AS-186 COMPOUNDS, NEW INHIBITORS OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE FROM *Penicillium asperosporum* KY1635

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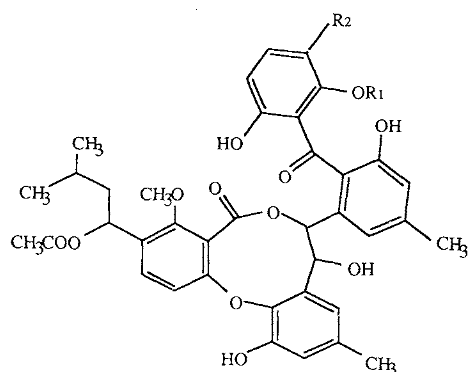
AS-186a, b, c, d, and g were isolated from the cultured broth of *Penicillium asperosporum* KY1635 as inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT). IC_{50} values for the effect of AS-186a, b, c, d, and g against ACAT activity of the microsomes from cholesterol-fed rabbit liver were calculated to be 22.9, 8.2, 11.5, 12.4, and 13.9 μM, respectively. Although AS-186a, and b were identical to penicillide and purpactin A, respectively, AS-186c, d, and g were found to be new compounds.

Acyl-CoA: cholesterol acyltransferase (ACAT, EC2.3.1.26) is a key enzyme responsible for cholesterol storage. We can expect a blockage of cholesterol absorbance in the small intestine, a decrease in cholesterol mass in blood, and a discharge of cholesterol from the body through the inhibition of this enzyme. From the viewpoint of prevention and improvement of atherosclerosis, there are many synthetic inhibitors of ACAT[1]. However, there are only 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]. So we have done a microbial screening work to obtain novel and potent ACAT inhibitors from a natural source. In the present investigation, we have isolated five compounds, designated AS-186a, b, c, d, and g from the culture broth of *Penicillium asperosporum* KY1635. Structural elucidation shows that, though AS-186a and b are identical with penicillide[6] and purpactin A[2], respectively, AS-186c, d, and g are found to be new compounds which are structurally related to purpactin A. This paper deals with the fermentation, purification and isolation of AS-186c, d, and g. Structural elucidation studies will be described in a separate paper.

Fig. 1. The structures of AS-186 compounds.



AS-186c $R_1=H$ $R_2=CH_2CH=C(CH_3)_2$
AS-186g $R_1=H$ $R_2=CH_2CH=C(CH_3)_2$
(diastereomer of AS-186c)
AS-186d $R_1=CH_3$ $R_2=CH(OH)CH_2CH_2CH(CH_3)_2$

Materials and Methods

Materials

[^{14}C] or [^{3}H] compounds were purchased from New England Nuclear. All other chemicals were commercially available and analytical grade.

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Microorganisms and Taxonomy

Penicillium asperosporum KY1635 (FERM BP-3888), isolated from a soil picked up in Kanagawa Prefecture, was used in the present investigation. Most of the taxonomic studies were carried out in accordance with published methods[7].

