Biological Activities of Novel Zaragozic Acids, the Potent Inhibitors of Squalene Synthase, Produced by the Fungus, *Mollisia* sp. SANK 10294

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Four novel zaragozic acids, F-10863A, B, C and D, were isolated from a culture broth of the fungus *Mollisia* sp. SANK 10294. F-10863 compounds contain a 4,6,7-trihydroxy-2,8-dioxyobicyclo-[3.2.1]octane-3,4,5-tricarboxylic acid core like previously reported zaragozic acids, but the structures of the side chains are different. Recently, it was found that F-10863A is identical to zaragozic acid D3, while the other three are novel compounds. F-10863 compounds are potent inhibitors of squalene synthase like previously reported zaragozic acids, and, furthermore, they exhibit serum cholesterol-lowering activity *in vivo*.

The treatment of hypercholesterolemia with pharmacological agents reduces the risk of developing arteriosclerosis. Several types of drugs, such as bile acid sequestrants or cholesterol biosynthesis inhibitors, are currently available. Pravastatin sodium, for one, suppresses cholesterol biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, one of the early stage enzymes in sterol biosynthesis[1]. Other enzymes involved in the later stage of sterol biosynthesis can also be targeted for inhibition. Squalene synthase (SQS, farnesylpyrophosphate: farnesylpyrophosphate farnesyltransferase, EC 2.5.1.21) catalyzes the reductive condensation of farnesylpyrophosphate to form squalene at the final branch point of the sterol biosynthetic pathway. The mammalian isoprenoid pathway produces several important compounds other than sterols, including dolichols, ubiquinones, heme A and the prenylated proteins. The synthesis of these essential isoprenoids branches from the sterol synthetic pathway at or before the synthesis of farnesylpyrophosphate (FPP), the substrate for squalene synthase. Thus, inhibitors of SQS will be selective inhibitors of sterol biosynthesis and will not affect the synthesis of other essential isoprenoids[2]. Many different types of SQS inhibitors have been discovered[3]. Zaragozic acids (ZAs) are produced by fungi and have very potent SQS inhibitory activities[4~6]. While screening microbial fermentation products for inhibitors of squalene synthase, we previously identified a novel compound, schizostatin, from *Schizophyllum commune*[7~9]. Subsequently, we discovered four novel ZAs, F-10863A, B, C and D from *Mollisia* sp. SANK 10294. Later we determined that F-10863A is identical to zaragozic acid D3 (ZAD3), which was produced by *Libertella* sp. (BERGSTROM *et al.*[10]), but no biological activity was reported. In this paper, we describe the biological activities of F-10863A (ZAD3) and three analogues, F-10863B, C and D. Taxonomy of the fungus, methods of fermentation and isolation of the compounds will be described elsewhere by HOSOYA *et al.* (in preparation).

Materials and Methods

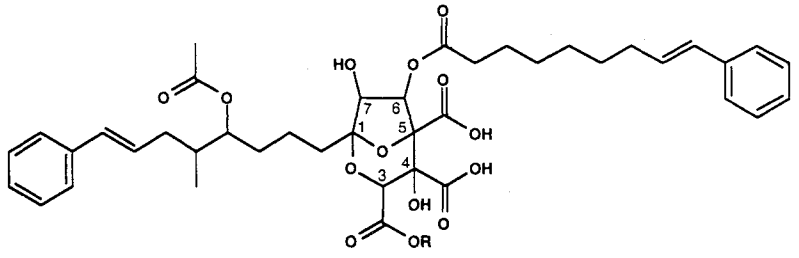
Materials

[4-^{14}C]-Isopentenyl pyrophosphate (IPP, 56 μCi/μmol) and [1-^{14}C]sodium acetate (58 μCi/μmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.)

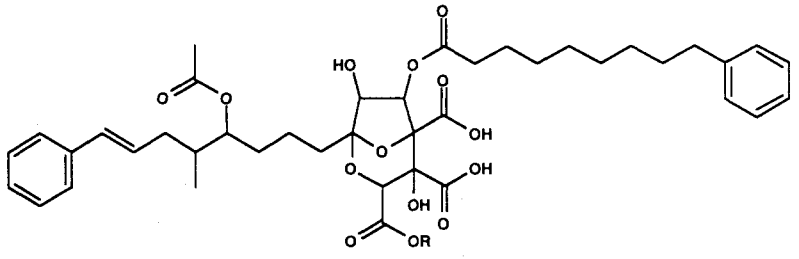
Animals

Male Wistar-Imamichi rats (180~250 g) and male golden hamsters (100~120 g) were purchased from Japan SLC Co., Ltd. They were fed a normal diet (Funabashi Farm, Chiba, Japan) under lighted conditions from 7:00 a.m. to 7:00 p.m. Common marmosets (300~350 g) were purchased from Cler Japan Inc. They were fed an MD7 diet (Funabashi Farm, Chiba, Japan) supplemented with fruit under lighted conditions from 7:00 a.m. to 7:00 p.m.

