AS-183, A NOVEL INHIBITOR OF ACYL-CoA : CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Scedosporium* sp. SPC-15549

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A novel compound, AS-183, which inhibits acyl-CoA : cholesterol acyltransferase (ACAT), was isolated from the culture broth of a fungus, *Scedosporium* sp. SPC-15549. AS-183 inhibited ACAT activity in an enzyme assay system using rabbit liver microsomes with an IC_{50} value of 0.94 μM. AS-183 also inhibited cholesterol ester formation in HepG2, CaCo2, and THP-1 cells with IC_{50} values of 18.1, 25.5, and 34.5 μM, respectively.

Acyl-CoA : cholesterol acyltransferase (ACAT, EC2.3.1.26) is a key enzyme responsible for cholesterol ester formation in atherogenesis and in cholesterol absorption from the intestines. ACAT inhibitors are expected to be effective for treatment of atherosclerosis and hypercholesterolemia. Although a wide variety of synthetic inhibitors of ACAT are now under clinical or preclinical evaluation[1], there are only a few papers showing the isolation and purification of ACAT inhibitors of microbial origin. Examples are the purpactins[2], glisoprenins[3], acaterin[4], and cyclodepsipeptide antibiotics[5].

During the course of our screening work to obtain new ACAT inhibitors from a microbial source, we isolated a novel compound, AS-183 (Fig. 1), which inhibits the ACAT activity of rabbit liver microsomes, from the culture broth of *Scedosporium* sp. SPC-15549 (FERM BP-3392). In this paper, we report the fermentation, isolation, and characterization of AS-183. Structural elucidation studies will be reported in a separate paper.

Materials and Methods

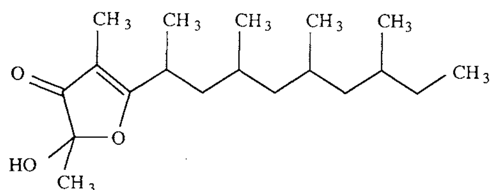
Materials

All of the radioactive compounds were purchased from New England Nuclear. All of the other chemicals were analytical grade.

Microorganisms and Taxonomy

Scedosporium sp. SPC-15549 was isolated from a soil sample collected in Kanagawa, Japan. Most of the taxonomic studies of strain SPC-15549 were carried out in accordance with the methods adopted by the Genera of Fungi Sporulating in Pure Culture (2nd Ed. CRAMER, VADUZ, J. A. VON ARX, 1974).

Fig. 1. The structure of AS-183.



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Fermentation

A 50-ml test tube containing 10 ml of a seed medium composed of vegetable juice (Campbell, 20%) and dextrin (3%, pH 6.5 before sterilization) was inoculated with a loopful of spores of the strain grown on an agar slant composed of malt extract (2%), glucose (2%), peptone (0.1%, Kyokuto), agar (2%, pH 6.5 before sterilization). After cultivation for five days on a reciprocating shaker (300 rpm) at 25°C, 5 ml of the culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the seed medium and the flask was incubated for two more days on a rotary shaker (200 rpm) at 25°C. A 10%-inoculation from the above vegetative medium was added to a 300-ml Erlenmeyer flask containing 50 ml of a fermentation medium composed of sucrose (5%), beef extract (0.5%), corn steep liquor (0.5%), pharmamedia (1.5%), $Mg_3(PO_4)_2 \cdot 8H_2O$ (0.05%, pH 6.0 before sterilization) and incubated for 7 days at 25°C. The production of AS-183 was traced by a HPLC method with an ODS column (Gasukurokogyo, unisil-NQ, C18, i.d. 4.6 × 150 mm), developing with 85% acetonitrile, by measuring UV 280 nm absorbance. The retention time of AS-183 in this HPLC method was 6.9 minutes from sample injection. For this measurement, 2 ml of the culture broth was withdrawn and centrifuged. The precipitated mycelium was extracted with 2 ml of methanol and the extract was diluted to 5 times its volume by H_2O before applying to a C18 Sep-Pak cartridge (Waters). After washing the cartridge with 50% methanol, AS-183 was eluted with methanol and the eluate was subjected to the HPLC method.

During the isolation process, AS-183 was detected by a TLC method using a silica gel plate (Merck, Art. No. 5715) developed with ethylacetate - *n*-hexane (4 : 6, Rf value: 0.52) or chloroform - methanol (20 : 1, Rf value: 0.50) in combination with visualization with I_2-vapor or UV 254 nm.