Fermentation

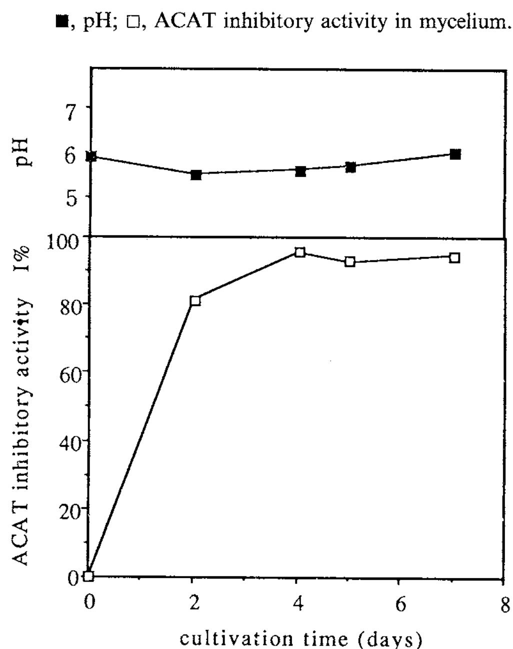
A 50-ml test tube containing 10 ml of a seed medium composed of vegetable juice (Campbell, 20%) and dextrin (3%, pH 6.5 before sterilization) was inoculated with a loopful of spores of the strain grown on an agar slant. After cultivation for five days on a reciprocating shaker (300 rpm) at 25°C, 5 ml 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 vegetable medium was added to a 300-ml Erlenmeyer flask containing 50 ml of a fermentation medium composed of glucose (0.5%), dried mashed potato (2%), peptone (0.5%, Kyokutou), KH_2PO_4 (0.5%), $Mg_3(PO_4)_2 \cdot 2H_2O$ (0.05%, pH 6.0 before sterilization) and incubated for 7 days at 25°C. The production of AS-186 compounds was traced by measurement of the inhibition of ACAT activity. For this measurement, 2 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 2 ml of methanol and the extract was concentrated to one-fifth volume *in vacuo*. Ten microliters of the methanol solution was provided for the assay.

ACAT Assay Using Rabbit Liver Microsomes

The methods used for a preparation of rabbit liver microsomes[8] and the measurement of ACAT activity[9] were described in the previous paper[10]. Briefly, the assay 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 plates (LK6D, Whatman) using *n*-hexane - diethyl ether - 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 a liquid scintillation counter.

Fig. 2. Cultivation of *Penicillium asperosporum* KY-1635 in 300-ml Erlenmeyer flask.

■, pH; □, ACAT inhibitory activity in mycelium.



HPLC Analysis of AS-186 Compounds

AS-186 compounds were detected by an HPLC system consisting of a Gilson Model 303 pump, Gilson Model 1001 UV detector, and Shimadzu C-R6A Chromatopac. AS-186 compounds dissolved in solvent was injected onto an octadecylated silica gel column (YMC, AM-313, C18, i.d. 6 × 250 mm) and developed with 85% methanol solution at a flow rate of 1.5 ml/minute at room temperature, monitoring UV absorbance at 254 nm.

Results

Production of AS-186 Compounds by Fermentation

The time course of AS-186 compounds production by *Penicillium asperosporum* in 300-ml Erlenmeyer flasks is shown in Fig. 2, AS-186 compound were produced equally in mycelia and in culture supernatant. The amount of AS-186

Fig. 3. Isolation and purification of AS-186a and b.

Culture broth 1.5 liters (300-ml Erlenmeyer flask × 30)
- centrifuged
  - Mycelial cake
    - extracted with CH_3OH (1.5 liters)
    - diluted with H_2O to 6 liters
  - Supernatant

Diaion HP-20 column chromatography (300 ml)
- washed with 50% MeOH (1.5 liters)
- eluted with MeOH (1.5 liters)
- concentrated *in vacuo*
- extracted with EtOAc (300 ml × 3)

EtOAc layer
- concentrated *in vacuo*

Oily material (2.2 g)

SiO_2 gel column chromatography (Merck, Art 7734, 200 ml)
- eluted with 10, 20, 40% acetone in *n*-hexane (1 liter each)
  - Crude AS-186a
  - Crude AS-186b

Prep. HPLC (ODS, i.d. 40 × 250 mm, 80% CH_3OH - H_2O)
- AS-186a 41.5 mg
- AS-186b 24.0 mg

compounds in the cultured broth reached a maximum on day 3 to 4.

Fig. 4. The structure of AS-186a and b.

