aqueous acetone, and then eluted with 60% aqueous acetone. The eluate (43 liters) was combined with 43 liters of water and loaded onto a column (30 liters) of Diaion SP-207 (Mitsubishi Chemical Ind. Co., Ltd.). After washing with 30% aqueous acetonitrile containing 0.1% of phosphoric acid and 35% aqueous acetonitrile containing 0.1% of phosphoric acid, the active fractions were eluted with 40% aqueous acetonitrile containing 0.1% of phosphoric acid and 45% aqueous acetonitrile containing 0.1% of phosphoric acid. The eluate (22.5 liters) was concentrated *in vacuo* to an aqueous solution, adjusted to pH 4.0 with 1 N NaOH and extracted with an equal volume of ethyl acetate. The extract was dehydrated with Na_2SO_4 and concentrated to dryness under reduced pressure. The resultant oily substance was dissolved small volume of dichloromethane : methanol (25 : 1) solution and applied to a 4-liter silica gel column for chromatography. The column was developed with dichloromethane : methanol (25 : 1) and (10 : 1). Fractions containing compound **1** were collected and concentrated *in vacuo* to dryness and dissolved in ethyl acetate. The solution of compound **1** was washed twice with acidic water (pH 4.0), dehydrated with Na_2SO_4 and concentrated *in vacuo* to give compound **1** in the form of a colorless crystal (27.8 g).

Compound **2** (5.5 mg) was originally isolated from 70 liters of cultured broth using essentially similar methods. After the structure elucidation, compound **2** was prepared from compound **1** using a conventional lactonization method.

Measurement of Cholesterol Synthesis in Hep G2 Cells

Culture of Human hepatoma cell line Hep G2 was described previously[10]. Cholesterol synthesis in Hep G2 cells was determined according to the method of BROWN *et al.*[11] with some modifications. In brief, Hep G2 cells were cultured 35 mm dishes with 1 ml of fresh medium

containing 10% human lipoprotein-deficient serum. The cells were preincubated for 2 hours at 37°C with various concentrations of compounds **1** or **2**. Then, 1 mM [1-^{14}C]acetic acid, sodium salt (37 MBq/mmol, DuPont/NEN Research Products) was added and the culture was incubated at 37°C for 2 hours. After incubation, the cells were washed with phosphate-buffered saline (PBS), pH 7.4 and then dissolved in 1 ml of aqueous 15% KOH. 1 ml of 15% KOH in 95% ethanol was added to each dissolved cell lysate and then samples were saponified for 1 hour at 75°C. The non-saponifiable lipids were extracted twice with 2 ml of petroleum ether, evaporated to dryness, resuspended in 100 μl of diethyl ether, and subjected to thin layer chromatography on a silica gel plate with a solvent system consisting of benzene/ethyl acetate (9 : 1, v/v). The spot corresponding to authentic cholesterol was scraped into a vial and the radioactivity was counted with a liquid scintillation counter (TRI-CARB 1500, Packard Instrument Co., Inc.).

Assay of HMG-CoA Reductase Activity in Solubilized Hep G2 Cell Extracts

HMG-CoA reductase activity was determined as described according to the method of BROWN *et al.*[12] with some modifications. Hep G2 cells in 75-cm^2 flasks were washed three times with cold phosphate buffered saline and scraped with a policeman. After centrifugation (500×*g*, 5 minutes at 4°C), the resultant cell pellet was frozen and kept at −80°C until use. Cell extracts were prepared by suspending the pellet in 400 μl of buffer containing 0.1 M potassium phosphate, pH 7.4, 5 mM EDTA, 0.2 M KCl and 0.25% Emulgen 409P (Kao Chemicals Co., Ltd.). After incubation for 10 minutes at 37°C, the suspension was centrifuged at 12,000×*g* for 15 minutes at 4°C. Aliquots of the supernatant (25 μg protein) were incubated at 37°C for 2 hours in a final volume of 50 μl containing 0.1 M potassium phosphate, pH 7.4, 20 mM glucose 6-phosphate, 2.5 mM NADP, 4 mM dithiothreitol, 0.5 unit of glucose 6-phosphate dehydrogenase and 40 μM DL-3-hydroxy-3-methyl [3-^{14}C]glutaryl coenzyme A (1040 MBq/mmol, DuPont/NEN Research Products). Compounds **1** and **2** were dissolved in 1 μl of DMSO and added to the assay mixture. The reaction mixture containing DMSO (1 μl) was served as a control. The reaction was terminated by adding 10 μl of 2 N HCl to the assay mixture. The mixture was further incubated for 15 minutes at 37°C for lactonization of mevalonic acid. The [^{14}C]-mevalonolactone formed was isolated by thin layer chromatography on a silica gel plate with a solvent system consisting of toluene/ethanol (1 : 1, v/v). The spot corresponding to authentic mevalonolactone was scraped into a vial and counted with a liquid scintillation counter.

