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Polyacetylenic Compounds, ACAT Inhibitors from the Roots of *Panax ginseng*

MUN-CHUAL RHO, HYUN SUN LEE, SEUNG WOONG LEE, JONG SUN CHANG, OH EOK KWON, MI YEON CHUNG, AND YOUNG KOOK KIM*

Laboratory of Lipid Metabolism, Korea Research Institute of Bioscience and Biotechnology, 52 Eoundong, Yusong-gu, Taejon 305-333, Korea

Acyl-CoA: cholesterol acyltransferase (ACAT), which plays a role in the absorption, storage, and production of cholesterol, has been explored as a potential target for pharmacological intervention of hyperlipidemia and atherosclerotic disease. In our search for ACAT inhibitors from natural sources, the petroleum ether extract of *Panax ginseng* showed moderate inhibition of ACAT enzyme from rat liver microsomes. Bioactivity-guided fractionations led to the isolation of one new polyacetylenic compound, (9*R*,10*S*)-epoxy-16-heptadecene-4, 6-diyne-3-one (**1**), in addition to the previously reported polyacetylenic compounds **2** and **3**. Their chemical structures were elucidated on the basis of spectroscopic evidence (UV, IR, NMR, and MS). The compounds **1**, **2**, and **3** showed significant ACAT inhibition with IC_{50} values of 35, 47, and 21 μM, respectively.

KEYWORDS: ***Panax ginseng;*** **Acyl-CoA: cholesterol acyltransferase (ACAT); Polyacetylenes; atherosclerosis; cholesteryl ester**

INTRODUCTION

Atherosclerosis is one of the leading causes of death in industrialized nations. It is a progressive disease characterized by the accumulation of lipids and fibrous elements in the arteries (*1*). In the earliest stages of atherosclerosis, large amounts of cholesterol accumulate in several sites (e.g., macrophages, smooth muscle cell) within vessel walls as cholesteryl esters through an esterification process. The accumulation of cholesteryl ester leads to the formation of foam cells, a hallmark of atherosclerotic lesions. Because this esterification process is regulated by acyl-CoA: cholesterol acyltransferase enzyme (ACAT), the inhibition of this enzyme activity may help to prevent the atherosclerotic process (*2*).

ACAT (EC 2.3.1.26) is the enzyme which catalyzes the conversion of free cholesterol and fatty acyl CoA to the storage form of cholesterol, cholesteryl ester (*3*). ACAT is an integral membrane protein located in the rough endoplasmic reticulum (ER) and plays pivotal roles in intestinal absorption of cholesterol, hepatic production of lipoproteins, and accumulation of cholesteryl ester within macrophages and smooth muscle cells of the atheroma. Two ACAT enzymes, ACAT1 and ACAT2, have been identified (*4*). ACAT1 is ubiquitously distributed in human atherosclerotic lesions, macrophages, and steroidogenic tissues, and ACAT2 is expressed only in liver and intestine (*5*). Therefore, it has been proposed that ACAT1 plays a critical role in foam cell formation in macrophage and ACAT2 plays a critical role in the cholesterol absorption process in intestinal enterocytes. In hepatocytes, the total pool of cholesteryl esters is produced by both ACAT1 and ACAT2 (*6*). The inhibition of ACAT enzymes by pharmacological agents decreased lesion size and macrophage content in the lesion and thus ACAT inhibition was extensively investigated as a potential intervention of atherosclerosis. There are evidences that ACAT1 and ACAT2 have distinct functions. ACAT1 has been suggested to be important for the homeostasis of intracellular cholesterol rather than the atherosclerosis. The complete deficiency of ACAT1 in LDL-deficient mice led to an increase in atherosclerotic lesions. ACAT2 localizes in the tissues that can express apoB and is suggested to play a role in the production of cholesteryl esters. Therefore, ACAT2 has been indicated as a target for improvement of plasma lipoprotein profiles in hyperlipoproteinemia (*7*).

Panax ginseng C. A. Meyer (Araliaceae) is one of the most important oriental medicinal plants and has been employed as an analeptic, stomachic, and erythropoietic agent in Korea, China, and Japan (*8*). Bioactivity-guided fractionations of the petroleum ether extracts of *Panax ginseng* were therefore undertaken to isolate compounds, elucidate their structures and determine their activities.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were determined on a Jasco DIP-370 polarimeter. IR spectra were recorded on a Jasco A-102 spectrophotometer. UV spectra were obtained on a Hewlett-Packard model 8453 spectrophotometer. ^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz), ^{1}H-^{1}H COSY, HMQC, and HMBC spectra were obtained on a Varian Unity 300 spectrometer using benzene-d_6 as solvent. HRFAB-MS was measured on a JEOL HX 110A/HX 100A

* To whom correspondence should be addressed. Tel: +82-42-860-4556. Fax: +82-42-861-2675. E-mail: kimyk@kribb.re.kr.