AS-186b	R = AcO
Purpactin A (Acetylpenicillide)	R = AcO
AS-186a	R = OH
Penicillide	R = OH

Isolation and Purification of AS-186a and b

The purification procedure for AS-186a and b is outlined in Fig. 3. The cultured broth (total 1.5 liters) was centrifuged at 8,000 rpm for 10 minutes. The resulting mycelial cake was extracted with 1.5 liters of methanol. The methanol-extracted solution was diluted with 6 liters of de-ionized water and applied to a Diaion HP-20 resin (Mitsubishi Chemical Industries Ltd., 300 ml) column. The supernatant of the cultured broth was also applied to another HP-20 resin column of the same size. The columns were washed with 50% methanol, and the AS-186 compounds were eluted with methanol. The eluate was combined and concentrated under reduced pressure *in vacuo* to yield an aqueous solution. The resultant aqueous solution was extracted three times with the same volume of ethyl acetate. The ethyl acetate layer was concentrated *in vacuo* to yield an oily

black material (2.2 g). This oily material was applied to silica gel (Merck, Art. 7734, Si60, 200 ml) column for chromatography. The adsorbed materials were successively eluted with 1 liter each of 10%, 20%, 40% acetone in *n*-hexane, and acetone. Individual fractions containing AS-186a and b were concentrated *in vacuo* to yield yellow oily materials (45 mg and 56 mg). The oily materials containing AS-186a (4 mg/chromatography) and AS-186b (10 mg/chromatography) were then subjected to preparative HPLC

Fig. 5. Detection of AS-186-related minor compounds.

(A): HPLC charts of side fractions of silica gel column chromatography peaks. Conditions for HPLC were shown in the figure.

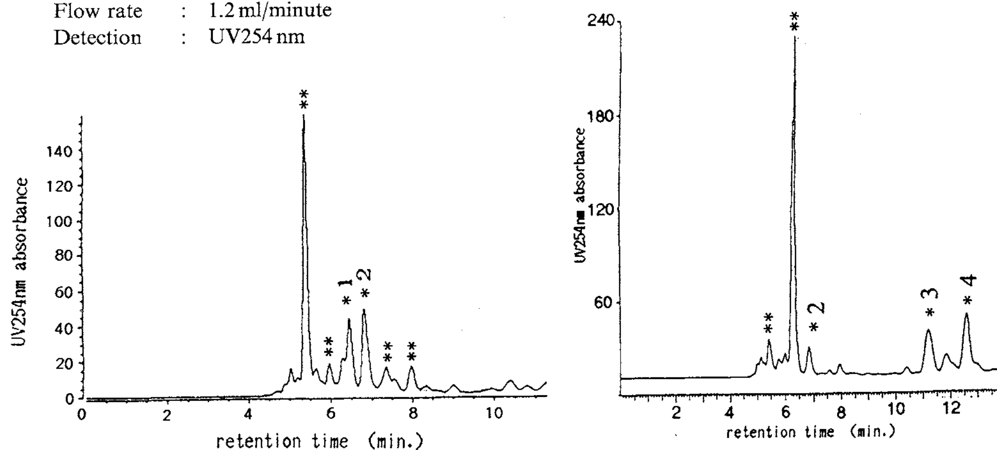
*, Peaks having similar UV spectra to those of AS-186a and b; **, peaks having different UV spectra from those of AS-186a and b.

(B): UV spectra of peaks 1, 2, 3, and 4.

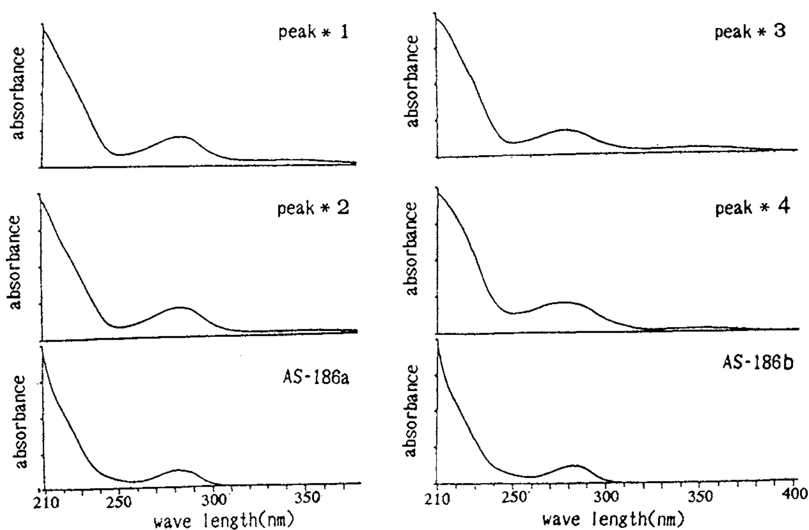
(A)