Results

Characteristics of Producing Strain No. 14919

This organism grew very rapidly on various culture media, and formed white-edged and dark gray colonies. After one month of incubation, the strain formed neither teleomorph nor anamorph. The differentiated hyphal structures (*e.g.* scleotia, bulbilis, chlamydospores and clamp connections) were also not observed. These characteristics showed that strain No. 14919 was classified in the Mycelia Sterilia[13].

Cultural characteristics on various agar media are summarized in Table 1. Culture growth on malt extract agar spread broadly, attaining more than 7.5 cm in diameter after two weeks at 25°C. The colony surface was plane, cottony, dark gray and yellowish white at the edge. Reverse of the colonies was greenish gray and yellowish white at the edge. Conidial structures were not observed. Colonies grown under the same conditions on potato dextrose agar spread broadly, attaining 6.5～7.0 cm in diameter with plain, felty, pale gray to black surface and white at the edge. Reverse of the colonies was black and yellowish white at the center. Conidial structure was not produced.

Vegetative hyphae were smooth, septate, hyaline and branched. The hyphal cells were cyrindical and 1.5～5.0 μm in diameter.

For inducing the strain to sporulate, we attempted two tests: (1) inoculating to a steam sterilized flat piece of leaf affixed to a corn meal agar plate (Matsushima's method) and (2) throwing a piece of cultures into a sterile water; *etc.* But we were not able to observe the characteristic morphogenesis of strain No. 14919, after all. Strain No. 14919 was able to grow at the temperature range from 9 to 33°C with the growth optimum at 24 to 27°C.

From above mentioned characteristics, the producing strain is an agonomycetous fungus strain No. 14919. It was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, as FERM BP-3752.

Fermentation

Figure 2 shows the time course of compound **1** production by strain No. 14919 in a 200-liter jar fermenter, along with the pH and the packed cell volume. The maximal yield of compound **1** was observed after 96 hours of cultivation at a concentration of 260 μg/ml.

Table 1. Cultural characteristics of strain No. 14919.

Medium	Cultural characteristics
Malt extract agar	G: Spreading broadly, >7.5cm
	S: Circular, plane, cottony, formed no conidial structures, dark gray (1F1), yellowish white (3A2) at the edge
	R: Greenish gray (30F2), yellowish white (3A2) at the edge
Potato dextrose agar (Difco 0013)	G: Spreading broadly, 6.5-7.5cm
	S: Circular,plane, felty, formed no conidial structures, yellowish gray (4B2), white (1A1) at the edge
	R: Black, yellowish white (4A2), at the center
Czapeck's solution agar	G: Rapidly, 4.0-4.5cm
	S: Circular to irregular, plane, raised, felty, formed no conidial structures, yellowish gray (4B2), white (1A1) at the edge
	R: Dark brown (6F4), brownish oranga (6C5) at the edge
Saburaud dextrose agar (Difco 0190)	G: Spreading broadly, >7.5cm
	S: Raised, felty, formed no conidial structures, brownish gray (6C2), yellowish white (4A2) at the center, produced brown exudations and brown soluble pigment
	R: Dark brown (6F4)
Oatmeal agar (Difco 0552)	G: Spreading broadly, >7.5 cm
	S: Felty to cottony, formed no conidial structures, dark gray (1F1) to black
	R: Brownish gray (5E2), orange gray (5B4) at the center
Emerson Yp Ss Agar (Difco 0739)	G: Spreading broadly, 6.0-6.5 cm
	S: Circular, plane, felty, formed no conidial structures, black, white (1A1) at the edge
	R: Yellowish white (3A2)
Corn meal agar (Difco 0386)	G: Spreading broadly, 5.5-6.0 cm
	S: Circular, raised, cottony, formed no conidial structures, yellowish white (4A2), partly dark gray (1F1)
	R: Yellowish white (4A2), partly dark gray (1F1)
YM 20 agar	G: Spreading broadly, >7.5 cm
	S: Circular, raised, floccose, formed no conidial structures, yellowish white (4A2), partly dark gray (1F1)
	R: Light yellow (4A5)
Abbreviation	G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

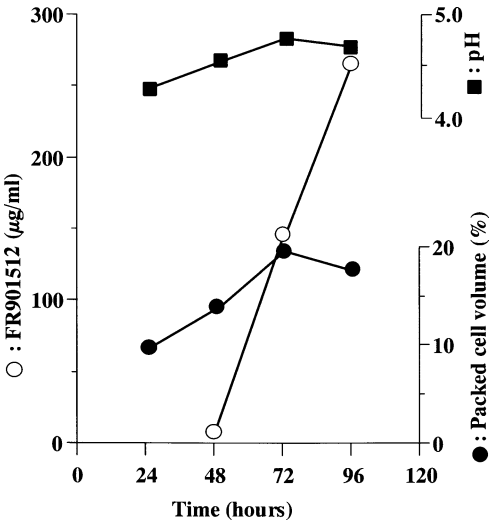
Isolation

The procedure used to isolation of **1** is summarized in Figure 3. During the isolation procedure, the pH of sample was controlled to slightly acidic condition to maintain the free acid from of compound **1**. The overall yield of purification was 47%.