Preparation of Rabbit Liver Microsomes

Male white rabbits fed a diet containing cholesterol (2%) and cholic acid (0.5%) for 1 to 2 months were killed by decapitation and their livers were rapidly removed. The microsomal fractions of rabbit liver were prepared according to the method of C. MARCO *et al.*[6]. Briefly, livers were cut into small pieces and then homogenized with a motor driven glass-Teflon homogenizer in 5-times their volume of ice-cooled potassium-phosphate buffer (pH 7.4, 100 mM) containing EDTA (1 mM) and dithiothreitol (2 mM). The homogenate was then centrifuged for 10 minutes at 800 × *g* and the resulting supernatants were again centrifuged for 10 minutes at 15,000 × *g*. The microsomal pellet was resuspended in the potassium-phosphate buffer and centrifuged for 60 minutes at 120,000 × *g*. The pellet was washed with the potassium-phosphate buffer, homogenized, recentrifuged for 60 minutes at 120,000 × *g*, and preserved at −80°C till experiments were done. All operations were done at 4°C.

Enzyme Assay

ACAT Assay Using Rabbit Liver Microsomes

The activity of the microsomal ACAT was measured according to the method of P. BRECHER *et al.*[7] with some modifications. The reaction mixture containing potassium-phosphate buffer (pH 7.4, 0.1 M), [1-^{14}C]oleoyl-CoA (0.1 μCi, 50 μM), dithiothreitol (2 mM), bovine serum albumin (fatty acid free, 50 μM), microsomal fraction (50 μg protein), and test-sample (dissolved in 10 μl of methanol) in a final volume of 0.2 ml was incubated for 45 minutes at 37°C. After which, the reaction was stopped by addition of 4 ml of chloroform - methanol (2 : 1). The chloroform extracts were developed by thin layer chromatography (TLC) on silica gel 60 plate (LK6D, Whatman) using *n*-hexane - diethylether - acetic acid (150 : 50 : 4) as a solvent system. The cholesteryl ester spot was scraped off and transferred into each scintillation vial and the radioactivity was measured using liquid scintillation counter.

Assay for Cholesterylester Formation in Clonal Cell Lines

Clonal cell lines used in the present study were purchased from ATCC. Cellular ACAT activity was measured according to the method of GOLDSTEIN *et al.*[8] with some modifications. The cells (5×10^5/well) were incubated overnight at 37°C in DULBECCO's modified EAGLE's medium containing lipoprotein-deficient serum (5%). The medium was changed to the same volume of fresh medium, and then 3 μl of an ethanol solution containing 25-hydroxycholesterol (5 μg/ml), cholesterol (10 μg/ml), and various amounts of inhibitor (dissolved in 10 μl of ethanol) was added to the medium. After incubation at 37°C for 4 hours, [1-^{14}C]oleic acid-BSA complex was added, and the incubation was continued for an additional 2 hours.

After which time, the cells were extracted with *n*-hexane - 2-propanol (3 : 2). The lipids were separated by TLC as described above. The cholesteryloleate spot was scraped off and its radioactivity was measured using a liquid scintillation counter.

Lecithin : cholesterol Acyltransferase (LCAT) Assay

LCAT activity was measured according to the method of STOKKE and NORUM[9]. The final volume of the incubation mixture was 0.2 ml. To 100 μl of human plasma, [^{3}H]cholesterol (0.3 μCi) was added as a suspension stabilized with albumin. The reaction mixture was then permitted to equilibrate for 4 hours at 37°C during which time LCAT was inhibited by 5,5′-dithio-bis-2-nitro-benzoic acid 10 mM. Next, 20 μl of 2-mercaptoethanol (100 mM) was added to reverse the inhibition of LCAT. After incubation for 40 minutes at 37°C, 4 ml of chloroform - methanol (2 : 1) was added to stop the reaction. The acylated cholesterol was separated by TLC using *n*-hexane - diethylether - acetic acid (150 : 50 : 4) as the solvent system, and the radioactivity was measured using a liquid scintillation counter.

Acyl CoA : sn-glycerol-3-phosphate Acyltransferase (AGAT) Assay

AGAT activity was measured according to the method of K. YAMADA and H. OKUYAMA[10] with some modifications. The reaction was started by addition of 4 mM [2-^{3}H(N)]glycerol-3-phosphate (26 μl) to the reaction mixture (174 μl) containing phosphate buffer (100 mM), oleoyl CoA (50 μM), BSA (1.25 mg/ml), and 100 μg of microsomal protein. After incubation at room temperature for 5 minutes, the reaction was stopped by adding ice-cooled chloroform acidified with HCl. The radioactivity of the chloroform extracts was measured using a liquid scintillation counter.

