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FR171456, a Novel Cholesterol Synthesis Inhibitor Produced by *Sporormiella minima* No. 15604

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties

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FR171456 and FR173945, novel and potent cholesterol synthesis inhibitors, have been isolated from the fermentation broth of a fungal strain No. 15604. This strain was identified *Sporormiella minima* from its mycological characteristics. FR171456 and FR173945 strongly inhibited cholesterol synthesis in human hepatoma cell line Hep G2. These compounds also have *in vitro* antifungal activity against *Candida albicans* and *Aspergillus fumigatus*.

An elevated level of blood low density lipoprotein-cholesterol (LDL-C) has been established as the most important risk factor for atherosclerosis and coronary artery disease[1~5]. It is well known that inhibitors of cholesterol biosynthesis such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) inhibitors are effective in lowering the level of blood plasma cholesterol, especially LDL-C[6]. HMG-CoA reductase is one of the early stage enzymes in the cholesterol biosynthetic pathway and catalyses the conversion of HMG-CoA to mevalonic acid. Mevalonic acid is a precursor for biologically important non-steroidal isoprenoids *e.g.* dolichol, ubiquinone (coenzyme Q10) isopentenyl tRNA and prenylated proteins[12], which play an important role in the regulation of cellular processes. So other enzymes involved in steps in the sterol biosynthetic pathway later than HMG-CoA reductase, could also be targeted by lipid lowering agents, hence avoiding cellular depletion of biologically important nonsteroidal isoprenoids. In the process of screening for microbial fermentation products as inhibitors of cholesterol biosynthesis post mevalonic acid (which is the product of HMG-CoA reductase) we identified FR171456 (**1**) and FR173945 (**2**) (Fig. 1) substances from the culture broth of *Sporormiella minima* No. 15604. In this paper, we describe the taxonomy of the producing organism, production, purification, Physico-chemical properties and *in vitro* biological activities of compounds **1** and **2**.

Materials and Methods

Taxonomic Studies

The fungus, strain No. 15604, was originally isolated from a soil sample collected at Mt. Kiyosumi, Chiba Prefecture, Japan. Cultural characteristics of strain No. 15604 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

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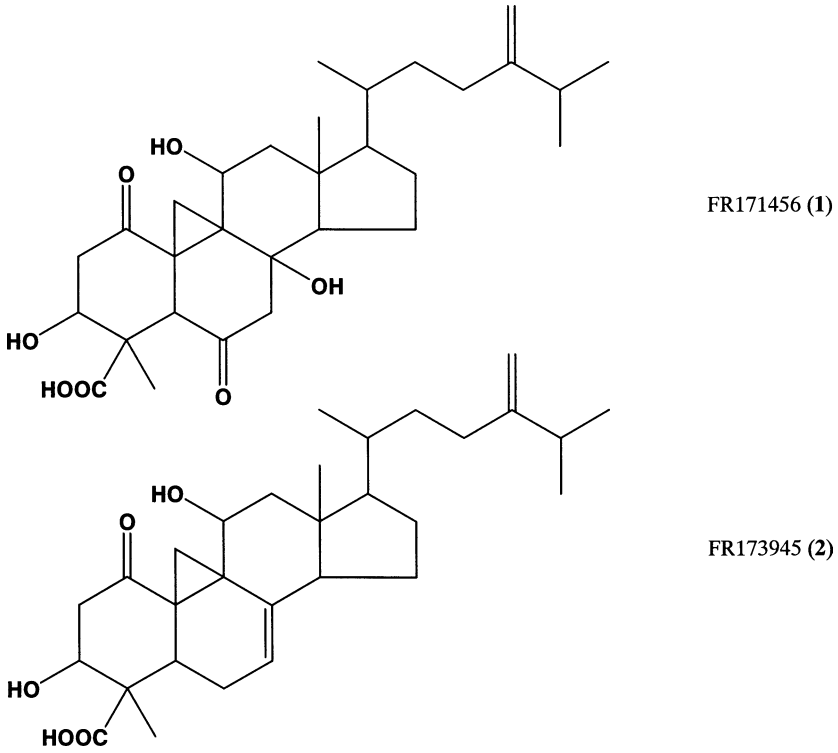
Fig. 1. Structures of FR171456 (**1**) and FR173945 (**2**).



solution agar and MY20 agar are based on the JCM Catalogue of Strains[7].

