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# Phenylpyropene A and B, New Inhibitors 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 the primary enzyme responsible for intracellular esterification of cholesterol in intestinal mucosal cells, synthesis of the cholesteryl esters packaged into very-low-density lipoproteins (VLDL) secreted by liver, accumulation of cholesteryl esters within macrophages and smooth muscle cells of the atheroma, and plays a key role in formation of atherosclerotic lesions[1,2]. Since elevated plasma level of cholesterol is related to an increased risk of coronary heart disease, massive accumulation of cholesteryl esters in macrophage-derived foam cells is a hallmark of atherosclerotic plaques[3]. Therefore, controlling ACAT activity may be of importance in prevention and treatments of hypercholesterolemia and atherosclerosis[4], and inhibition of ACAT would be expected to retard the progression of atherosclerosis ether by reducing serum cholesterol levels or by directly preventing the accumulation of cholesterol in arterial tissues[5].

In the course of our screening for inhibitors of ACAT, we isolated two new potent compounds named phenylpyropene A (**1**) and B (**2**) from a fermentation broth of *Penicillium griseofulvum* F1959 (Fig. 1). In addition, the related compound, phenylpyropene C (**3**), was isolated from the same culture broth as we reported previously[6]. In this study, we report isolation, physico-chemical properties, structure determination, and biological activities of **1** and **2**.

The fungal strain *P. griseofulvum* F1959 was originally isolated from a soil sample collected at Ulsan, Korea, and it was deposited in the Korean Collection for Type Culture (KCTC), Korea, as KCTC 0387BP. Taxonomic studies of the fungal strain, and fermentation procedure were described in our previous report[6].

The fermentation broth (2 liters) of *P. griseofulvum* F1959 was extracted with EtOAc (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 $CH_3CN$; flow rate, 8 ml/minute; UV, 322 nm). **1** and **2** were eluted with retention times of 8 minutes and 31 minutes, respectively. White powders of **1** and **2** were obtained in quantities of 30.2 mg and 3.4 mg, respectively.

The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formulae were determined to be $C_{32}H_{38}O_{10}$ for **1** and $C_{30}H_{36}O_7$ for **2** by high-resolution fast atom bombardment mass spectra (HRFAB-MS), respectively. The IR spectrum of **1** showed absorption bands due to carbonyl groups (1740 and 1702 $cm^{-1}$) and hydroxyl group (3350 $cm^{-1}$). **1** and **2** showed the same UV maxima at 238 ($\varepsilon$ 208,000) and 322 ($\varepsilon$ 137,000) nm in MeOH, indicating the presence of the same chromophore.

The $^1H$ and $^{13}C$ NMR spectra of **1** showed 38 protons and 32 carbons, and the spectra of **2** showed 36 protons and 30 carbons, supporting the molecular formula. In the DEPT spectrum, **1** was classified as six methyls, three methylenes, one oxy methylene, two methines, three oxy methines, three quaternary carbons, six $sp^2$ methines, four $sp^2$ quaternary carbons and four carbonyl carbons, and **2** was classified as five methyls, five methylenes, one oxy methylene, two methines, one oxy methine, three quaternary carbons, six $sp^2$ methines, four $sp^2$ quaternary carbons and three carbonyl carbons.

In the $^1H$ NMR spectrum of **1**, two aromatic protons at 7.36 ppm appeared as a doublet (H-2′ and H-6′) and three other aromatic protons (7.71 ppm; H-3′, H-4′ and H-5′)