3-Hydroxy-3-methylglutaryl Coenzyme A(HMG-CoA) Reductase Assay

HMG-CoA reductase assay was measured by the method of SHAPIRO *et al.*[11] with some modifications. On a reaction mixture (95 μl) consisted of 100 μg of microsomal protein, dithiothreitol (12 mM), NADP (4 mM), glucose-6-phosphate (G-6-P, 40 mM), glucose-6-phosphate dehydrogenase (5 units/ml), and potassium-phosphate buffer (pH 7.2, 200 mM). The reaction was started by addition of [^{14}C]-HMG-CoA (5 μl, 28.4 nCi) and incubated at 30°C for 30 to 60 minutes. After which time, 5 N HCl (10 μl) was added to stop the reaction, and then 20 μl of non-labeled mevalonolactone (0.1 M) and 10 μl of DL-[2-^{3}H]mevalonolactone (5,500 dpm) were added to the mixture. After an additional 30 minutes incubation at 30°C the reaction mixture, along with a few milligrams of Na_2SO_4, was transferred into a glass tube. The reaction mixture was extracted twice with 3 ml each of diethylether, and the extract was developed on a TLC plate by using toluene - acetone (1 : 1) as the solvent system. The radioactivity of the separated mevalonolactone spot was scraped off and the radioactivity was measured using a liquid scintillation counter.

Cholesterol 7α-Hydroxylase Assay

Cholesterol 7α-hydroxylase activity was measured by the method of STRAKA *et al.*[12] with some modifications. The reaction mixture containing 0.5 mg microsomal protein, [1,2-^{3}H(N)]cholesterol-BSA (1 μCi, 19.6 nmol-1%BSA) complex, and potassium-phosphate buffer (pH 7.4, 70 mM) was pre-incubated for 2 hours on ice, and subsequently incubated at 37°C for 5 minutes. The reaction was started by addition of the potassium-phosphate buffer (pH 7.4, 70 mM) containing NADP (1.25 mM), glucose-6-phosphate (2.5 mM), $MgCl_2$ (10 mM), and glucose-6-phosphate dehydrogenase (5 units). After 4 minutes incubation at 37°C, the reaction was stopped and extracted with 2 ml of chloroform - methanol (2 : 1). The extract was developed on a TLC plate by using petroleum ether - diethylether - acetic acid (50 : 50 : 1) as the solvent system. The radioactivity of the separated 7α-hydroxycholesterol was measured using a liquid scintillation counter.

Results

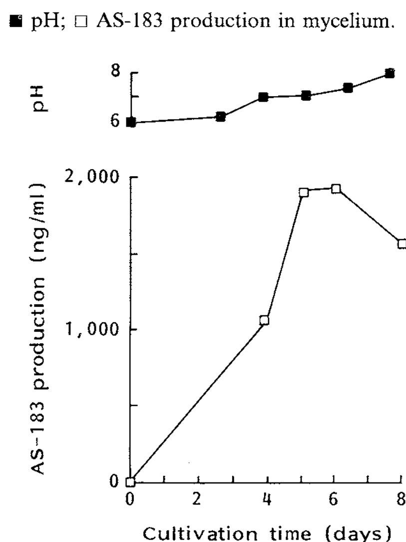
Production of AS-183 by Fermentation

Numerous attempts to increase the yield of AS-183 were made; the resultant defined medium and

optimum conditions for production are described under Materials and Methods. The time course of AS-183 production by *Scedosporium* sp. SPC-15549 in 300 ml Erlenmeyer flasks is shown in Fig. 2. AS-183 was produced mainly in the mycelia; the amount of AS-183 produced in the mycelia is approximately 10-fold higher than in the broth filtrate. The amount of AS-183 in the mycelia significantly increased on day 4 and reached its maximum on day 5 to 6.

Fig. 2. Time course of AS-183 production in 300-ml Erlenmeyer flask.

■ pH; □ AS-183 production in mycelium.