HPLC conditions

Column	:	YMC, ODS, AM-313, i.d. 6 × 250 mm
Mobile phase	:	80% CH_3OH
Flow rate	:	1.2 ml/minute
Detection	:	UV254 nm



(B)



(YMC, SH-363-10, C18, i.d. 40 × 250 mm, 80% methanol). The elution of the AS-186 compounds were monitored with UV254 nm absorption. The main peak fractions were collected and concentrated to dryness *in vacuo*, yielding white powdered AS-186a and b. Structural elucidation studies based on [^{13}C and ^{1}H] NMR and MS measurements showed that AS-186a and b were penicillide and purpactin A, respectively.

Screening and Detection of AS-186 Minor Compounds

TOMODA *et al.*[2] have already isolated purpactins which are identical to AS-186a and b, as ACAT inhibitors from the cultured broth of *Penicillium purpurogenum.* The production of AS-186a and b by *Penicillium asperosporum* KY1635 in the present study is 10-fold higher than that by *Penicillium purpurogenum.* We therefore thought there was a possibility that minor compounds related to purpactins are also produced in the same cultured broth. Thus side fractions of the silica gel column chromatography peaks were subjected to HPLC (YMC, AM-313, C18, i.d. 6 × 250 mm, 80% methanol, UV 254 nm), and their UV spectra were monitored with a photo diode array UV-VIS detector (Hewlett Packard, HP-1090). By this method, we detected at least 4 additional compounds, which have similar UV spectra to those of

Fig. 6. Purification and isolation of AS-186c, d, g.

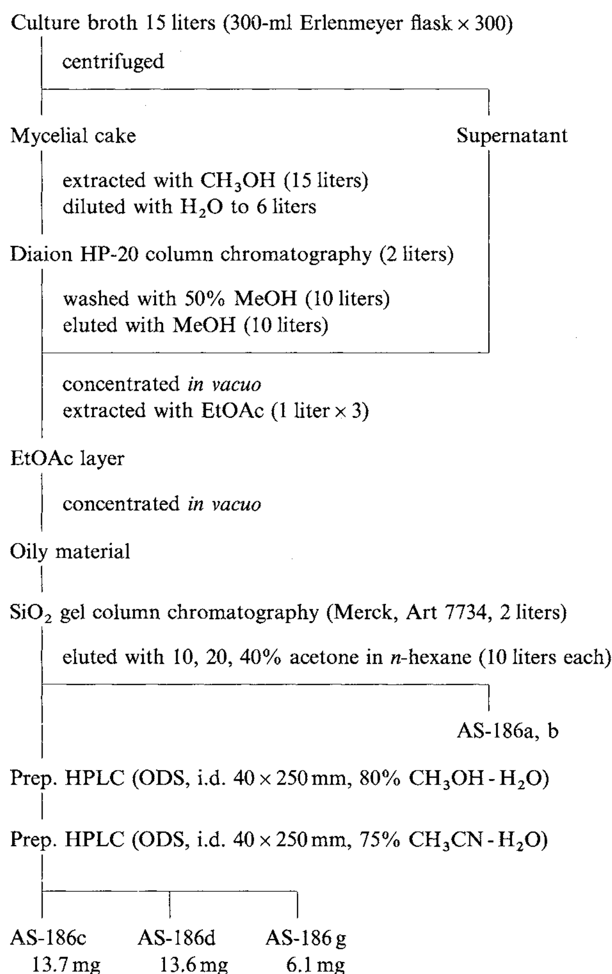


Table 1. The physico-chemical properties of AS-186 compounds.

