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Phenylpyropene C, a New Inhibitor of Acyl-CoA: Cholesterol Acyltransferase Produced by *Penicillium griseofulvum* F1959

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Acyl-CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.26) is responsible for intracellular esterification of cholesterol and plays a key role in intestinal absorption of cholesterol, hepatic production of lipoproteins[1,2] and accumulation of cholesteryl esters within macrophages and smooth muscle cells of the atheroma[3]. Therefore, ACAT is an attractive target for new treatments of hypercholesterolemia and atherosclerosis[4]. In the course of our search for ACAT inhibitors from microbial sources, phenylpyropene C was isolated from the fermentation broth of *Penicillium griseofulvum* F1959. It was deposited in the Korean Collection for Type Culture (KCTC), Korea, as KCTC 0387BP.

The fungal strain F1959 was originally isolated from a soil sample collected at Ulsan, Korea. Taxonomic studies of the strain were carried out by the method of Samson[5]. Czapek's medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 3.0% sucrose, 1.5% agar) and MEA medium (2.0% malt extract, 0.1% peptone, 2.0% glucose, 2.0% agar) were used in the identification of strain F1959. Colonies on Czapek agar at 25°C were restricted, attaining a diameter of 15~20 mm within 7 days, reverse yellowish to orange-brown. Growth on MEA was rather faster, 20~25 mm in diameter within 7 days, colored yellowish green due to a heavy spolulation. Conidiophores were mononematous or loosely synnematous, especially in the marginal areas, smooth walled, hyaline, irregular terverticilate to quaterverticillate, with branches strongly divergent. Stipes were undulate, phialides were ampulliform, closely packed and exceptionally short, 4.5~6.5 μm, abruptly tapering to short collula. Conidia were ellipsoidal, 3.0~3.5×2.2~2.5 μm, smooth-welled green in color, and borne in closely packed, discovered chains. From these characteristics, strain F1959 was identified as a strain of *Penicillium griseofulvum*.

Penicillium griseofulvum F1959 was inoculated into a 1-liter baffled flask containing 100 ml of seed medium composed of 0.5% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.8 before autoclaving. The culture was incubated for 18 hours at 29°C on a rotary shaker (150 rpm). Twenty milliters of the seed culture was transferred to 5-liter baffled flask containing 1 liter of a medium consisting of 2% soluble starch, 0.4% soytone, 0.3% Pharmamedia, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$ and 0.2% NaCl, pH 6.0 before autoclaving. *Penicillium griseofulvum* F1959 was cultivated at 29°C for 5 days on a rotary shaker at 150 rpm. The fermentation broth (2 liters) of *Penicillium griseofulvum* F1959 was extracted with ethyl acetate (2 liters). The extract was concentrated and then chromatographed on a silica gel column (Kieselgel 60, 230~400 mesh, 50 ml, E. Merck) eluting with $CHCl_3$-MeOH (99 : 1). The active fractions were concentrated *in vacuo* and then subjected to Sephadex LH-20 column with MeOH. Final purification was achieved by HPLC (column, YMC-pack ODS column 20×250 mm; solvent, 75% aqueous acetonitrile; flow rate, 8 ml/minute; UV, 322 nm). Phenylpyropene C was eluted with a retention time of 49 minutes. Pure phenylpyropene C (20.5 mg) was obtained as a pale yellow powder.

The physico-chemical properties of phenylpyropene C are summarized in Table 1. The molecular formula of phenylpyropene C was established as $C_{28}H_{34}O_5$ by high resolution FAB-MS. The IR spectrum of phenylpyropene C suggested the presence of –CO–O– (1740 and 1702 cm^{-1}) residues. Phenylpyropene C showed UV maxima at 238 (208,000) and 322 (137,000) nm in MeOH. The ^1H and ^{13}C NMR spectra of phenylpyropene C showed 34 protons and 28 carbons, supporting the molecular formula. They were classified as five methyls, five methylenes, three methines, three quaternary carbons, six sp^2 methines, four sp^2 quaternary carbons and two carbonyl carbons in the DEPT spectrum. In the ^1H NMR spectrum, the fact that phenylpyropene C displayed two aromatic protons at 7.43 ppm appearing as a doublet (H-2′ and H-6′) and three other

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Table 1. Physico-chemical properties of phenylpyropene C.