Fig. 1. Structures of F-10863A (ZAD3) and three analogues, B, C and D.



R=H; F-10863A (Zaragozic acid D3)
R=Me; F-10863B



R=H; F-10863C
R=Me; F-10863D

Cells

Rat hepatocytes and spleen cells were freshly isolated from male Wistar-Imamichi rats and cultured using the method of TSUJITA *et al.*[1]. Bovine aortic smooth muscle cells were obtained from medial explants of bovine aorta and cultured by the method of NEMECEK *et al.*[11]. Hep G2 cells, normal human skin fibroblasts, mouse L-cells and HeLa cells were cultured as described by TSUJITA *et al.*[1].

Assay Method for Squalene Synthase (SQS) Activity

Assays of SQS was done as described previously[7]. Rat liver microsomes prepared from the liver of male Wistar-Imamichi rats were used as the enzyme source for the SQS assay. [4-^{14}C]-FPP enzymatically synthesized from [4-^{14}C]-IPP and [cold]-geranyl pyrophosphate was used as the substrate for the SQS assay.

Assays of Sterol Synthesis in Several Cultured and Isolated Cells

All freshly isolated and cultured cells used were maintained at 37°C in a 5% CO_2 incubator. Sterol synthetic activity in cells was determined by the incorporation of [1-^{14}C]sodium acetate into digitonin-precipitable sterols as described by TSUJITA *et al.*[1].

Methods for Animal Experiments

Cholesterol-lowering Effect in Hamsters

F-10863A and B were administered orally to hamsters (n=5) for 7 days. After overnight fasting, blood samples were collected and serum was obtained by centrifugation at 2,150×*g* for 10 minutes at 4°C. The levels of serum total cholesterol were determined using a Hitachi type 736 automatic analyzer (Tokyo, Japan).

Cholesterol-lowering Effect in Marmosets

F-10863A was administered orally to marmosets (n=2, one male and one female) for 7 days. After overnight fasting, blood samples (1 ml) were collected from the femoral vein and serum was obtained by centrifugation at 2,150×*g* for 10 minutes at 4°C. The levels of serum total cholesterol were determined using a Hitachi type 736 automatic analyzer (Tokyo, Japan).

Statistical Analysis

Data from these experiments were statistically analyzed by Student's t-test. Values are expressed as mean±S.E.M.

Results

Structures of F-10863A(Zaragozic acid D3), B, C and D

Structures of F-10863A (ZAD3) and three analogues B, C and D are shown in Fig. 1. They contain a 4,6,7-trihydroxy-2,8-dioxyobicyclo[3.2.1]octane-3,4,5-tricarboxylic acid core like previously reported zaragozic acids, but the structures of the 1-alkyl and 6-acyl side chains, are different from them. F-10863B is a monomethyl ester derivative of the carboxyl group at the C3 position of F-10863A, and F-10863D is the same derivative of F-10863C.

Biological Activities

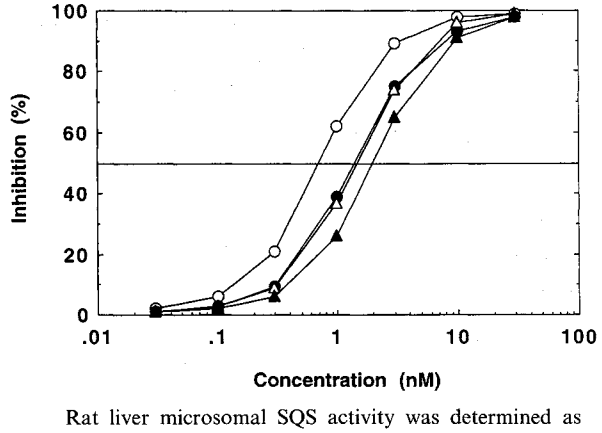
In Vitro SQS Inhibitory Activities

SQS activity was measured in the presence of various concentrations of the F-10863 compounds. All 4 compounds have very potent SQS inhibitory activities, and act in a dose dependent manner. The IC_{50} values of F-10863A, B, C and D are 0.7, 1.3 , 1.6 and 2.0 nM, respectively (Fig. 2). From kinetic analysis, F-10863A inhibited SQS activity competitively with respect to farnesylpyrophosphate with a *Ki* value of 1.6 nM (Fig. 3).