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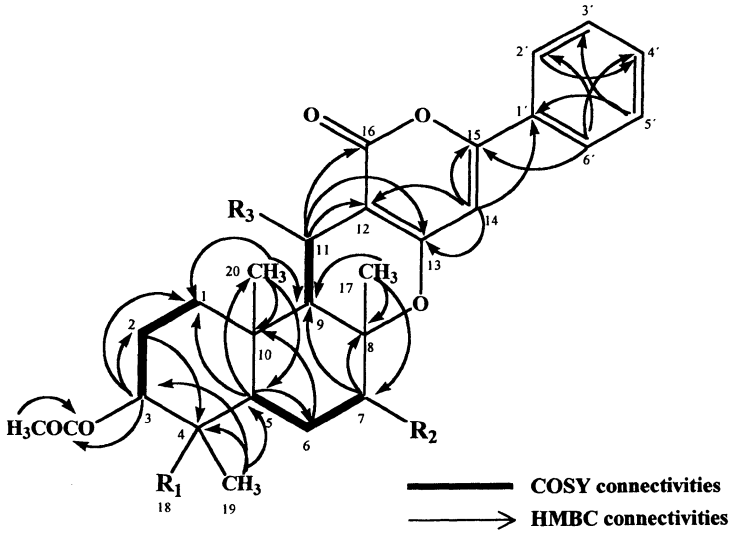
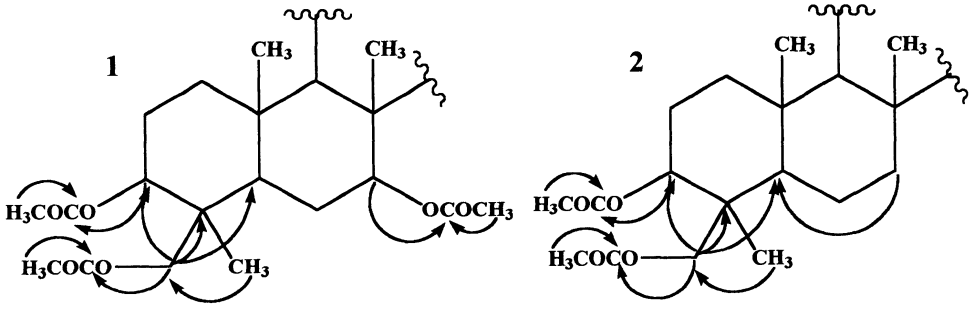
Fig. 1. Structures and NMR-derived connectivities of phenylpyropenes A (**1**), B (**2**), and C (**3**).




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **Phenylpyropene A (1)** | $-CH_2-OCOCH_3$ | $-OCOCH_3$ | -OH |
| **B (2)** | $-CH_2-OCOCH_3$ | -H | -H |
| **C (3)** | $-CH_3$ | -H | -H |

Table 1. Physico-chemical properties of phenylpyropene A (**1**) and B (**2**).

| | **1** | **2** |
|---|---|---|
| Appearance | White powder | White powder |
| Molecular formula | $C_{32}H_{38}O_{10}$ | $C_{30}H_{36}O_7$ |
| HRFAB - MS (*m/z*) | | |
| Found | 605.2358 $[M+Na]^+$ | 509.2543 $[M+H]^+$ |
| Calcd. | 605.2363 | 509.2539 |
| UV $\lambda_{max}$nm (ε, MeOH) | 238 (208,000), 322 (137,000) | 238 (208,000), 322 (137,000) |
| IR $\nu_{max}$ ($cm^{-1}$, KBr) | 3350, 1740, 1702 | 1740, 1702 |

