Epi-cochlioquinone A, a Novel Acyl-CoA : Cholesterol Acyltransferase Inhibitor Produced by *Stachybotrys bisbyi*

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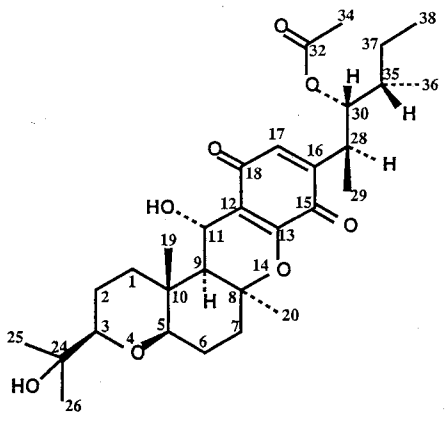
A novel acyl-CoA : cholesterol acyltransferase (ACAT) inhibitor, designated epi-cochlioquinone A has been isolated from the fermentation broth of *Stachybotrys bisbyi* SANK 17777. The molecular formula, physicochemical properties, NMR spectroscopic analysis and X-ray crystallographic analysis revealed that this compound was a stereoisomer of cochlioquinone A, which has been previously reported as a nematocidal agent. It inhibited ACAT activity in an enzyme assay using rat liver microsomes with an IC_{50} value of 1.7 μM. However, it showed about 10-fold less potent inhibitory effect on plasma lecithin cholesterol acyltransferase (LCAT) than on ACAT. In addition, it inhibited *in vivo* cholesterol absorption in rats by 50% at 75 mg/kg.

Acyl-CoA : cholesterol acyltransferase (ACAT) plays a critical role both in cholesterol absorption in the intestine and cholesterol ester formation in the liver and peripheral tissues[1]. Therefore, ACAT inhibitors would be expected to reduce serum cholesterol levels by means of inhibition of cholesterol absorption in the intestine and/or VLDL cholesterol secretion from the liver, and to regress the arterial lesions by means of inhibition of lipid accumulation. In the course of screening of compounds which inhibit cholesteryl ester formation using rat liver microsomes, we isolated a new ACAT inhibitor, named epi-cochlioquinone A, from the fermentation broth of *Stachybotris bisbyi*. In this paper, we describe the taxonomy and fermentation of the producing organism, as well as the isolation, physicochemical properties, structure determination, and biological properties of epi-cochlioquinone A.

Fig. 1. The structure of epi-cochlioquinone A.



Materials and Methods

Materials

[1-^{14}C]oleoyl-CoA (2.2 Gbq/mmol), [1, 2, 6, 7-^{3}H]cholesteryl oleate (3.7 Tbq/mmol), [1-^{14}C]oleate (2.2 Gbq/mmol), [1,2-^{3}H]cholesterol (3.7 Tbq/mmol) were obtained from Dupont NEN. DALBECCO's modified EAGLE's medium (DMEM) was from Nissui Pharmaceutical Corp., and fetal bovine serum (FBS) was from Gibco. Fatty acid-free bovine serum albumin (BSA) was obtained from Sigma. All other chemicals were of the highest grade commercially available.

Spectroscopic Studies

The UV spectrum was recorded on a Shimadzu UV-200S spectrophotometer. IR spectral data was recorded using a JASCO FT-IR 5SXC infrared spectrometer. Optical rotation was obtained with a JASCO DIP-370 digital polarimeter. The EI-MS spectrum recorded on a

JEOL JMS-D100 mass spectrometer at 20 eV. NMR spectra were obtained on a JEOL JNM-GX-400 spectrometer at 400 and 125 MHz field strengths for ^{1}H and ^{13}C nuclei, respectively.

Single Crystal X-Ray Analysis

Epi-cochlioquinone A was recrystalized from isopropylether. The yellow plate crystal having an approximate dimension of $0.7 \times 0.3 \times 0.15$ mm was mounted on a glass fibre . All measurements were made on a Rigaku AFC-5R diffractometer with graphite monochromated Cu*K*a radiation. The data were collected at a temperature of 25°C using a w-2q scan technique to a maximum 2q value of 130°. Pertinet crystal, data collection, and refinement parameters are summarized in Table 4. The structure was solved by direct methods using MULTAN 78[2], and refined by the block diagonal least-squares technique with anisotropic thermal parameters. Atomic scattering factors were taken from the previous report[3]. Calculations were carried out with the DIRECT-SEARCH program system.