Inhibition of Sterol Synthesis in Several Cultured and Isolated Cells

F-10863A and B were determined to inhibit sterol synthesis in several cultured and isolated cells. In freshly isolated rat hepatocytes, F-10863A and B inhibited sterol synthesis potently with the IC_{50} values of 6.7 and 11 nM, respectively. However, these compounds only weakly inhibited sterol synthesis in other cells, suggesting that they show cellular specificity in their activitiy (Table 1).

Fig. 2. Inhibition of SQS by F-10863A (ZAD3), B, C and D.



Rat liver microsomal SQS activity was determined as described previously (Reference 7). Symbols represent F-10863A (○), B (●), C (△) and D (▲).

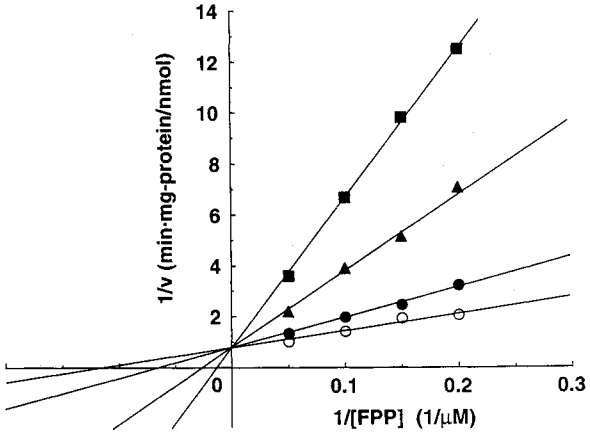
In Vivo Serum Cholesterol-lowering Effects in Hamsters

As shown in Fig. 4, F-10863A reduced serum total cholesterol levels in hamsters by 18.1% and 22.3% compared to the control group at doses of 30 mg/kg and 100 mg/kg, respectively. F-10863B also reduced serum total cholesterol levels by 18.5% at a dose of 100 mg/kg ($P<0.001$). Neither of these two compounds showed significant toxic effects at these dosages.

In Vivo Serum Cholesterol-lowering Effects in Marmosets

F-10863A reduced serum total cholesterol levels by

Fig. 3. Lineweaver-Burk plot for the inhibition of SQS by F-10863A (ZAD3).



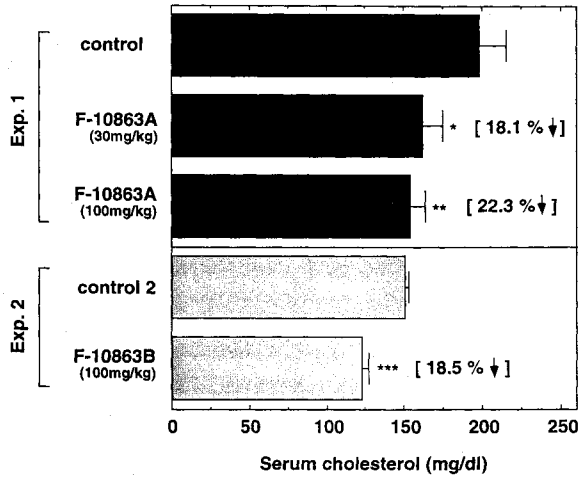
The enzyme assay was carried out as described previously, except that the concentration of the substrate was varied as indicated. The analysis was performed so that the enzyme reaction was a linear function of time. The points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fit of these data to the rate equation for competitive inhibition. The concentrations of F-10863A in this experiment were 0 (○), 1.25 (●), 5.0 (▲) and 12.5 (■) nM.

Table 1. Effects of F-10863A and B on sterol synthesis in various isolated and cultured cells.

