ACATERIN, A NOVEL INHIBITOR OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Pseudomonas* sp. A92

SHIN NAGANUMA, KAORU SAKAI, KEIJI HASUMI
and AKIRA ENDO

Department of Applied Biological Science, Tokyo Noko University,
Fuchu-shi, Tokyo 183, Japan

(Received for publication March 13, 1992)

Acaterin, a novel inhibitor of acyl-CoA : cholesterol acyltransferase (ACAT), was isolated from a culture broth of *Pseudomonas* sp. A92 by Diaion HP-20 column chromatography, solvent extraction and reverse phase HPLC. Spectroscopic analyses of the compound yielded 3-(1-hydroxyoctyl)-5-methyl-2(5*H*)-furanone as the proposed structure. In the presence of oxidized low density lipoprotein, acaterin inhibited the synthesis of cholesteryl ester in macrophage J774 by 50% at a concentration of 45 μM. Acaterin also inhibited ACAT activity in the rat liver microsomes by 50% at a concentration of 120 μM. Kinetic studies suggested that inhibition of ACAT by acaterin was noncompetitive with respect to oleoyl-CoA.

Macrophages play an important role in the accumulation of cholesteryl esters in arterial wall.[1,2] The oxidized low density lipoprotein (LDL) is involved in the process which results in the formation of foam cells.[3~5] In the course of our screening program for inhibitors of cholesteryl ester synthesis in macrophages in the presence of oxidized LDL, we isolated an active compound designated acaterin (Fig. 1) from culture broth of *Pseudomonas* sp. A92. In the present report, taxonomy of the producing strain, fermentation, isolation, characterization and biological activities of acaterin are described.

Taxonomy of Producing Organism

The producing strain *Pseudomonas* sp. A92 was isolated from a soil sample collected in Itsukaichi Town, Tokyo and identified by taxonomic studies which were based on the volume 1 of BERGEY'S Manual of Systematic Bacteriology.

Fermentation

A medium for both inoculation and production was composed of glucose 1%, corn starch 3%, peptone 0.5%, soybean meal 1%, yeast extract 0.5%, $CaCO_3$ 0.2%. Seed medium (100 ml in 500-ml Sakaguchi flasks) was inoculated with cell scrapings

Fig. 1. Structure of acaterin.

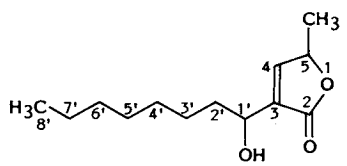
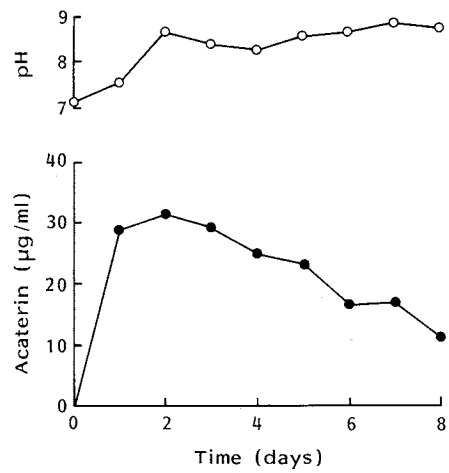


Fig. 2. Time course of the fermentation of acaterin. (○) pH, (●) acaterin.



from slant culture and incubated on a reciprocal shaker (200 rpm) at 28°C for 2 days. One ml portion of the seed culture was inoculated into the same medium (100 ml in 500-ml Sakaguchi flasks), which was then incubated under the same conditions and harvested after 5 days. A typical time course of the fermentation is shown in Fig. 2, where production of acaterin was measured by reverse phase HPLC under the following conditions: Column; Inertsil PREP-ODS (6 × 250 mm), solvent; CH_3CN - H_2O (3 : 2), flow rate; 1 ml/minute, detection; UV at 220 nm. Under these conditions, acaterin was eluted at Rt 14.5 minutes.