Assay of Microsomal ACAT Activity

Microsomes prepared from rat liver were used as a source of ACAT and the activity was determined as described elsewhere[4] with modifications. Experimentally, rat liver microsomes (0.3~0.5 mg protein) and 1 mg of BSA in 0.1 M potassium-phosphate buffer (pH 7.4) containing 1 mM EDTA (final volume 0.5 ml) were preincubated for 5 minutes at 37°C. The reaction was started by the addition of 25 nmol (53.7 kBq) of [^{14}C]oleoyl-CoA. The assay was stopped after 6 minutes by adding 0.5 ml of ethanol and [^{3}H]cholesteryl oleate (0.37 kBq) was added as internal standard to estimate recovery. Lipids were extracted with 4 ml of *n*-hexane. The extract was subjected to thin-layer chromatography together with unlabeled cholesteryl oleate as a marker. The chromatogram was developed in petroleum ether-diethyl ether-acetic acid 130:10:1 (v/v/v). The cholesteryl oleate zone was visualized with iodine vapor and scraped off. The radioactivity was counted using a liquid scintillation counter.

Cholesteryl Ester Formation in J774 Macrophage

Human LDL ($1.019 < d < 1.063$) and lipoprotein-deficient serum ($d > 1.25$) were prepared from plasma of healthy normolipidemic subjects by sequential ultracentrifugation[5]. J774 cells were maintained in monolayer in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% heat-inactivated FBS. On day 0, 1.0×10^{6} cells were inoculated into 6-well plates (Corning) in 2 ml medium supplemented with 10% FBS and incubated at 37°C for 18 hours in a humidified 5% CO_2/95% air atomosphere. On day 1, each monolayer was washed with phosphate-buffered saline, and incubated in DMEM (supplemented with 5% lipoprotein-deficient serum) with 200 nmol [1-^{14}C]oleate (5,000 dpm/nmol) in complex with 2.5 mg BSA by sonication[6], 100 μg/ml LDL and various amounts of epi-cochlioquione A dissolved in dimethylsulfoxide. After incubation for 3 hours, the cells were extracted with hexane-isopropanol (3:2) and the amount of cholesteryl [1-^{14}C]oleate formed was determined using thin-layer chromatography as described elsewhere[6].

Assay of Plasma LCAT Activity

LCAT activity was assayed by the method of STOKKE and NORUM[7].

Measurement of Cholesterol Absorption in Rats

Groups of 4 male Sprague-Dawley rats (6 weeks old, Charles River, Japan) were fed on a 1.5% cholesterol and 0.5% cholate-supplemented chow diet for 3 days. On day 3, varying doses of epi-cochlioquinone A were orally administered as a suspension in a 1% Tween 80 solution. [1,2-^{3}H]Cholesterol (1.85 MBq) was orally administered at 30 minutes after the drug dosage. Blood was withdrawn by cardiac puncture at 4 hours after the isotope dosing. Serum was prepared by centrifugation, and radioactivity in serum was determined.

Results

Taxonomy

Epi-cochlioquinone A producing strain SANK 17777 was isolated from soil sample collected in Okinawa. The mycological properties of the strain SANK 17777 were observed to be as follows. Growth on oat meal agar plate was rapid, covering a Petri dish plate in 2 weeks at 25°C. The texture of the colony was velvety to a fine powdery form, salmon-pink in color; at the center of the colony a downy white mycelium was developed. Conidiophores were determinate, produced solitary or in groups. They were straight, simple or branched, composed of 2 to 5 cells. The surface of the conidiophores were sometimes minutely rough-walled. Phialides were born at the tip of condiohiores, produced in a whorl of 3 to 8 in number. They were unicellular, subclavate, hyaline, smooth-walled, $10 \sim 17 \times 4 \sim 6$ μm, with conspicuous collarettes. Phialoconidia were produced in slimy masses on phialides and were hyaline, smooth-walled, one-celled, lemon-shaped to fusiform. Based on the properties stated above, SANK 17777 was identified as *Stachybotrys bisbyi* (Srinivasan) Barron[8].