Physico-chemical Properties

The physico-chemical properties of compound **1** and **2** are summarized in Table 2. The Rf value of compound **1** and **2** on silica gel TLC (silica gel 60 F254, E. Merck) developed with chloroform : methanol : acetic acid (100 : 10 : 1, v/v) were 0.30 and 0.57, respectively. By a

Fig. 2. Time course of FR901512 (**1**) production in a 200-liter jar fermentor.



combination of physico-chemical and spectral evidence, the planer structure of compound **1** and **2** were elucidated (Fig 1). Full details of the structure elucidation of these compounds will be published elsewhere.

Inhibition of Sterol Synthesis in Hep G2 Cells

Compounds **1** and **2** inhibited the incorporation of [^{14}C]-acetic acid into cholesterol in Hep G2 cells with IC_{50} values of 1.0 nM and 0.59 nM, respectively (Table 3). These compounds did not inhibit cholesterol synthesis from mevalonic acid, which is product of HMG-CoA reductase (data not shown). Therefore the inhibition point of these compounds in cholesterol synthesis is at an earlier step than mevalonic acid formation.

Fig. 3. Purification procedure of compound **1**.

Culture broth (230L, 59.3g)
- extracted with acetone (230L)

Acetone extract
- diluted with water, adjusted to pH4.0

HP-20 column chromatography (25L)
- washed with 30% and 40% acetone
- eluted with 60% acetone
- diluted with water, adjusted to pH4.0

SP-207 column chromatography (30L)
- washed with 30% acetonitrile-0.1% H_3PO_4 and 35% acetonitrile-0.1% H_3PO_4
- eluted with 40% acetonitrile-0.1% H_3PO_4, 45% acetonitrile-0.1% H_3PO_4

Active fractions
- concentrated *in vacuo* to an aqueous residue
- adjusted to pH4.0
- extracted with EtOAc, dehydrated with Na_2SO_4
- concentrated *in vacuo* to an oily residue
- dissolve in dichloromethane : methanol (25:1)

Silica gel 60 column chromatography (4L)
- eluted with dichloromethane : methanol (25:1) and (10:1)
- concentrated to dryness, dissolve in EtOAc
- wash with acidic water (pH 4.0)
- dehydrated with Na_2SO_4
- concentration, crystallization from EtOAc

Compound **1** crystal (27.8g)

Table 2. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Colorless needles	Colorless powder
Molecular formula	$C_{21}H_{28}O_6$	$C_{21}H_{26}O_5$
Elementary analysis		
Calcd for $C_{21}H_{28}O_6$:	C 67.00, H 7.50	
Found:	C 66.80, H 7.63	
Molecular weight	376.46	358.44
FAB-MS (*m/z*)	399 $(M+Na)^+$	381 $(M+Na)^+$
MP	136-137℃	167-168℃
$[\alpha]_D^{23}$	+20 (*c* 0.68, MeOH)	-16 (*c* 0.4, MeOH)
UV λ_{max} nm (MeOH)	210, 240, 280	210, 240, 280
IR ν_{max} (KBr) cm^{-1}	3300, 2950, 1710, 1670, 1440, 1380, 1270, 1250, 1090, 980	3300, 2960, 1730, 1720, 1370, 1250, 1240, 1090, 1050, 960

Table 3. Inhibitory effect of **1** and **2** on cholesterol synthesis in Hep G2 cells.

Compound	Inhibiton of cholesterol synthesis (IC_{50}, nM)
1	1.0
2	0.59

Table 4. Inhibitory effect of **1** and **2** on HMG-CoA reductase activity in Hep G2 cell lysate.

Compound	Inhibiton of HMG-CoA reductase (IC_{50}, nM)
1	0.95
2	23.0

Inhibition of HMG-CoA Reductase Activity

Compounds **1** and **2** inhibits HMG-CoA reductase activity with IC_{50} value of 0.95 nM and 14.0 nM, respectively (Table 4). These compounds are potent and strong inhibitors of HMG-CoA reductase.

Discussion

In this paper we have presented novel and potent HMG CoA reductase inhibitors, FR901512 (**1**) and FR901516 (**2**), isolated from the fermentation broth of fungal strain No. 14919. This strain did not sporulate at tested culture conditions. The morphological characteristics showed that strain No. 14919 was classified as a mycelia sterilia[13].