The morphological characteristics were determined from the cultures on a 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 (160 ml) containing sucrose 4%, cotton seed flour 2%, dried yeast 1%, peptone 1%, KH_2PO_4 0.2%, $CaCO_3$ 0.2% and Tween80 0.1% was poured into a 500 ml Erlenmeyer flask and sterilized at 120°C for 30 minutes. The flask was inoculated with a loopful of *Sporormiella minima* No. 15604 from a slant culture. The flask was shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 4 days. The resultant seed culture was used to inoculate 20 liters of sterile production medium consisting of starch acid hydrolysates 6%, cotton seed flour 1%, peanut powder 1%, dried yeast 2%, $Na_2HPO_4 \cdot 12H_2O$ 0.5%, Adecanol LG-109 (defoaming agent, Asahi Denka Co. Ltd.) 0.05% and Silicone KM-70 (defoaming agent, Shin-Eteu Chemical Co. Ltd.) 0.05% in a 30-liter jar fermenter. Fermentation was carried out at 25°C for 6 days under aeration of 20 liters per minutes and agitation of 250 rpm.

Cell Cultures

Hep G2 cells (HB 8065, a human hepatoma cell line) were obtained from the American Type Culture Collection. Hep G2 cells were grown in 75-cm^2 flasks containing Eagle's modified minimum essential medium with non-essential amino acids supplemented with pyruvate (1 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) in a humidified incubator (5% CO_2) at 37°C.

Measurement of Cholesterol Synthesis in Hep G2 Cells

Cholesterol synthesis in Hep G2 cells was determined according to the method of BROWN *et al.*[10] with some modifications. Hep G2 cells in 35 mm dishes were cultured with 1 ml of fresh medium containing 10% human lipoprotein-deficient serum, and the cells were preincubated for 2 hours at 37°C with various concentration of compound **1** or **2**. Then 1 mM [2-^{14}C] mevalonic acid,

DBED salt (37 MBq/mmol) was added to the medium and the cells were incubated at 37°C for 2 hours. After incubation, the cells were washed with phosphate-buffered saline (pH 7.4) and then dissolved in 1 ml of 15% - KOH aqueous solution. To each dissolved cell lysate was added same volume of 15% KOH - 95% ethanol solution, 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 and resuspended in 1 ml of 50% acetone in 95% ethanol containing 0.1% cold cholesterol as a carrier. Sterols were precipitated by the addition of 0.5% digitonin in 50% ethanol, incubated at 37°C 30 minutes and filtered on a glass filter. The radioactivity of digitonin-precipitable [^{14}C]-labeled sterols formed was counted with a liquid scintillation counter.

Antifungal Activities

Antifungal activities of compound **1** and **2** were measured by micro-broth dilution method in 96-well culture plate employing yeast nitrogen base dextrose medium. To a 50 μl sample solution with serial 2-fold dilutions was added a 50 μl of microorganism suspension in saline to yield a final concentration of 1×10^5 colony forming units/ml. The *Candida* and *Aspergillus* cultures were incubated at 37°C for 22 hours. After incubation, the growth of microorganism in each well was determined by measuring the turbidity. The results were shown as IC_{50} value in which concentration the turbidity was half of that in the well without sample.

Results

Taxonomy of Producing Strain No. 15604

The fungal strain No. 15604 grew very rapidly on various culture media, and formed dark green to brown colonies. This strain formed teleomorph, consisting of ascomata, on some agar media. The asci were bitunicate and cylindrical, the ascospores had gelatinous sheath, and each cells of ascospore had an elongated germ slit. On the basis of its morphological characteristics, the strain appears to belong to the ascomycetes genus Sporormiella Ellis et Everhart 1892. Its mycological characteristics were as follows.

Cultural characteristics on various agar media are summarized in Table 1. Culture on Emerson Yp Ss agar grew spreading broadly, attaining more than 7.5 cm in diameter after two weeks at 25°C. This colony surface was plane, thin, felty, and greenish gray to orange gray. The reverse was dark green to orange white. Ascomata were observed. Colonies on malt extract agar grew spreading broadly, attaining 5.5～6.5 cm in diameter under the same condition. The surface was plane, thin, and dark green. The reverse was dark green. Ascomata was not observed.