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

| No. | 1 | | 2 | |
|---|---|---|---|---|
| | $^{13}C$ (ppm) | $^{1}H$ (ppm) | $^{13}C$ (ppm) | $^{1}H$ (ppm) |
| C-1 | 36.16 | 1.31 (m), 1.81 (m) | 36.75 | 1.18 (m), 1.82 (m) |
| C-2 | 22.72 | 1.77 (m), 2.12 (m) | 22.86 | 1.70 (m), 1.84 (m) |
| C-3 | 73.60 | 4.72 (dd, *J*= 5.4, 11.4) | 73.69 | 4.80 (dd, *J*= 4.5, 11.7) |
| C-4 | 40.29 | | 40.53 | |
| C-5 | 45.38 | 1.56 (m) | 47.62 | 1.47 (dd, *J*= 1.6, 12.0) |
| C-6 | 25.18 | 1.72 (m) | 18.97 | 1.45 (m), 1.66 (m) |
| C-7 | 77.79 | 4.95 (m) | 39.99 | 2.14 (dt, *J*= 3.2, 11.7), 1.63 (m) |
| C-8 | 82.86 | | 80.38 | |
| C-9 | 54.74 | 1.47 (m) | 51.57 | 1.57 (dd, *J*= 4.8, 12.9 ) |
| C-10 | 37.82 | | 36.67 | |
| C-11 | 60.28 | 4.92 (m) | 17.27 | 2.55 (dd, *J*= 4.8, 17.1), 2.32 (dd, *J*= 17.1, 12.9) |
| 11-OH | | 2.92 (br. s) | | |
| C-12 | 102.11 | | 99.25 | |
| C-13 | 162.52 | | 163.20 | |
| C-14 | 98.21 | 6.32 (s) | 98.19 | 6.37 (s) |
| C-15 | 159.93 | | 158.34 | |
| 3-O-CO-***CH*** $_3$ | 21.13 | 1.97 (s) | 21.16 | 2.04 (s) |
| 3-O-***C***O-$CH_3$ | 170.49 | | 170.51 | |
| 7-O-CO-***CH*** $_3$ | 21.22 | 2.08 (s) | | |
| 7-O-***C***O-$CH_3$ | 170.04 | | | |
| 18-O-CO-***CH*** $_3$ | 20.79 | 2.02 (s) | 20.88 | 2.06 (s) |
| 18-O-***C***O-$CH_3$ | 170.93 | | 170.91 | |
| C-16 | 164.42 | | 164.45 | |
| C-17 | 16.24 | 1.62 (s) | 20.66 | 1.27 (s) |
| C-18 | 64.84 | 3.67 (dd, *J*= 11.7, 47.5) | 65.10 | 3.82, 3.77 (d, *J*= 12.0) |
| C-19 | 13.24 | 0.81 (s) | 13.08 | 0.87 (s) |
| C-20 | 17.45 | 1.37 (s) | 15.56 | 0.98 (s) |
| C-1′ | 131.01 | | 131.43 | |
| C-2′ | 125.58 | 7.71 (m) | 125.38 | 7.78 (m) |
| C-3′ | 128.88 | 7.36 (m) | 128.83 | 7.43 (m) |
| C-4′ | 130.96 | 7.36 (m) | 130.53 | 7.43 (m) |
| C-5′ | 128.88 | 7.36 (m) | 128.83 | 7.43 (m) |
| C-6′ | 125.58 | 7.71 (m) | 125.38 | 7.78 (m) |

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

appeared as a multiplet exhibited existence of 1-substituted phenyl. The hydroxyl proton of **1** was exhibited at 2.92 ppm appearing as a broad singlet (11-OH). 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 partial overlap of signals in the high field region ($\delta$ 1.10 to 1.90). Therefore, the proton sequences were determined by differential selective proton decoupling spectra. The irradiation at $\delta$ 4.72 (3-H) and $\delta$ 1.31 (1-Hb) simplified the two signals of $\delta$ 1.77 (2-Ha) and $\delta$ 2.12 (2-Hb). These results indicated the sequence of O–CH–$CH_2$–$CH_2$– as shown in Fig. 1. The irradiation at $\delta$ 1.47 (9-H) and $\delta$ 2.92 (11-OH) gave the positive signal at $\delta$ 4.92 (11-H), showing the presence of –CH–CH–OH sequence. The remaining protons and carbons were assigned by heteronuclear multiple-bond correlation (HMBC) spectroscopy experiments. The presence of 3,4-disubstituted 6-

(1-phenyl)-$\alpha$-pyrone in **1** was demonstrated by the coupling of methine proton of 6.32 (14-H) to four carbons of $\delta$ 102.11 (C-12), 131.01 (C-1'), 159.93 (C-15) and 162.52 (C-13). A direct coupling between 9-H and 11-H was detected. Long-range couplings between 9-H ($\delta$ 1.47) and C-11 ($\delta$ 60.28) revealed that C-9 ($\delta$ 54.74) was connected to C-11. The $^{1}H$-$^{13}C$ long-range coupling were observed from 11-H ($\delta$ 4.92) to C-13 ($\delta$ 162.52), C-16 ($\delta$ 164.42) and C-12 ($\delta$ 102.11), which indicated that the methine connected with C-12 and the $\alpha$-pyrone connected with sesquiterpene moieties. One unit of $>C(CH_3)CH_2CH_2CH$ and their connection to C-9 was defined by the $^{1}H$-$^{1}H$ COSY and HMBC experiments. The structure of sesquiterpene moiety was suggested by several H-C connectivities; the respective protons of three methyl groups $\delta$ 0.81 (19-$H_3$), 1.37 (20-$H_3$) and 1.62 (17-$H_3$) had four coupling carbons at $\delta$ 73.60 (C-3), 45.38 (C-5) 40.29 (C-4) and 64.84 (C-18), four carbons at $\delta$ 45.38 (C-5), 54.74 (C-9), 36.16 (C-1) and 37.82 (C-10), and three carbons at $\delta$ 82.86 (C-8), 54.74 (C-9) and 77.79 (C-7). The acetoxy positions (–$OCOCH_3$) were also determined by HMBC experiments. Two oxymethine protons at $\delta$ 4.72 (3-H) and $\delta$ 4.95 (7-H), and two methyl protons at $\delta$ 1.97 and $\delta$ 2.08 coupled the carbonyl carbons of $\delta$ 170.49 and $\delta$ 170.04, respectively. The oxymethylene proton at $\delta$ 3.67 (18-H) and methyl proton at $\delta$ 2.02 coupled the carbonyl carbon of $\delta$ 170.93. The partial structure was supported by a good agreement of the $^{1}H$ and $^{13}C$ chemical shifts with those of phenylpyropene C reported previously[6].