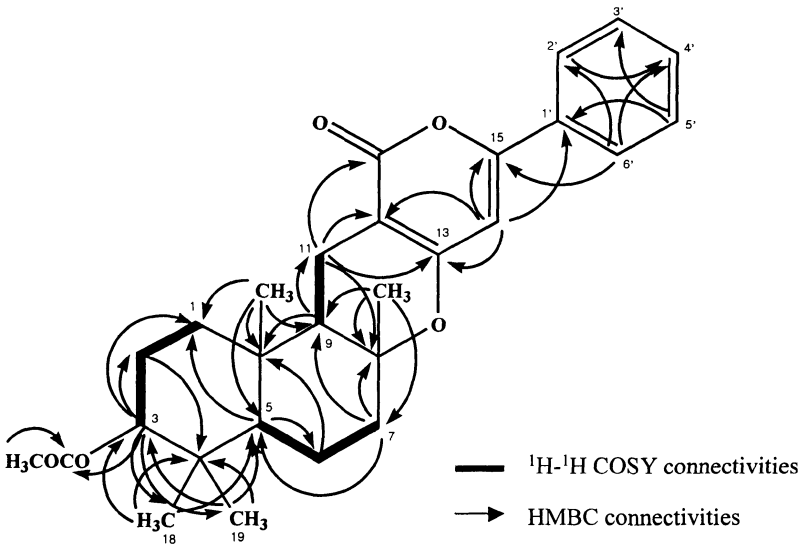
Appearance	Pale yellow powder
Molecular formula	$C_{28}H_{34}O_5$
HRFAB-MS (m/z)	
Found	451.2487 $[M+H]^+$
Calcd.	451.2484
UV λ_{max} nm (ε, MeOH)	238 (208,000), 322 (137,000)
IR ν_{max} (KBr)	1740, 1702 cm^{-1}

Table 2. ^1H and ^{13}C chemical shifts of phenylpyropene C in $CDCl_3$.

No.	^{13}C (ppm)	^1H (ppm)
C-1	37.42	1.18 (ddd, J = 4.8,15.0, 10.5), 1.82 (m)
C-2	23.77	1.78 (m), 1.68 (m)
C-3	80.38	4.51 (dd, J = 4.8, 11.2)
C-4	38.00	
C-5	55.31	1.10 (dd, J = 1.6, 12.0)
C-6	19.57	1.79 (m)
C-7	40.53	2.14 (dt, J = 3.2, 12.4), 1.71 (m)
C-8	80.74	
C-9	51.74	1.52 (dd, J = 4.4, 17.2)
C-10	37.03	
C-11	17.57	2.53 (dd, J = 4.4, 17.2), 2.25 (dd, J =17.2, 17.2)
C-12	99.57	
C-13	163.36	
C-14	98.50	6.37 (s)
C-15	158.38	
3-O-CO-CH_3	21.53	2.06 (s)
3-O-CO-CH_3	170.97	
C-16	164.62	
C-17	28.36	1.27 (s)
C-18	20.98	0.92 (s)
C-19	16.91	0.89 (s)
C-20	15.44	0.95 (s)
C-1'	131.65	
C-2'	125.52	7.79 (m)
C-3'	128.98	7.43 (m)
C-4'	130.63	7.43 (m)
C-5'	128.98	7.43 (m)
C-6'	125.52	7.79 (m)

^{13}C and ^1H NMR spetra were recored at 100 MHz and 400 MHz, respectively.

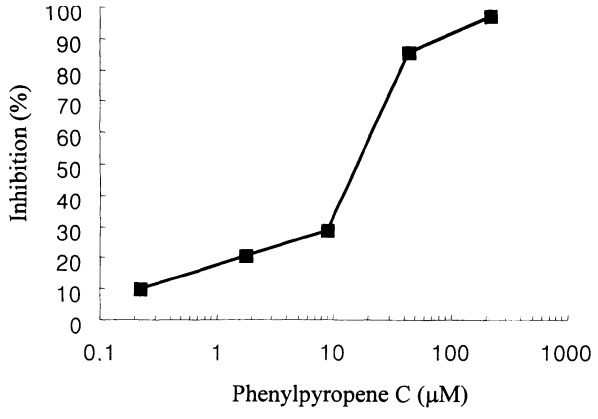
Fig. 1. Structure of phenylpyropene C by NMR analysis.