Fermentation

A slant culture of *Stachybotrys bisbyi* grown on WSH agar (crushed oat meal 1.0%, $NaNO_3$ 0.1%, KH_2PO_4 0.1%, $MgSO_4$ ($7H_2O$ 0.25% and agar 2.0% without adjustment of pH) was used to inoculate 500-ml buffled Erlenmeyer flasks containing 100 ml of the seed medium

(GPMY; glycerol 5%, potatoes 5%, malt extract 0.5% and yeast extract 0.5% in tap water without adjustment of pH). The flasks were shaken on a rotary shaker for 3 days at 26°C. Two milliliters of the seed culture were transferred into 100 ml of a GPMY medium in 500-ml buffled Erlenmeyer flasks. The flasks were shaken on a rotary shaker for 7 days at 26°C. The production of epi-cochlioquinone A was measured by HPLC under the following conditions: Waters Radial-Pak ODS 8NVC184, 4.6 mm × 150 mm; 55% acetonitrile-H_2O, at 2 ml/minute; UV detection at 210 nm. Epi-cochlioquinone A was eluted with a retention time of 7.0 minutes.

Isolation

After filtration of the cultured broth (1.3 liters), the filtrate was extracted with ethyl acetate in an acidic condition. The mycelium obtained was extracted with 1.3 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was also extracted with ethyl acetate in an acidic condition. The combined organic phase was washed with 3% $NaHCO_3$ and dried over Na_2SO_4. The extracts were concentrated *in vacuo* to dryness to yield a brown oil (294 mg). The crude extract was chromatographed with reverse phase HPLC (Senshu Pak ODS-H-5251, 20 × 250 mm) using acetonitrile-H_2O (65:35) as the eluent at 6.0 ml/minute. The peak of epi-cochlioquinone A was collected, evaporated and recrystallized with a mixture of *n*-hexane and acetone to yield a 90 mg crystal.

Characterization and Structure Determination of Epi-cochlioquinone A

Epi-cochlioquinone A appears as yellow crystal and its physico-chemical properties are summarized in Table 1. The compound is soluble in alcoholic solvents, acetone, ethylacetate, dimethylsulfoxide and chloroform, partially soluble in *n*-hexane, but is insoluble in water.

Table 1. Physico-chemical properties of epi-cochlioquinone A.

Appearance		Yellow crystals
Melting Point		158 ~ 161 °C
$[\alpha]_D$		+43.4° (*c* 1.16, EtOH)
Molecular formula		$C_{30}H_{44}O_8$
EI-MS (m/z)		532 [M^+]
HR-EIMS (m/z)	Calcd	532.3004 (for $C_{30}H_{44}O_8$)
	Found	532.3035
Elemental analysis	Calcd	C 67.65 H 8.33 (for $C_{30}H_{44}O_8$)
	Found	C 68.39 H 8.59
UV λ_{max} nm (log ε) in EtOH		270 (4.01), 386 (3.05)
IR ν_{max} (KBr)cm^{-1}		2971, 1738, 1679, 1650, 1604, 1373, 1240, 1103

The molecular formula of epi-cochlioquinone A was determined to be $C_{30}H_{44}O_8$ on the basis of HREI-MS measurement (*m/z*, found 532.3004, calcd 532.3035). The ^{13}C NMR spectrum ($CDCl_3$) showed 30 resolved peaks (Table 2), which were classified into seven $-CH_3$, five $-CH_2$, three –CH–, four –O–CH–, one –CH= and ten quaternary carbons by analysis of DEPT spectra. The ^1H NMR spectrum displayed 44 proton signals (Table 3). These results supported the molecular formula.