The $^{1}H$ and $^{13}C$ NMR spectra of **2** are very similar to those of **1** except for the presence of 11-OH signal and 7-acetoxy position (Table 2). The detailed analysis of the $^{1}H$-$^{1}H$ COSY and HMBC experiment proved the partial structures shown in Fig. 1.

ACAT activity was measured according to a modified method of M.-C. RHO, *et al.*[6] In brief, the reaction mixture, containing 4 $\mu$l of rat liver microsomes (10 mg/ml protein), 20 $\mu$l of 0.5 M potassium phosphate buffer (5X assay buffer; pH 7.4, 10 mM dithiothreitol), 15 $\mu$l of bovine serum albumin (BSA; fatty acid free, 40 mg/ml), 2 $\mu$l of cholesterol in acetone (20 mg/ml, added last), 41 $\mu$l of water, and 10 $\mu$l of test sample in a total volume of 92 $\mu$l, was preincubated for 20 minutes at 37°C. The reaction was initiated by the addition of 8 $\mu$l of [1-$^{14}C$] oleoyl-CoA solution (0.05 $\mu$Ci: final concentration 10 $\mu$M). After 25 minutes of incubation at 37°C, the reaction was stopped by the addition of 1 ml of isopropanol-heptane (4:1, v/v) solution. A mixture of 0.6 ml of heptane and 0.4 ml of 0.1 M assay buffer was then added to the terminated reaction mixture, agitated for 2 minutes, and allowed to separate into phases. Cholesterol oleate was recovered in the upper (heptane) phase. The radioactivity in 100 $\mu$l of the upper phase was measured in a 7 ml liquid scintillation vial with 3 ml of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (Packard Delta-2000). ACAT inhibitory activities were calculated as following method. Background values were obtained by preparing heat inactivated microsomes.

$$\text{Inhibition } \% = 100 \times [1 - \{T - C_2/C_1 - B\}]$$

T: CPM value that treated with both sample and enzyme

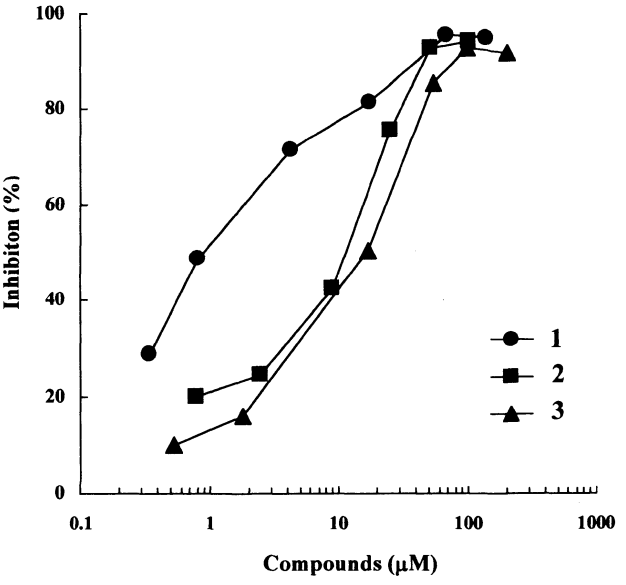
$C_1$: CPM value that treated with enzyme alone