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Table 1. NMR data of **1**, **2**, and **3** (75 MHz for ^{13}C, 300 MHz for ^{1}H in Benzene-*d*6)

	1		**2**		**3**	
no.	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	8.07(q)	0.79 t (J = 7.5 Hz)	67.42(t)	3.33 t (J = 6.0 Hz)	8.09(q)	0.79 t (J = 7.5 Hz)
2	39.09(t)	2.08 q (J = 7.5 Hz)	45.96(t)	2.38 t (J = 6.0 Hz)	39.11(t)	2.06 q (J = 7.5 Hz)
3	186.40		184.20(s)		186.40(s)	
4	85.29(s)		85.67(s)		85.37(s)	
5	74.04(s)		74.02(s)		74.00(s)	
6	66.43(s)		66.42(s)		66.41(s)	
7	74.75(s)		75.20(s)		74.81(s)	
8	20.11(t)	1.88 (dd (J = 6.7, 18.0 Hz) 2.15 (dd (J = 5.7, 18.0 Hz)	20.13(t)	2.14 dd (J = 5.7, 18.0 Hz) 1.87 dd (J = 6.7, 18.0 Hz)	20.15(t)	2.17 dd (J = 5.7, 18.0 Hz) 1.92 dd (J = 6.6, 18.0 Hz)
9	53.86(d)	2.68 ddd (J = 4.0, 5.7, 6.7 Hz)	53.85(d)	2.68 ddd (J = 5.7, 6.6, 4.0 Hz)	53.91(d)	2.70 ddd (J = 5.7, 6.6, 4.0 Hz)
10	56.60(d)	2.51 m	56.67(d)	2.53 m	56.69(d)	2.55 m
11	29.41(t)	1.21 m	28.08(t)	1.21 m	30.02(t)	1.21 m
12	29.41(t)	1.21 m	29.90(t)	1.21 m	29.91(t)	1.21 m
13	28.08(t)	1.21 m	28.01(t)	1.21 m	28.11(t)	1.21 m
14	26.94(t)	1.21 m	27.14(t)	1.21 m	27.16(t)	1.21 m
15	34.34(t)	1.95 br qt (J = 1.4, 6.6 Hz)	32.47(t)	1.21 m	32.48(t)	1.21 m
16	139.31(d)	5.77 m	23.39(t)	1.21 m	23.39(t)	1.21 m
17	115.06(t)	5.04 m	14.63(q)	0.91 t (J = 6.9 Hz)	14.68(q)	0.90 t (J = 6.9 Hz)
1-OCH_3			58.70(q)	2.96 s		

spectrometer. The HPLC system consisted of a Shimadzu model LC-6AD pump and SPD-10A detector.

Materials. Silica gel (230−400 mesh, 0.040−0.063 mm; Merck) was used for column chromatography and silica gel 60 F_{254} TLC plates (Merck). Bovine serum albumin (BSA, essentially fatty acid free), cholesterol, D,L-dithiothreitol, and potassium phosphate were obtained from Sigma Chemical Co (St. Louis, MO). [1-^{14}C] Oleoyl-CoA was purchased from Amersham Pharmacia Biotech Inc (Little Chalfont, Buckinghamshire, England).

Plant Material. The roots of 4-year-old *Panax ginseng* were purchased at Keumsan Ginseng Market, Korea, in May 2001. The authenticity of the plant was confirmed by Prof. K. H. Bae, College of Pharmacy, Chungnam National University. A voucher specimen, CA-03-041, was deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation. Fresh *Panax ginseng* roots (7.5 kg) were finely crushed and extracted twice with petroleum ether (15 L) at room temperature for 3 days. The petroleum ether extracts were concentrated in vacuo. The crude samples (22 g) were chromatographed on a silica gel (400 g) eluting with hexane/ethyl acetate (100:1, 70:1, 50:1, 30:1, 10:1, each 2 L, v/v). Based on the TLC profile, 18 fractions were collected, and each fraction was monitored by the in vitro ACAT enzyme assay. Fractions (F-8 ∼ F-10, hexane/ethyl acetate = 50:1, 30:1 fractions, 5.72 g) showed the highest inhibitory effect in our assay system and were further separated by low-pressure liquid chromatography on a 310 × 25 mm, 40−63 μm Lichroprep CN Lobar column, at a flow rate of 5 mL/min, with UV detection at 254 nm, eluted with of hexane/$CHCl_3$ (90:10) to give three sub-fractions. The second fraction (2.4 g) with the highest inhibitory effect was successively separated by semipreparative HPLC on a 20 × 250 mm i.d., 4 μm YMC J'sphere ODS H-80 column, at a flow rate of 7 mL/min, with UV detection at 254 nm, at elution with 88% aqueous MeOH. The active compounds **1** (157.7 mg), **2** (50 mg), and **3** (250 mg) were eluted with retention times of 19, 21, and 25 min, respectively.

Compound 1. Light yellow oil; $[\alpha]^{25}_{D}$: −73° (*c* 1.0, $CHCl_3$); IR (neat): ν_{max} ($CHCl_3$) 1220 (C−O), 1644 (C═O), 2235 (C≡C) cm^{-1}; UV (MeOH): λ_{max} = 210, 240, 253, 266, 280 nm; HRFAB-MS: *m*/*z* = 281.1516 (calcd. for $C_{17}H_{22}O_2Na$ $[M+Na]^+$: 281.1552); ^{1}H NMR (300 MHz, C_6D_6), ^{13}C NMR (75 MHz, C_6D_6): see **Table 1**.