The structure of epi-cochlioquinone A is shown in Fig. 1. Physico-chemical properties of epi-cochlioquinone A were similar to those of cochlioquinone A[9]. However, epi-cochlioquinone A had a higher melting point; 158 ~ 161°C for epi-cochlioquinone A versus 130 ~ 132°C for cochlioquione A. In addition, compared with NMR data of cochlioquinone A[8], the methyl protons at the 19-position (10-CH_3, δ_H 0.64) of epi-cochlioquione A showed higher field shift than those of cochlioquinone A (δ_H 1.02). From these data, the two compounds described above seemed to be stereoisomers. The structure of epi-cochlioquinone A was confirmed by single crystal X-ray analysis. X-Ray crystallographic analysis of epi-cochlioquinone A is summarized in Table 4. The non-hydrogen atoms were refined anisotropically. The final cycle of block-diagonal least-squares refinement was based on 2359 observed reflections and 519

Table 2. ^{13}C NMR chemical shifts of epi-cochlioquinone A.

Carbon No.	^{13}C chemical shifts (ppm, 125MHz)[a]	Carbon No.	^{13}C chemical shifts (ppm, 125MHz)[a]
1	36.6	12	119.3
2	21.6	13	152.6
3	84.5	15	181.3
24	71.8	16	149.0
25	26.1	17	133.2
26	23.8	18	188.0
5	83.1	28	34.9
6	23.5	29	16.8
7	36.8	30	78.5
8	79.8	32	170.4
9	52.4	34	20.7
10	35.6	35	36.2
19	11.6	36	13.0
11	59.4	37	26.6
20	26.4	38	11.6

[a] The sample was dissolved in $CDCl_3$. Chemical shifts are shown with reference to TMS as 0 ppm.

Table 3. ^{1}H NMR chemical shifts of epi-cochlioquinone A.

Carbon No.	^{1}H chemical shifts (ppm, 400MHz)[a]		
1	1.35 (1H, dd)	28	3.40 (1H, dd)
	1.99 (1H, dd)	29	1.18 (3H, s)
2	1.46 (1H, m)	16	5.02 (1H, dd)
	1.61 (1H, m)	34	1.92 (3H, s)
	3.20 (1H, dd)	35	1.65 (1H, m)
25	1.18 (3H, s)	36	0.91 (3H, d)
26	1.14 (3H, s)	37	1.18 (1H, m)
5	3.14 (1H, dd)		1.35 (1H, m)
6	1.58 (1H, ddd)	38	0.89 (3H, t)
	1.82 (1H, ddd)	24-OH	
7	1.70 (1H, dd)	11-OH	
	2.36 (1H, dd)		
20	1.37 (3H, s)		
9	1.60 (1H, s)		
19	0.64 (3H, s)		
11	4.61 (1H, s)		
17	6.56 (1H, s)		

[a] The sample was dissolved in $CDCl_3$. Chemical shifts are shown with reference to TMS as 0 ppm.

Table 4. Single crystal X-ray crystallographic analysis of epi-cochlioquinone A.

Crystal parameters		
Emporocal formula		$C_{30}H_{44}O_8$
Formula weight		532.7
Crystal dimensions (mm)		0.7 × 0.3 × 0.15
Crystal system		Monoclinic
Lattice Parameters:		a = 34.337 (6) Å
		b = 6.283 (2) Å
		c = 14.699 (3) Å
		β = 108.94(2) °
		V = 2999 (1) Å^3
Space group		*C*2 with Z=4
Density calc (g/cm^3)		1.18
Linear absorption factor (cm^{-1})		7.0
Refinement parameters		
No. of reflections measured		2820
Nonzero reflections (I>3.00σ)		2359
R-index	Residuals: R[a]	0.044
	Residuals: Rw[b]	0.083

[a] $\Sigma||Fo|-|Fc||/\Sigma|Fo|$, [b] $[\Sigma w(|Fo|-|Fc|)^2/\Sigma wFo)]^{1/2}$.

Fig. 2. Molecular structure of epi-cochlioquinone A in crystalline state.