Isolation

The fermentation broth (2.8 liters) was centrifuged at 12,000 × *g* for 15 minutes. The supernatant was adjusted to pH 8.0 by the addition of 28 ml of 1 M sodium phosphate buffer (pH 8.0) and applied to a Diaion HP-20 column (150 ml). The column was washed with 1.5 liters of 10 mM sodium phosphate buffer (pH 8.0) and then eluted with 1.5 liters of methanol. The eluted fraction was evaporated and lyophilized to give 2.85 g of a brown solid. The solid was dissolved in 500 ml of water, adjusted to pH 3.0 with HCl and then extracted with 500 ml of ethyl acetate (three times). The organic solvent extract was concentrated under reduced pressure to yield 714 mg of brown oil. The oil was dissolved in CH_3CN - H_2O (3 : 2) and purified by reverse phase HPLC under the following conditions: Column; Inertsil PREP-ODS (30 × 250 mm), solvent; CH_3CN - H_2O (3 : 2), flow rate; 25 ml/minute, detection; UV at 220 nm. The active fractions were extracted with ethyl acetate. The solvent layer was dried with sodium sulfate and evaporated to yield 20.7 mg of pure acaterin.

Physico-chemical Properties and Structural Elucidation

Acaterin was obtained as light brown oil. The physico-chemical properties of acaterin are summarized in Table 1. The molecular formula of acaterin was determined to be $C_{13}H_{22}O_3$ from the results of SI-MS (*m/z* 227, $(M+H)^+$) and HREI-MS (calcd: *m/z* 208.1464, found: *m/z* 208.1425, $(M-H_2O)^+$).

Table 1. Physico-chemical properties of acaterin.

Appearance	Light brown oil
Molecular formula	$C_{13}H_{22}O_3$
SI-MS (*m/z*)	227 $(M+H)^+$
HREI-MS (*m/z*)	
Calcd:	208.1464
Found:	208.1425 $(M-H_2O)^+$
UV λ_{max}^{EtOH} nm (ε)	207 (28,800), 280 (800)
IR ν_{max} (KBr) cm^{-1}	3450, 2920, 2850, 1740, 1450, 1370, 1320, 1200, 1110, 1080, 1020

Fig. 3. ^{13}C NMR spectrum of acaterin (67.9 MHz, pyridine-d_5).

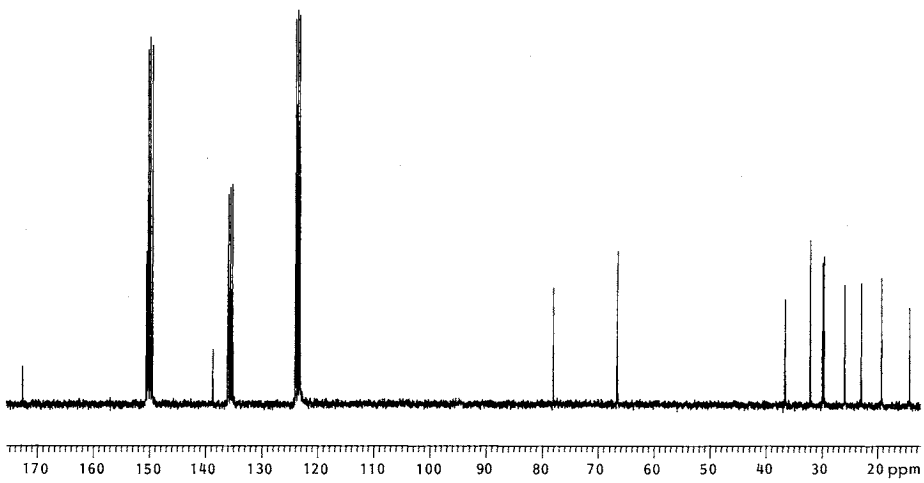
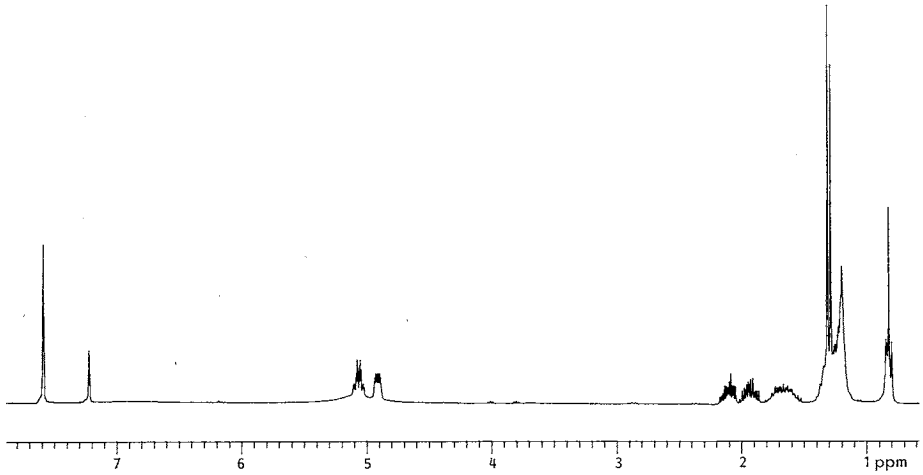


Fig. 4. ^{1}H NMR spectrum of acaterin (270 MHz, pyridine-d_5).