The morphological characteristics of this strain were determined on the culture of LCA agar[8]. Perithecia were scattered or loosely aggregated, immersed or superficial, subglobose to nearly pyriform, 130～200×110～180 μm, smooth, and dark brown to black. Asci were eight-spored, cylindrical, and 60～90×20～30 μm. Ascospore were obliquely bi- or tri-seriate, four-celled, cylindrical, 23～30×4～5 μm, broadly rounded at end, straight or curved, ranging from hyaline when young through brown, and transversely septate. Constrictions at septa were broad and deep. Segments were readily separate at the central septum and easily separable at the other septa. Cells of ascospore were nearly equal in size. Terminal cells were very slightly narrow toward the ends. Germ slits were nearly parallel with a kink near the middle. Gelatinous sheath were hyaline. The vegetative hyphae were smooth, septate, hyaline to brown, and branched. The hyphal cells were cylindrical and 1～4 μm in diameter. This strain was able to grow at the temperature range from 6 to 36°C with the growth optimum at 23 to 31°C.

According to the taxonomic criteria of the genus *Sporormiella*, strain No. 15604 resembled *Sporormiella minima* Ahmed et Cain 1972. And above characteristics corresponded with this species description by AHMED et CAIN[11] without ascus size. Then strain No. 15604 was identified as a strain of *Sporormiella minima* and has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4507.

Fermentation

Figure 2 shows the time course of compound **1** production by strain No. 15604 in a 30-liter jar fermenter, along with the pH and the packed cell volume. The maximal production of **1** was observed after 144 hours of cultivation at a concentration of 120 μg/ml.

Isolation

The culture broth (360 liters) was extracted with 360 liters of acetone by intermittent mixing. The acetone extract was filtered with an aid of diatomaceous earth and 600 liters of water was added. The resultant cake was re-extracted with 60 liters of acetone. The acetone extract was filtered with an aid of diatomaceous earth and 180 liters of

Table 1. Cultural characteristics of strain No. 15604.

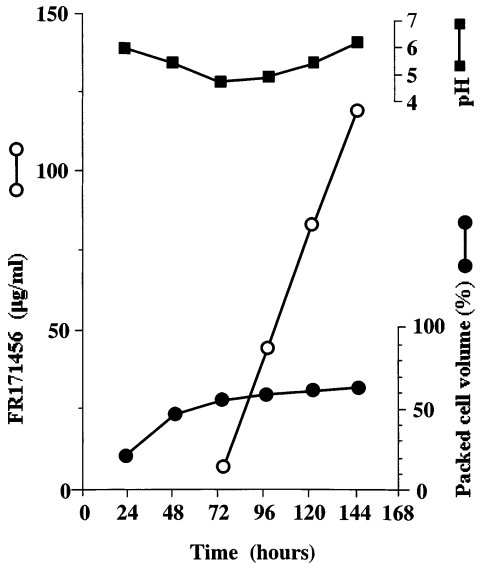
Medium	Cultural characteristics
Malt extract agar	G: Spreading broadly, 5.5-6.5cm
	S: Irregular, plane, thin, did not form teleomorph, dark green (30F4), yellowish white (2A2) at the edge
	R: Dark green (30F5), yellowish white (1A2) at the edge
Potato dextrose agar (Difco 0013)	G: Spreading broadly, 6.5-7.5cm
	S: Circular, sulcate, thin, formed immature ascomata, yellowish brown (5F4), yellowish white (3A2) at the edge
	R: Grayish brown (5F3), yellowish white (1A2) at the edge
Czapeck's solution agar	G: Spreading broadly, 5.0-6.0cm
	S: Irregular, plane, thin, did not form teleomorph, dark green (30F4), yellowish white (2A2) at the edge
	R: Yellowish white (1A2)
Saburaud dextrose agar (Difco 0190)	G: Spreading broadly, 6.5-7.5cm
	S: Irregular, raised, sulcate, wrinkly at the center, did not form teleomorph, light yellow (4A4)
	R: Grayish yellow (4B5)
Oatmeal agar (Difco 0552)	G: Spreading broadly, >7.5 cm
	S: Sulcate, felty to cottony, did not form teleomorph, gray (1E1), pale orange (5A3) at the edge
	R: Brownish gray (4D2), pale yellow (4A3) at the edge
Emerson Yp Ss Agar (Difco 0739)	G: Spreading broadly, >7.5 cm
	S: Plane, thin, felty, formed ascomata, greenish gray (30C2), orange gray (5B2) at the center, greenish white (30A2) at the edge
	R: Dark green (30F3), orange white (5A2) at the center, greenish white (30A2) at the edga
Corn meal agar (Difco 0386)	G: Spreading broadly, >7.5 cm
	S: Circular, plane, thin, felty, did not form teleomorph, dark green (30F4)
	R: Dark green (30F4)
YM 20 agar	G: Spreading broadly, >7.5 cm
	S: Irregular, raised, felty, did not form teleomorph, dull green (30E3)
	R: Dull green (30F4)
Abbreviation	G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

water was added.