From the mycological characteristics, we called the producing strain an agonomycetous fungus strain No. 14919.

Compounds **1** and **2** were identified during a screening program for cholesterol synthesis inhibitor targeted the early stage of cholesterol synthesis. These compounds have potent HMG-CoA reductase inhibitory activity (Table 4). Based on physico-chemical data and 2D-NMR spectra, these compounds have novel structure (Fig. 1). Compounds **1** and **2** were structurally unique compared to previously reported naturally occurring HMG-CoA reductase inhibitors such as mevastatin or lovastatin[6]. Compounds **1** and **2** have unique tetralin nucleus instead of hexahydronaphthalene ring in mevastatin and lovastatin. And the moiety derived from 3,5-dihydroxy-heptanoic acid in the mevastatin and lovastatin was replaced by 3,5-dihydroxy-heptaenoic acid in compounds **1** and **2**.

As noted and shown in Table 4, compound **1** inhibited HMG-CoA reductase activity with an IC_{50} value of 0.95 nM. While inhibitory activity for HMG-CoA reductase of **2** is less potent than **1**, compound **2** was not completely hydrolyzed to acid form under the assay conditions. Compounds **1** and **2** potently inhibited cholesterol synthesis in Hep G2 cells from ^{14}C-acetic acid with an IC_{50} value of 1.0 and 0.59 nM, respectively (Table 3). But compounds **1** and **2** did not inhibit the cholesterol synthesis from ^{14}C-mevalonate in Hep G2 cells (data not shown).

These data suggests that compounds **1** or **2** inhibits HMG-CoA reductase actinity in Hep G2 cells, and would possess lipid-lowering effects in animal model or in human. We describe in our adjoining paper the *in vivo* hypolipidemic effects of compound **1**[14].

References

1) SUPERKO, H. R. & R. M. KRAUSSE: Coronary artery disease regression. Convincing evidence for the benefit of aggressive lipoprotein management. Circulation 90: 1056～1069, 1994
2) LEVINE, G. N.; J. F. KEANEY Jr. & J. A. VITA: Cholesterol reduction in cardiovascular disease? Clinical benefits and possible mechanisms. N. Engl. J. Med. 332: 512～520, 1995
3) DOWNS, J. R.; M. CLEARFIELD, S. WEIS, E. WHTNEY, D. R. SHAPIRO, P. A. BEERE, M. S. A. LANGENDORFER, E. A. STEIN, W. KRUIER & A. M. GOTTO Jr.: Primary prevention of acute coronary events with lovastation in men and women with average cholesterol levels. JAMA 279: 1615～1622, 1998
4) JUNGCICKEL, P. W.: Cholesterol-lowering therapy: is there really a controversy: Ann. Pharmacother. 30: 539～542, 1996
5) SHEPHERD, J.; S. M. COBBE, I. FORD, C. G. ISLES, A. R. LORIMER, P. W. MCFARLANE, H. MCKILLOPJ & C. J. PACKARD: Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N. Engl. J. Med. 333: 1301～1307, 1995
6) ENDO, A.: The discovery and development of HMG-CoA reductase inhibitors. J. Lipid Res. 33: 1569～1582, 1992
7) NAKASE, T.: JCM catalogue of Strains, 6th edition., Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, (RIKEN), Toppan, 1995
8) MIURA, K. & M. KUDO: An agar-medium for aquatic Hyphomycetes. Trans. Mycol. Soc. Japan 11: 116～118, 1970
9) KORNERUP, A. & J. H. WANSCHER: Methuen Handbook of Color, 3rd ed., pp. 525, Methuen, London, 1978
10) HATORI, H.; T. SHIBATA, Y. TSURUMI, T. NAKANISHI, M. KATSUOKA, Y. OHTSU, K. SAKAMOTO, S. TAKASE, H. UEDA, M. HINO & T. FUJII: FR171456, a novel cholesterol synthesis inhibitor produced by *Sporormiella minima* No.15604. I. Taxonomy, fermentation, isolation, physico-chemical properties. J. Antibiotics 57: 253～259, 2004
11) BROWN, M. S.; J. R. FAUST & J. L. GOLDSTEIN: Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. J. Biol. Chem. 253: 1121～1128, 1978
12) BROWN, M. S.; S. E. DANA & J. L. GOLDSTEIN Jr.: Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblast. J. Biol. Chem. 249: 789～796, 1974
13) BARRON, G. L.: The Genera of Hyphomycetes from Soil. Williams & Wilkins Co., Baltikore, 1968
14) HATORI, H.; B. SATO, I. SATO, T. SHIBATA, H. UEDA, M. HINO & T. FIJII: FR901512, a novel HMG-CoA reductase inhibitor produced by agonomycetous fungus No. 14919. II. Biological profiles. Manuscript in preparation.