Absorption band at 1740 cm^{-1} in the IR spectrum and both intense UV absorption maximum at 207 nm and weak absorption at 280 nm (Table 1) suggested the presence of an α,β-unsaturated γ-lactone. The ^{13}C NMR and ^{1}H NMR spectra are shown in Figs. 3 and 4, respectively. The decoupled ^{13}C NMR spectrum of acaterin revealed the presence of 13 carbon atoms. The number of protons attached to each carbon was determined by measurement with DEPT. When ^{13}C NMR spectrum was measured in DMSO-d_6 after the addition of a drop of D_2O, the signal of δ_C 66.47-carbon was broadened. From these data as well as the physico-chemical properties, each signal in ^{13}C NMR spectrum was assigned as follows: 2 methyl carbons at δ_C 14.24 and 19.19, 6 methylene carbons at around δ_C 22.87~36.49, 1 methine carbon bearing a hydroxyl group at δ_C 66.47, 1 oxygen bearing methine carbon at δ_C 77.91, 1 olefinic methine carbon at δ_C 150.55, 1 olefinic quaternary carbon at δ_C 138.63 and 1 carbonyl carbon due to a γ-lactone ring at δ_C 172.71 (Table 2). The ^{1}H NMR spectrum exhibited resonances for 22 protons including 1 doublet and 1 triplet methyl protons at δ_H 0.82 and 1.30, 1 olefinic proton at δ_H 7.59 and 2 oxygen bearing methine protons at δ_H 4.91 and 5.06. Thus assignments of signals determined by ^{13}C and ^{1}H NMR spectra and ^{13}C-^{1}H COSY experiment are summarized in Table 2.

Table 2. ^{13}C NMR (67.9 MHz, pyridine-d_5) and ^{1}H NMR (270 MHz, pyridine-d_5) data for acaterin.

Carbon No.	Chemical shift (δ)	
	^{13}C	^{1}H
C-2	172.71	
C-3	138.63	
C-4	150.55	7.59 (1H, t, J=1.6)
C-5	77.91	5.06 (1H, ddq, J=1.6, 2.1, 6.9)
C-5-CH_3	19.19	1.30 (3H, d, J=6.9)
C-1′	66.47	4.91 (1H, dddd, J=1.6, 2.1, 3.9, 8.0)
C-1′-OH		~5.1 (1H, br s)
C-2′	36.49	2.11 (1H, dddd, J=3.9, 6.2, 10.1, 13.2)
		1.92 (1H, dddd, J=5.0, 8.0, 10.1, 13.2)
C-3′	25.82	1.66 (2H, m)
C-4′	29.81	~1.3 (2H, m)
C-5′	29.54	~1.25 (2H, m)
C-6′	32.03	~1.2 (2H, m)
C-7′	22.87	1.20 (2H, m)
C-8′	14.24	0.82 (3H, t, J=6.6)

Spectra were measured in pyridine-d_5 at 25°C. TMS was used as an internal reference (δ 0.00). J=Hz.

The partial structures were determined by ^{1}H-^{1}H COSY and heteronuclear multiple-bond correlation (HMBC) experiments. The lower field resonances of olefinic signals (δ_H 7.59, δ_C 150.55) in the NMR spectra suggested that this proton is attached to the β-carbon on the double bond to a lactonic carbonyl

Fig. 5. Long-range coupling observed in the HMBC experiment of acaterin.