These extracts were combined and passed through a column (30 liters) of Diaion HP-20 (Mitsubishi Chemical Ind. Co., Ltd.). The column was washed with water, methanol - water (1 : 1) solution, methanol - water (7 : 3) solution and eluted with methanol.

To the eluate (260 liters), an equal volume of water was added and the solution was passed through a column (12 liters) of Diaion SP-207 (Mitsubishi Chemical Ind. Co., Ltd.). After washing the column with methanol - water (1 : 1) solution and water, the column was eluted with a stepwise gradient of acetonitrile - water (containing 0.05% H_3PO_4) v/v, 2 : 3, 1 : 1, 3 : 2, 7 : 3, 4 : 1. Compounds **1** and **2** were eluted with the last two gradient solvents, respectively.

Fig. 2. Time course of FR171456 (**1**) production in a 30-liter jar fermentor.



The eluate containing compound **1** was adjusted to pH 6.0 with 1 N-NaOH and an equal volume of water was added. The mixture was loaded onto a column (1 liter) of YMC gel (ODS-AM 120-S50, YMC Co., Ltd.). The column was washed with methanol - water (1 : 1) solution, methanol - water (7 : 3) solution and eluted with methanol - water (4 : 1) solution. An active fraction was concentrated *in vacuo* to an aqueous solution, adjusted to pH 2.0 with 1 N-HCl and extracted with an equal volume of ethyl acetate, twice. The combined extract was washed with water and concentrated to dryness under reduced pressure. The resultant pale yellowish product was dissolved in a small volume of methanol. After standing at 4°C overnight, compound **1** was obtained as colorless prisms (869 mg).

The eluate containing compound **2** was adjusted to pH 6.0 with 1 N NaOH and concentrated *in vacuo* to an aqueous solution. This solution was loaded onto a column (1 liter) of YMC gel. The column was washed with acetonitrile - methanol - 0.015 M $(NH_4)_2HPO_4$-H_3PO_4buffer (pH 6.0) (40 : 10 : 50) solution and eluted with acetonitrile - methanol - 0.015 M $(NH_4)_2HPO_4$-H_3PO_4buffer (pH 6.0) (50 : 10 : 40) solution.

This eluate was diluted with water and loaded onto a column (1 liter) of YMC gel. After washing the column with acetonitrile - methanol - water (40 : 15 : 45) containing 0.05% H_3PO_4, acetonitrile - methanol - water (50 : 15 : 35) containing 0.05% H_3PO_4 and acetonitrile - methanol - water (60 : 15 : 25) containing 0.05% H_3PO_4, the active fraction was eluted with acetonitrile - methanol - water (70 : 15 : 15) containing 0.05% H_3PO_4. This active fraction was concentrated under reduced pressure to an aqueous solution. The solution was adjusted to pH 2.0 with 1 N HCl and extracted with an equal volume of ethyl acetate, twice. The combined extract was washed with water and concentrated to dryness under reduced pressure. The resultant pale yellowish product was dissolved in a small volume of methanol. After standing at 4°C 16 hours, compound **2** was obtained as colorless needles (46 mg).

Physico-chemical Properties

The Physico-chemical properties of compound **1** and **2** are summarized in Table 2. Compound **1** and **2** are acidic substances soluble in pyridine and DMSO. The Rf values of FR171456 and FR173945 on silica gel TLC (silica gel 60W, E. Merck) developed with dichloromethane : methanol (10 : 1, v/v) were 0.38 and 0.45, respectively. The molecular formula of compound **1** and **2** were determined as $C_{30}H_{44}O_7$ and $C_{30}H_{44}O_5$, respectively, by FAB-MS and elementary analysis.

The determination of structure of **1** and **2** were accomplished primarily by a series of 2-D NMR techniques. Full details of the structure elucidation of **1** and **2** will be described elsewhere.

Inhibition of Sterol Synthesis in Hep G2 Cells

Compound **1** and **2** were shown to inhibit the incorporation of [^{14}C]-mevalonic acid into cholesterol in Hep G2 cells. The IC_{50} value of cholesterol synthesis inhibition of compound **1** and **2** were shown in Table 3. Similar IC_{50} values were obtained from [^{14}C]-acetic acid or [^{3}H]-farnesyl-diphosphate incorporation into cholesterol in Hep G2 cells (data not shown). The inhibition point of compound **1** and **2** in cholesterol synthesis pathway is later than squalene synthetase catalyzed committed step in the sterol synthesis pathway.