aromatic protons (7.79 ppm; H-3′, H-4′ and H-5′) as a multiplet exhibits existence of 1-substituted phenyl. The connectivity of proton and carbon atoms was assigned by DEPT and HMQC spectrum (Table 2). Detailed analysis of the ^{1}H-^{1}H COSY experiment proved the partial structures shown Fig. 1. There was a partial overlap of signals in the high field region (δ 1.10 to 1.90). Therefore, the proton sequences were determined by differential selective proton decoupling spectra. The irradiation at δ 4.51 (3-H) and δ 1.18 (1-Hb) simplified the two signals of δ 1.78 (2-Hb) and 1.68 (2-Ha). These results indicated the sequence of –O–CH–CH_2–CH_2– as shown Fig 1. The remaining protons and carbons were assigned by heteronuclear multiple-bond correlation (HMBC) spectroscopy experiments. The presence of 3,4-disubstituted 6-phenyl-α-pyrone was shown because the methine proton of δ 6.37 (14-H) was coupled to four carbons of δ 99.57 (C-12), 131.65 (C-1′), 158.38 (C-15) and 163.36 (C-13). A proton spin system from 9-H to 11-H was detected in ^{1}H-^{1}H COSY spectrum and long-range couplings between 9-H (δ 1.52) and C-11 (δ 17.57) revealed that the carbon at δ 51.74 (C-9) was connected to C-11. The ^{1}H-^{13}C long-range couplings were observed from 11-H_2 (δ 2.53 and 2.25) to C-13 (δ 163.36), C-16 (δ 164.62) and C-12 (δ 99.57), which indicated that the methylene connected with C-12 and the α-pyrone connected with sesquiterpene moieties. Two units of >C(CH_3)CH_2CH_2CH– and their connection to C-9 were defined by the ^{1}H-^{1}H COSY and HMBC experiments. The structure of sesquiterpene moiety was suggested by the following evidences; the respective four methyl protons δ 0.89 (19-H_3), 0.92 (18-H_3), 0.95 (20-H_3) and 1.27 (17-H_3) had three coupling carbons of δ 80.38 (C-3), 38.00 (C-4) and 28.36 (C-18), two ones of δ 80.38 (C-3) and 38.00 (C-4), four ones of δ 55.31 (C-5), 51.74 (C-9), 37.42 (C-1) and 37.03 (C-10), and three ones of δ 80.74 (C-8), 51.74 (C-9) and 40.35 (C-7). The one acetoxy position was also determined by HMBC experiments. The oxymethine proton at δ 4.51 (3-H) and methyl proton at δ 2.06 long range coupled the carbonyl carbon of δ 170.97. The partial structure was supported by a good agreement of the ^{1}H and ^{13}C chemical shifts with those of pyripyropene E reported by TOMODA *et al.*[6]. The structure elucidation described in this paper revealed that phenylpyropene C consists of three parts, phenyl, α-pyrone and sesquiterpene moieties. Thus the structure of phenylpyropene C was determined as shown in Fig. 1.

ACAT activity was assayed as reported previously[7]. In brief, the reaction mixture, containing 10 μl of rat liver microsomes (10 mg/ml protein), 20 μl of 1 M potassium phosphate buffer (pH 7.4, 10 mM dithiothreitol), 10 μl of bovine serum albumin (fatty acid free, 180 mg/ml), 2.0 μl of cholesterol in acetone (20 mg/ml, added last), 130 μl of water, and 10 μl of test sample in a total volume of 190 μl, was preincubated for 30 minutes at 37°C. The reaction was initiated by the addition of 10 μl of [1-^{14}C]oleoyl-CoA solution (0.05 μCi: final concentration 10 μM). After 30

Fig. 2. ACAT inhibitory activity of phenylpyropene C.



minutes of incubation at 37°C, the reaction was stopped by the addition of 1.0 ml of isopropanol - heptane (4 : 1, v/v) solution. A mixture of 0.6 ml of heptane and 0.2 ml of 0.1 M assay buffer was then added to the terminated reaction mixture. This was mixed for 2 minutes and allowed to separate into phases. Cholesterol oleate was recovered in the upper (heptane) phase. The radioactivity in 100 μl of the upper phase was measured in a 7 ml liquid scintillation vial with 4 ml of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (Packard Delta-2000). Background values were obtained by preparing heat inactivated microsomes.

Phenylpyropene C inhibited ACAT activity with the IC_{50} value of 16.0 μM in a dose dependent fashion (Fig. 2). Though the structure of phenylpyropene C is similar to the pyripyropene E[6] produced by *Aspergillus fumigatus*, the pyridine ring of pyripyropene E was replaced by the benzene ring in phenylpyropene C. Furthermore, phenylpyropene C was isolated from *Penicillium griseofulvum* F1959, pathologically safer strain rather than *Aspergillus fumigatus*. The structural modification and its analogues are now in progress.

Acknowledgments

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