	AS-186c	AS-186g	AS-186d
Appearance	Yellow powder	Yellow powder	Yellow powder
Molecular formula	$C_{43}H_{46}O_{12}$	$C_{43}H_{46}O_{12}$	$C_{44}H_{50}O_{13}$
SI-MS (*m/z*)	777 $(M+Na)^+$	777 $(M+Na)^+$	769 $(M-H_2O+H)^+$
$[\alpha]_D$ (CH_3OH)	−8.5° (*c* 0.2)	−80.1° (*c* 0.1)	−142.8° (*c* 0.1)
Color reaction			
Positive:	I_2, H_2SO_4, anisaldehyde	I_2, H_2SO_4, anisaldehyde	I_2, H_2SO_4, anisaldehyde
Negative:	Aniline-phthalate, Ninhydrin, Rydon-Smith	Aniline-phthalate, Ninhydrin, Rydon-Smith	Aniline-phthalate, Ninhydrin, Rydon-Smith
Solubility			
Soluble:	MeOH, EtOAc, acetone, $CHCl_3$, CH_3CN, DMSO	MeOH, EtOAc, acetone, $CHCl_3$, CH_3CN, DMSO	MeOH, EtOAc, acetone, $CHCl_3$, CH_3CN, DMSO
Insoluble:	*n*-Hexane, H_2O	*n*-Hexane, H_2O	*n*-Hexane, H_2O
UV λ_{max} (CH_3OH, nm)	286 (ε=18,400), 365 (ε=3,100)	285 (ε=17,600), 370 (ε=2,900)	278 (ε=13,800) 351 (ε=4,300)
IR (KBr, cm^{-1})	3438, 1743, 1616, 1603, 1471, 1425, 1257, 1232, 1045	3437, 1743, 1604, 1600, 1471, 1421, 1254, 1047	3425, 1739, 1614, 1587, 1469, 1271, 1230, 1194, 1049

purpactins, designated AS-186c, d, e, and f (Fig. 5).

Purification and Isolation of AS-186 Minor Compounds

A large-scale cultivation of *Penicillium asperosporum* KY1635 in 300-ml Erlenmeyer flasks was done. AS-186-related minor compounds were extracted and purified from 15 liters of the cultured broth firstly with HP-20 and silica gel column chromatography as described above. The active fractions from silica gel column chromatography were subjected to HPLC analysis. AS-186a, b, c, and d were detected and another new compound that had similar UV spectrum to those of AS-186c and d, designated AS-186g, was also detected. AS-186e and f were not detected in this cultivation. Further purification of AS-186c, d, and g was performed by a repetition of the preparative HPLC using 80% methanol in H_2O and 75% acetonitrile in H_2O as solvent systems. The purification procedure is summarized in Fig. 6.

Physico-chemical Properties

The physico-chemical properties of AS-186 compounds are summarized in Table 1. AS-186 compounds are readily soluble in methanol, acetone, ethyl acetate, chloroform, acetonitrile and dimethyl sulfoxide, and are virtually insoluble in water and *n*-hexane. The structures of the AS-186 compounds were elucidated (Fig. 1) on the basis of [^1H and ^{13}C] NMR spectra data and physico-chemical analysis. AS-186c, d, and g are found to be new compounds sharing a nine-membered ring structure. Details of these structural studies will be described in a separate paper.

Biochemical Properties

AS-186a, b, c, d, and g inhibited microsomal ACAT activity with IC_{50} values (the concentration causing 50% inhibition of maximally stimulated enzyme activity) of 22.9, 8.2, 11.5, 12.4, and 13.9 μM, respectively.

Discussion

In this paper, we described that *Penicillium asperosporum*, produced a series of ACAT inhibitors designated AS-186a, b, c, d, and g. Though AS-186a and b were identical to penicillide[6] and purpactin A[2], respectively, AS-186c, d, and g were found to be new compounds that have nine-membered ring

structures.

There are many synthetic ACAT inhibitors having urea or amide moieties[1], and some of these have been under clinical or pre-clinical evaluation, but only a few compounds are known as metabolites of microorganisms[2~5] or are other types of natural products[11]. It is noteworthy that the structures of the AS-186 compounds are 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 walls, from the viewpoint of prevention and improvement of arteriosclerosis. AS-186 compounds will be good tools for chemical synthetic works to aim at development of therapeutic agents.

Acknowledgment

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