$C_2$: CPM value that treated with both sample and heat inactivated enzyme

B: CPM value that treated with heat inactivated enzyme

ACAT inhibitory activities of **1** and **2** are shown in Fig. 2. The concentrations of **1** and **2** required for 50% ACAT inhibition ($IC_{50}$) were determined to be 0.86 and 12.83 $\mu$M, respectively, and ACAT inhibitory activity of **1** and **2** was

Fig. 2. ACAT inhibitory activities of **1**, **2**, and **3**.




| | $IC_{50}$ (μM) | $IC_{Max}$ μM (Inhibition %) |
|---|---|---|
| **1** | **0.8 ± 0.04** | **68.72 ± 5.32 (95.31)** |
| **2** | **12.8 ± 0.08** | **101.04 ± 7.82 (93.94)** |
| **3** | **16.0 ± 0.09** | **99.80 ± 4.64 (92.63)** |

Values represent the mean±S.E. (n=3) and are representative of three independent experiments.

more effective than **3**[6].

The structure elucidation described in this study reveal that **1** and **2** consist of common three part, phenyl ring, $\alpha$-pyrone and sesquiterpene motif, to form a steroid-like structure. These structures are similar to **3**[6]. Though, the structures of **1** and **2** share to $\alpha$-pyrone and sesquiterpene moieties of pyripyropene A[7] and E[8] produced by *Aspergillus fumigatus*, the pyridine ring of pyripyropene A and E is replaced by the phenyl ring in **1** and **2**. From comparison of the chemical shifts for **1**, **2**, and **3**, it was concluded that they contain three-, two $-OCOCH_3$, and one acetoxy moieties (Fig. 1). Among them, **1** was the most potent ACAT inhibitor. Thus, we considered that ACAT inhibitory activity of these compounds depended on the number of acetoxy groups and the existence of 11-hydroxyl group. **1**, **2**, and **3** are expected to provide a novel type of lead compounds as potent ACAT inhibitors. Structure modification and their analog synthesis are now in progress.

Acknowledgments

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## References

1) SUCKING, K. E. & E. F. STANGE: Role of acyl-CoA: cholesterol acyltransferase in cellular cholesterol metabolism. J. Lipid Res. 26: 647~1671, 1985

2) STANGE, E. F. & J. M. DIETSCHY: Cholesterol absorption and metabolism by the intestinal epithelium. *In* New Comprehensive Biochemistry. Vol. 12. Sterols and Bile Acids. *Eds.*, H. DANIELSSON & J. SJÖVALL, pp. 121~149, Elsevier Science Publishers, 1985

3) KATHAWARA, F. G. & J. G. HEIDER: Acyl-CoA: cholesterol acyltransferase inhibitors and lipid-lipoprotein metabolism. *In* Pharmacochemistry library 17: Antilipidermic drugs. *Eds.*, D. T. WITIAK, H. A. I. NEWMAN & D. R. FELLER, pp. 159~395, 1991

4) SLISKOVIC, D. R. & A. D. WHITE: Therapeutic potential of ACAT inhibitors as lipid lowering and antiatherosclerotic agents. Trends in Pharmacol. Sci. 12: 194~199, 1991

5) TOMODA, H.; Y.-K. KIM, H. NISHIDA, R. MASUMA & S. ŌMURA: Pyripyropenes, novel inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Aspergillus fumigatus*. I. Production, isolation, and biological properties. J. Antibiotics 47: 148~153, 1994

6) RHO, M.-C.; H. S. LEE, K. T. CHANG, H. Y. SONG, O. E. KWON, S. W. LEE, J. S. KO, S. G. HONG & Y.-K. KIM: Phenylpyropene C, a new Inhibitor of acyl-CoA: cholesterol acyltransferase produced by *Penicillium griseofulvum* F1959. J. Antibiotics 55: 211~214, 2002

7) KIM, Y.-K.; H. TOMODA, H. NISHIDA, T. SUNAZUKZA, R. OBATA & S. ŌMURA: Pyripyropenes, novel inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Aspergillus fumigatus*. II. Structure elucidation of pyripyropenes A, B, C and D. J. Antibiotics 47: 154~162, 1994

8) TOMODA, H.; N. TABATA, D.-J. YANG, H. TAKAYANAGI, H. NISHIDA, S. ŌMURA & T. KANEKO: Pyripyropenes, novel ACAT inhibitors produced by *Aspergillus fumigatus*. III. Structure elucidation of pyripyropenes E to L. J. Antibiotics 48: 495~503, 1995