Antifungal Activity

The IC_{50} values of compound **1** and **2** for *Candida albicans* FP578 and *Aspergillus fumigatus* 1305 are listed in Table 4. These compound exhibited antifungal activity against clinically important pathogens *C. albicans* and *A. fumigatus*.

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

	1	**2**
Appearance	Colorless prisms	Colorless needles
Molecular formula	$C_{30}H_{44}O_7$	$C_{30}H_{44}O_5$
Elementary analysis	Calcd for $C_{30}H_{44}O_7.CH_3OH$: C 67.86, H8.82 (%) Found: C67.81, H9.15 (%)	Calcd for $C_{30}H_{44}O_5.1/2H_2O$: C72.99, H9.19 (%) Found: C73.35, H 9.54 (%)
FAB-MS (*m/z*)	539 $(M+Na)^+$	507 $(M+Na)^+$
HRFAB-MS (*m/z*)		
Calced:	539.2984$(M+Na)^+$	
Found:	539.2951$(M+Na)^+$	
Melting point	181-190℃ (dec.)	218.1-221.5℃
Specific rotation [α]D (23℃)	+115°(c=1.0 in pyridine)	-74°(c=0.54 in pyridine)
UV λ_{max}nm (methanol)	end absorption	end absorption, 240 nm (sh.)
IR $\nu_{max}cm^{-1}$ (KBr)	3470,2960,2880,2610,1720, 1640,1460,1380,1290,1270, 1240,1190,1140,1080,1020, 990,890	3420,3080,2960,2870,1700, 1640,1470,1380,1360,1340, 1300,1280,1260,1130,1090, 1060,1010,990,890

Table 3. Inhibition of cholesterol synthesis in HepG2 cells.

Compound	Inhibition of cholesterol synthesis (IC_{50}, ng/ml)
1	4.2
2	8.3

Table 4. Antifungal activities of compound **1** and **2**.

Organisms	IC_{50} (μg/ml)	
	1	**2**
Candida albicans FP578	50.0	1.6
Aspergillus fumigatus 1305	3.0	12.5

Discussion

In this paper we have presented novel and potent cholesterol synthesis inhibitors, FR171456 (**1**) and FR173945 (**2**), which were isolated from the culture broth of *Sporormiella minima* No. 15604. From the evidence of physico-chemical data and 2-D NMR spectra, these compounds have novel structures (Fig. 1).

These compounds were discovered during a screening program for cholesterol synthesis inhibitors acting at a

stage of cholesterol biosynthesis later than the HMG-CoA reductase catalyzed step. Compound **1** and **2** inhibited cholesterol biosynthesis in Hep G2 cells from mevalonic acid, which is the product of HMG-CoA reductase. Their IC_{50} values were 4.2 ng/ml and 8.3 ng/ml, respectively (Table 3).

Conversion of HMG-CoA to mevalonic acid is an early step in mammalian isporenoid pathway. This pathway not only produces sterols but also produces dolichol, ubiquinone, the farnesyl group of heme A, the farnesyl and geranylgeranyl group of prenylated proteins, and the isopentenyl side chain of isopentenyl adenine[12]. The pathways for the synthesis of these isoprenoids diverge from the synthesis of sterols either at or before the farnesyl diphosphate branch point. Thus squalene synythetase (farnesyl-diphosphate : farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) is the first committed step of sterol synthesis[13]. HMG-CoA reductase inhibitors could in principle suppress all post mevalonic acid biosynthetic steps and compromise supplies of biologically important non-steroidal isoprenoids[14,15]. In fact, several studies have implicated the inhibition of non-steroidal isoprenoids, to account for the side effect of observed with HMG-CoA reductase inhibitors. Mitochondrial dysfunction due to the deficiency of ubiquinone (Co-enzyme Q10) has been demonstrated to contribute significantly to the HMG-CoA reductase inhibitor induced complications[16].

Compound **1** and **2** also inhibited cholesterol biosynthesis from acetic acid or farnesyl-diphosphate in Hep G2 cells (data not shown). These results strongly suggested that the inhibition point(s) or target enzyme(s) of **1** and **2** are later than the squalene synthetase step in the sterol synthesis pathway.

These compound exhibited antifungal activity against clinically important pathogens *C. albicans* and *A. fumigatus*.

The details of *in vivo* evaluation of these compounds are described following paper[17]. The structural analysis will be described elsewhere.

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