Isolation and Purification of AS-183

The purification procedure for AS-183 is outlined in Fig. 3. The culture broth (total 50 liters) was centrifuged at 8,000 rpm for 10 minutes. The mycelial cake was extracted with methanol. The methanol extracts were diluted with 200 liters of de-ionized water and applied to a Diaion HP-20 (Mitsubishi Chemical Industries Ltd., 2 liters) resin column. The column was washed with water and then with 50% methanol, and AS-183 was eluted with methanol. The eluate was concentrated under reduced pressure *in vacuo* to yield an aqueous solution. The resultant aqueous solution was extracted with ethyl acetate. The ethyl acetate layer was concentrated *in vacuo* to yield an oily black material (36 g). Half of this oily material was applied to a silica gel (Merck, Si60, Art. 7734, 2 liters) column for chromatography. The adsorbed materials were successively eluted with 6 liters each of chloroform, chloroform - methanol (99 : 1), chloroform - methanol (98 : 2), and chloroform - methanol (95 : 5). The remaining ethyl acetate extracts were chromatographed in the same way. The fractions enriched with AS-183 from each column chromatography were combined and concentrated *in vacuo* to yield brown oily materials (3.5 g). These oily materials were subjected to silica gel (Merck, Si60, Art. 9385, 2 liters) column chromatography. The column was successively eluted with 10 liters each of 10, 15, 20, and 25% ethyl acetate in *n*-hexane, and the fractions containing AS-183 were collected and concentrated *in vauco*. Final purification of AS-183 was achieved by Sephadex LH-20 column chromatography by using methanol as the elution solvent. This LH-20 column chromatography was repeated once more and pure AS-183 was obtained as a colorless oil (19.7 mg).

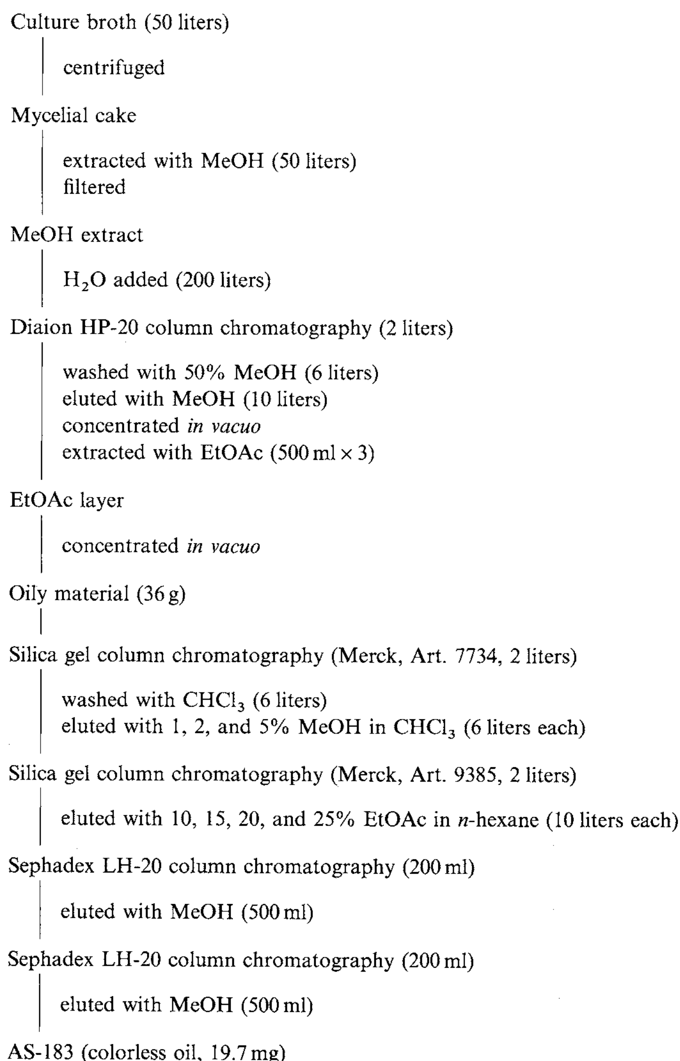
Physico-chemical Properties

The physico-chemical properties of AS-183 are summarized in Table 1. AS-183 is readily soluble in methanol, acetone, ethyl acetate, chloroform, acetonitrile, and dimethyl sulfoxide and is virtually insoluble in water and *n*-hexane. The molecular formula of AS-183 was determined to be $C_{19}H_{34}O_3$ on the basis of HREI-MS measurements. The structure of AS-183 was elucidated to be 2,4-dimethyl-2-hydroxy-5-(1,3,5,7-tetramethyl-nonyl)-3(2*H*)-furanone (Fig. 1), on the basis of ^{1}H and ^{13}C NMR spectral data and physico-chemical analyses. The details of these studies will be described in a separate paper.