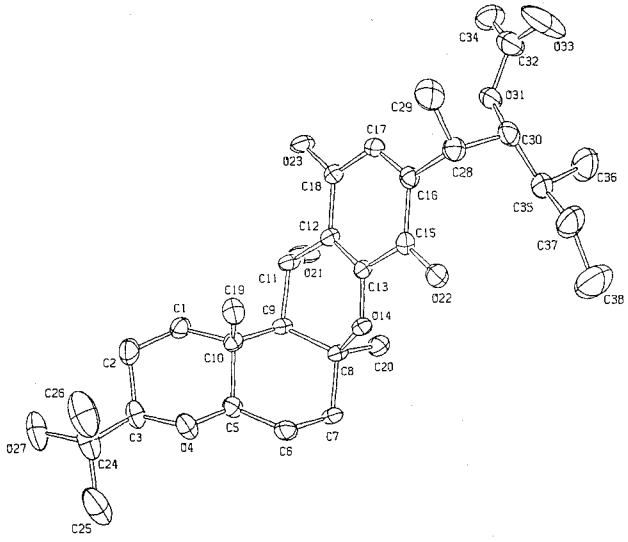
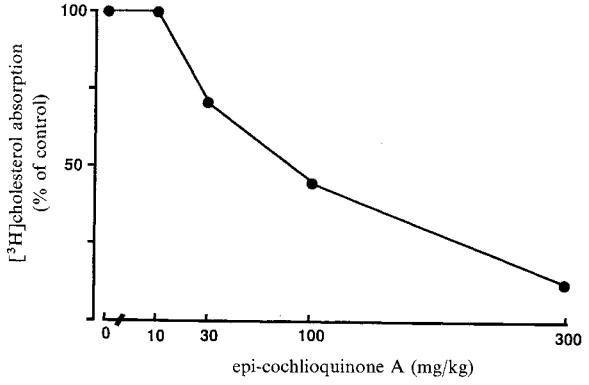


Table 5. Inhibitory effects of epi-cochlioquinone A on ACAT and LCAT activity.

Enzyme		IC_{50} (μM)
ACAT	rat liver microsome	1.7
	intact J774 macrophage	16.0
LCAT		15.8

Fig. 3. Effect of epi-cochlioquinone A on [^{3}H]cholesterol absorption in cholesterol-fed rats.



variable parameters of R = 0.044, Rw = 0.043. Thus, the structure of epi-cochlioquinone A described above has been confirmed and the relative configuration was also determined as shown in Fig. 2. From the result of X-ray crystallographic analysis, the methyl group attached to the C-8 atom of epi-cochlioquinone A was deduced to exist as the cis isomer in relation to the pendant C-9 hydrogen atom, while cochlioquinone A was confirmed

as the trans isomer[10]

Biological Properties

The biological properties of epi-cochlioquinone A are summarized in Table 5. Epi-cochlioquinone A inhibited microsomal ACAT activity by 50% at a concentration of 1.7 μM. It inhibited plasma LCAT activity by 50% at 15.8 μM. It was also evaluated in the cell ACAT assay in J774 macrophages. Cholesteryl ester formation was inhibited in a dose-dependent manner (data not shown) with an IC_{50} value of 16.0 μM. In cholesterol-fed rats, epi-cochlioquinone A inhibited cholesterol absorption with an ED_{50} value of 75 mg/kg (Fig. 3). No acute toxicity was observed up to 2000 mg/kg po in mice.

Discussion

Epi-cochlioquinone A is a stereoisomer of cochlioquinone A, which has a steroid-like structure and was reported to have nematocidal activity[11]. Whether or not cochlioquinone A has ACAT inhibitory activity is unknown, since cochlioquinone A was not isolated from the fermentation broth of *Stachybotrys bisbyi* SANK 17777. Epi-cochlioquinone A did not show nematocidal activity against *Caemarhabditis elegans* at 2 μM (data not shown).

Epi-cochlioquinone A showed about a 10-fold less potent inhibitory effect on plasma LCAT activity than on ACAT. Since LCAT is a critical enzyme for cholesterol reverse transport from peripheral tissues or arterial lesions to the liver[12], the selective inhibition shown by epi-cochlioquinone A is favorable for regression of arterial lesions. Another target organ of ACAT inhibitors is the intestine. Inhibition of ACAT activity in intestine results in suppression of cholesterol absorption followed by reduction of plasma cholesterol levels. Epi-cochlioquinone A inhibited cholesterol absorption in cholesterol-fed rats. An investigation of the plasma cholesterol-lowering effect through a mechanism of inhibition of intestinal ACAT by epi-cochlioquinone A is now in progress.

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