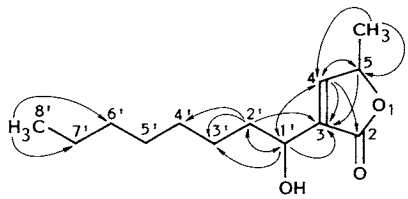
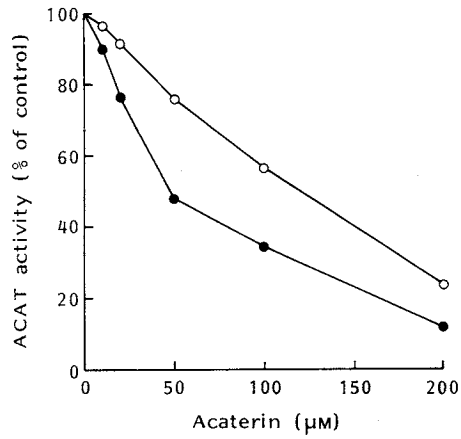


Fig. 6. Inhibition of ACAT activity by acaterin.

(●) macrophage assay, (○) microsomal assay. Control activities were: 1.75 nmol/mg protein for macrophage assay; 1.72 nmol/minute/mg protein for microsomal assay. Each value represents the average of duplicate determinations which varied less than 10%.



group. Furthermore, 4-H proton had an allylic coupling ($J=1.6$ Hz) with a methine signal at 1'-H (δ_H 4.91) and had a *vicinal* coupling ($J=1.6$ Hz) with an oxygen bearing methine proton at δ_H 5.06 which was substituted by a methyl group. These findings suggested that acaterin had a 3,5-disubstituted-furanone.

The structure of a long side chain attached to C-3 of the α,β-unsaturated γ-lactone ring was confirmed by HMBC experiment as shown in Fig. 5. The methylene protons of 2'-CH_2 (δ_H 1.92 and 2.11) showed couplings to C-3, C-1', C-3' and C-4' and the methyl signal of 8'-CH_3 (δ_H 0.82) was coupled to C-6' and C-7'. The remaining methylene carbon at δ_C 29.54 was therefore assigned to C-5'.

Based on the above informations, the structure of acaterin was established to be 3-(1-hydroxyoctyl)-5-methyl-2(5*H*)-furanone as illustrated in Fig. 1.

Biological Activities

Effect on Synthesis of Cholesteryl Ester in J774 Macrophages

Mouse macrophage cell line J774A.1 was obtained from Japanese Cancer Research Resources Bank. The cells were maintained in monolayer in medium A (DULBECCO's modified EAGLE's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% fetal bovine serum (FBS). On day 0, 1.5×10^5 cells were inoculated into 24-well dishes (Nunc) in 0.4 ml medium A supplemented with 10% FBS and incubated at 37°C for 24 hours. On day 1, the monolayers were washed with phosphate-buffered saline, received medium B (medium A supplemented with lipoprotein-deficient FBS) and incubated at 37°C for 24 hours. On day 2, the cells were incubated at 37°C in 0.3 ml of fresh medium B containing 0.1 mM [1-^{14}C]oleate (10,000 dpm/nmol) in complex with 1.2 mg/ml bovine serum albumin (BSA),[6] 100 μg protein/ml of oxidized LDL[7] and various amounts of acaterin. After incubation for 3 hours, the cells were extracted with hexane-isopropanol (3:2). The extracts were assayed for cholesteryl [^{14}C]oleate using thin-layer chromatography.[6]

The synthesis of cholesteryl [^{14}C]oleate in J774 cells was inhibited by 50% at a concentration of 45 μM acaterin (Fig. 6). Incorporation of [^{14}C]oleate into triacylglycerol under the same conditions and the metabolism, which included binding on the cell surface, internalization and lysosomal degradation, of oxidized ^{125}I-LDL were not inhibited by acaterin (data not shown).

Effect on ACAT Activity of Rat Liver Microsomes

ACAT activity was assayed using microsomes prepared from rat liver.[8] The standard reaction mixture

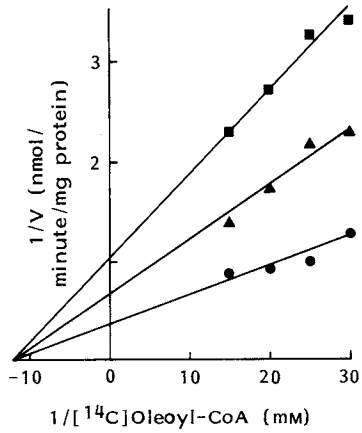
Table 3. Effects of washing on the ACAT inhibition by acaterin.