Biochemical Properties

The inhibitory activities of AS-183 against various cholesterol metabolic enzymes are shown in Table

Fig. 3. Purification procedure of AS-183.



2. AS-183 inhibited microsomal ACAT activity with an IC_{50} value (the concentration causing 50% inhibition of maximally-stimulated enzyme activity) of 0.94 μM. By contrast, AS-183 had no effect on the activities of acyl CoA:sn-glycerol-3-phosphate acyltransferase (AGAT), lecithin : cholesterol acyltransferase (LCAT), cholesterol 7α-hydroxylase (Chol. 7α-OHase), and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase at 288, 57, 65 and 65 μM, respectively. AS-183 also inhibited cholesterol eater formation through the inhibition of cellular ACAT activity. The IC_{50} values of AS-183 for cellular ACAT were calculated to be 18.1, 25.5, and 34.5 μM in HepG2, CaCo2, and THP-1 cells, respectively.

AS-183 had no antimicrobial activity against *Staphylococcus aureus* KY4779, *Enterococcus faecalis* KY4280, *Bacillus subtilis* KY4773, *Escherichia coli* KY4271, *Klebsiella pneumoniae* KY4275, *Proteus vulgaris* KY4277, *Shigella sonnei* KY4281, *Salmonella typhosa* KY4278, *Pseudomonas aeruginosa* KY4276, or *Candida albicans* KY5011 at concentrations up to 100 μg/ml.

Table 1. Physico-chemical properties of AS-183.

Appearance	Colorless oil
Molecular formula	$C_{19}H_{34}O_3$
TLC, Rf value	
$CHCl_3$ - MeOH (20 : 1)[a]	0.50
n-Hexane - EtOAc (6 : 4)[a]	0.52
80% MeOH[b]	0.47
80% CH_3CN[b]	0.57
Color reaction	
positive	I_2, H_2SO_4, anisaldehyde
negative	Aniline-phthalate, ninhydrin, Rydon-Smith
Solubility	
soluble	MeOH, EtOAc, acetone, $CHCl_3$, CH_3CN, DMSO
insoluble	*n*-Hexane, H_2O
UV λ_{max} (CH_3CN, nm)	278 (ε=11,000)
IR ($CHCl_3$, cm^{-1})	3573, 3313, 2960, 2927, 1701, 1610, 1462, 1379, 1043, 955

[a] Silica gel 60 F_{254} (Merck, Art. No. 5628).
[b] RP-18 F_{254} (Merck, Art. No. 13724).

Table 2. Effect of AS-183 on various cholesterol metabolic enzymes.

Enzyme	IC_{50} (μM)
ACAT	
Rabbit liver microsome	0.94
HepG2 cell	18.1
CaCo2 cell	25.5
THP-1 cell	34.5
AGAT	>288
LCAT	>57
Chol. 7α-OHase	>65
HMG-CoA reductase	>65

ACAT: Acyl-CoA : cholesterol acyltransferase.
AGAT: Acyl-CoA : sn-glycerol-3-phosphate acyltransferase.
LCAT: Lecithin : cholesterol acyltransferase.
Chol. 7α-OHase: Cholesterol 7α-hydroxylase.
HMG-CoA reductase: 3-Hydroxy-3-methylglutaryl coenzyme A reductase.

Discussion

In this paper, we demonstrate that a novel compound, AS-183, isolated from the culture broth of *Scedosporium* sp. SPC-15549, is a potent inhibitor of microsomal ACAT activity. By contrast, AS-183 has no inhibitory activity against two other acyltransferases, namely HMG-CoA reductase and Chol. 7α-hydroxylase. Further, AS-183 also inhibited cholesterol ester formation in cultured HepG2 (derived from human liver), CaCo2 (derived from human small intestine), and THP-1 (derived from human monocyte) cells, indicating the inhibition by AS-183 of ACAT activities in the living cells. Several synthetic ACAT inhibitors have been reported having urea or amide moieties and some of these have been under clinical or preclinical evaluation, but only a few compounds are known as metabolites of microorganisms or are other types of natural products[2~5,13]. It is noteworthy that the structure of AS-183 is not similar to those of the synthetic ACAT inhibitors reported so far. There is a great need for drugs that inhibit cholesterol absorption in gut, promote discharge of cholesterol from liver, and decrease cholesterol-mass in artery wall, from the viewpoint of prevention and improvement of arteriosclerosis. As such it is very important to study chemical modifications of AS-183 as a lead compound to attempt to develop novel anti-arteriosclerosis drugs.

Acknowledgment

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