	Inhibition of sterol synthesis, IC_{50} (nM)	
	F-10863A	F-10863B
Freshly isolated rat hepatocytes	6.7	11
Freshly isolated rat spleen cells	16,000	240
Hep G2 cells	16,000	5,200
Human skin fibroblasts	30,000	18,000
Bovine aortic smooth muscle cells	17,000	3,200
Mouse L cells	13,000	440
HeLa cells	38,000	18,000

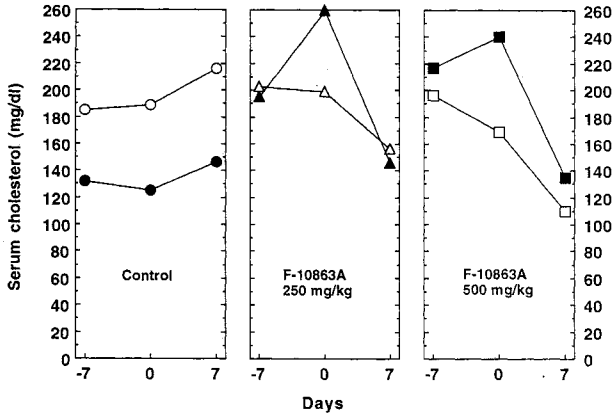
Sterol synthetic activity in cells was determined by the incorporation of [1-^{14}C]sodium acetate into digitonin-precipitable sterols.

Fig. 4. Effects of F-10863A and B on serum cholesterol levels in hamsters.



The assay method was described in "Materials and Methods". Results are expressed as the mean±S. E. M. $*P<0.05$, $**P<0.01$, $***P<0.001$

Fig. 5. Effects of F-10863A on serum cholesterol levels in marmosets.



The assay method was described in "Materials and Methods". Symbols, ○: Control (male), ●: control (female), △: 250 mg/kg (male), ▲: 250 mg/kg (female), □: 500 mg/kg (male), ■: 500 mg/kg (female).

about 20~30% compared to pretreatment levels at doses of 250 or 500 mg/kg. Although the doses were higher relative to those administered to hamsters, no significant toxicity was observed.

Discussion

F-10863A and three analogues, B, C and D, are novel ZAs having very potent SQS inhibitory activity similar to previously reported ZAs. F-10863A was found to be identical to ZAD3. F-10863B and D are the monomethyl ester of carboxyl group at the C3 position of F-10863A (ZAD3) and F-10863C, respectively (Fig. 1). Although one of their three carboxylic acid residues, the C3 carboxylic acid, is methylated, F-10863B and D are almost as potent as F-10863A and C. WATSON *et al.*[12] also reported that they chemically synthesized three monomethyl esters at either the C3, C4 or C5 carboxylic acid in zaragozic acid A (ZAA) and showed that free carboxylic acid at the C3 or C4 positions of ZAA is not required for SQS inhibitory activity, but the free carboxylic acid at C5 of ZAA is crucial for the activity. F-10863B and D are supposed to be synthesized from F-10863A and C, respectively, in the cultivation of *Mollisia* sp. SANK 10294. This methylation was site-specific and C4 or C5 methylesters were not detected in the culture broth.

F-10863A inhibited SQS activity competitively with respect to farnesylpyrophosphate. Similar observations using ZAA were done by BERGSTROM *et al.*[5] and HASUMI *et al.*[6]. Although F-10863A and B are strong inhibitors of SQS *in vitro*, they very weakly inhibited sterol synthesis in almost all the cells tested, except freshly isolated hepatocytes. It is known that hydrophilic compounds hardly permeate the lipid bilayer membrane, plasma membrane, because they have low affinity for the membrane. Since F-10863 compounds have 2 or 3 free carboxylic acid residues and show relatively hydrophilic in nature, it is probable that only small amount of the compounds were incorporated into the cells by passive diffusion. On the other hand, it has been reported that hepatocytes have transporter proteins for organic anions[13]. Pravastatin sodium, having a carboxyl group in its structure, is selectively incorporated into hepatocytes by transporter proteins[13]. Since F-10863A and B have three and two carboxylic acids in their molecules, respectively, they could also be incorporated into freshly isolated hepatocytes by transporter proteins. BERGSTROM *et al.* reported that ZAA has similar cellular specificity in its efficacy.[10] F-10863B exhibited lower cellular specificity than F-10863A. Since the methylation of the C3 carboxylic acid reduces the polarity of the compound, F-10863B is incorporated into cells easier than F-10863A by the diffusion mechanism.

F-10863 compounds reduced serum cholesterol levels in hamsters as well as marmosets without any significant toxicity *in vivo*. In addition, ZAA was also reported to markedly reduce serum cholesterol levels in marmosets.[4] Therefore, zaragozic acids, including F-10863 com-

pounds, are expected to have hypolipidemic effects in humans.

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