Microsomal washing (times)	ACAT activity in microsomes (nmol/mininute/mg protein)		Recovery of ACAT activity (%)
	Control	Acaterin-treated	
0	1.60	0.04	2.5
1	1.55	1.24	80.0
2	1.61	1.27	78.9
3	1.35	1.26	93.3

Each value represents the average of duplicate determinations which varied less than 10%.

Fig. 7. Lineweaver-Burk plot of acaterin inhibition of microsomal ACAT activity.

(●) No acaterin, (▲) 100 μM acaterin, (■) 200 μM acaterin. Each value represents the average of duplicate determinations which varied less than 10%.



contained the following components in a final volume of 100 μl: 150 mM potassium phosphate buffer (pH 7.4); 80 μM BSA (fatty acid-free); 100 μM [1-^{14}C]oleoyl-CoA (10,000 dpm/nmol); and 2 mg/ml microsomal protein. The mixture was preincubated for 5 minutes at 37°C. The reaction was initiated by adding microsomal protein which had been preincubated at 37°C for 5 minutes. After incubation for 1 minute, reaction was stopped by adding 500 μl of ethanol and then lipids were extracted with 1.5 ml of hexane. The extracts were assayed for cholesteryl [^{14}C]oleate as described above.

As shown in Fig. 6, ACAT activity in rat liver microsomes was inhibited by acaterin. The IC_{50} value was measured to be 120 μM.

Reversibility of Acaterin Inhibition of ACAT

Rat liver microsomes (2 mg) were incubated at 37°C in 800 μl of 150 mM potassium phosphate buffer in the absence or presence of 0.5 mM acaterin. After incubation for 15 minutes, 80 μl aliquots were removed (in duplicate) from both mixtures. These aliquots were added to 20 μl of 150 mM potassum phosphate buffer, 0.4 mM BSA and 0.5 mM [^{14}C]oleoyl-CoA and then assayed for ACAT activity as described above. The remaining mixtures were washed by combining with 2.5 ml of 150 mM potassium phosphate buffer and 80 μM BSA, incubating for 5 minutes at 37°C, and then centrifuging at 100,000 $\times g$ for 1 hour at 4°C. The pellet was resuspended in 640 μl of 150 mM potassium phosphate buffer, and aliquots (80 μl) were removed and assayed for ACAT activity. The washing procedure was repeated three times.

As shown in Table 3, ACAT activity in microsomes previously incubated with acaterin was recovered by washing. The result indicated that the inhibition of ACAT by acaterin was reversible.

Kinetic Analysis of Inhibition of ACAT by Acaterin

ACAT assays were carried out as described above, except that concentrations of [^{14}C]oleoyl-CoA were varied as indicated. A Lineweaver-Burk plot of this experiment is shown in Fig. 7, which suggested that the inhibition of ACAT by acaterin was noncompetitive with respect to the substrate oleoyl-CoA. However, this result should be carefully interpreted, since kinetic analysis of ACAT using crude microsomal preparation bears some difficulties.[9]

Discussion

In the present experiments, we have isolated a novel compound, designated acaterin, from *Pseudomonas* sp. A92 as an inhibitor of ACAT. Its structure was identified to be a furanone bound to a hydrophobic side chain. Acaterin is the first inhibitor of ACAT isolated from bacteria.

Several compounds have been reported as inhibitors of ACAT which include 57-118,[10] DL-melinamide,[11] CL 277,082[9] and purpactins.[12] Of these compounds, 57-118 and DL-melinamide is a competitive inhibitor and an uncompetitive inhibitor of the enzyme, respectively, while acaterin as well as CL 277,082 is a noncompetitive inhibitor.

Acaterin has been shown to inhibit cholesteryl ester synthesis in macrophage J774 which was incubated with oxidized LDL. Since oxidized LDL is implicated in the accumulation of cholesteryl esters and foam cell formation of macrophages, it is proposed that acaterin may be effective in suppressing foam cell formation and thus atherosclerotic lesion development in arterial wall.

Acknowledgment

The skilful technical assistance of Messrs C. SHINOHARA, K. NUMATA and K. TAKIZAWA and Misses J. KOSHIZAWA and R. OHIRA is gratefully acknowledged. The authors thank Dr. S. MURAKAWA for helpful comments and suggestions. This work was supported in part by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture and a Research Grant for Cardiovascular Diseases from the Ministry of Health and Welfare.

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