Compound 2. Light yellow oil; $[\alpha]^{25}_{D}$: −75.5° (c 0.5, $CHCl_3$); IR (neat): ν_{max} ($CHCl_3$) 1220, 1700 (C═O), 2250 (C≡C) cm^{-1}; UV (MeOH): λ_{max} = 208, 228 (sh), 243, 253, 269, 285 nm; HRFAB-MS: *m*/*z* = 291.1655 (calcd. for $C_{18}H_{26}O_3$ $[M+H]^+$: 291.1660); ^{1}H NMR (300 MHz, C_6D_6), ^{13}C NMR (75 MHz, C_6D_6): see **Table 1**.

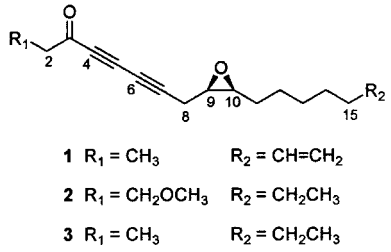
Compound 3. Light yellow oil; $[\alpha]^{25}_{D}$: −70.0° (*c* 1.0, $CHCl_3$); IR (neat): ν_{max} ($CHCl_3$) 1680 (C═O), 2240 (C≡C) cm^{-1}; UV (MeOH): λ_{max} = 210, 228 (sh), 240, 253, 267, 283 nm; HRFAB-MS: *m*/*z* = 261.1852 (calcd. for $C_{17}H_{24}O_2$ $[M+H]^+$: 261.1855); ^{1}H NMR (300 MHz, C_6D_6), ^{13}C NMR (75 MHz, C_6D_6); see **Table 1**.

ACAT Enzyme Assay Using Rat Liver Microsomes. ACAT activity was assayed as reported previously (*9*). In brief, the reaction mixture, containing 10 μL of rat liver microsomes (10 mg/mL protein), 20 μL of 0.5 M potassium phosphate buffer (pH 7.4, 10 mM dithiothreitol), 10 μL of bovine serum albumin (180 mg/mL), 2.0 μL of cholesterol in acetone (20 mg/mL), 130 μL of water, and 10 μL of test sample in a total volume of 190 μL, was preincubated for 30 min at 37 °C. The reaction was initiated by the addition of 10 μL of [1-^{14}C] oleoyl-CoA (0.05 μCi: final concentration 10 μM). After 30 min of incubation at 37 °C, the reaction was stopped by addition of 1.0 mL of 2-propanol-heptane (4:1, v/v) solution. A mixture of 0.6 mL of heptane and 0.4 mL of 0.1 M potassium phosphate buffer was then added to the reaction mixture. This was mixed for 2 min 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 4 mL liquid scintillation vial with 3 mL of scintillation cocktail (Lipoluma, Lumac Co.) using a Wallac microbeta liquid scintillation counter (Boston, MA). Background values were obtained using heat inactivated microsomes.

Data Analysis. Each experiment was performed at least in triplicate. Results are expressed or plotted as the mean value ± standard error (SE).

RESULTS AND DISCUSSION

Due to its roles in the absorption, storage, and production of cholesterol, ACAT has been explored as a potential target for pharmacological intervention of hyperlipidemia and atherosclerotic disease (*1*). There are many known synthetic and naturally occurring inhibitors of ACAT; however, some of them have problems associated with oral bioavailability, adrenal and/or hepatic toxicity, drug interactions, and accumulation of un-



1 $R_1 = CH_3$ $R_2 = CH{=}CH_2$

2 $R_1 = CH_2OCH_3$ $R_2 = CH_2CH_3$

3 $R_1 = CH_3$ $R_2 = CH_2CH_3$

Figure 1. Structures of polyacetylenic compounds **1**, **2** and **3** isolated from the root of *Panax ginseng*.

esterified cholesterol (*10*). Recently, a few candidate drugs have reached the stage of clinical trials as potential lipid-lowering and atherosclerotic agents. In our search for ACAT inhibitors from natural sources, we found that the petroleum ether extracts of *Panax ginseng* inhibited ACAT enzyme activity. The extracts were fractionated by open column chromatography on silica gel and semipreparative HPLC to afford compounds **1**, **2,** and **3** (**Figure 1**).

Compounds **2** and **3** were recently reported as new acyl CoA: diacylglycerol acyltransferase (DGAT) enzyme inhibitors and their structures were also determinated by our group (*11*). Compounds **2** and **3** were obtained as light yellow oils which showed $[\alpha]_D$ −75.5° and −70.0°, respectively. Their UV spectra showed a typical absorption band for a diyne chromophore (*12*). They showed the presence of conjugated triple bonds and a carbonyl group in the IR spectra. Their molecular formulas were determined to be $C_{18}H_{26}O_3$ (**2**) and $C_{17}H_{24}O_2$ (**3**) by the HR−FAB mass spectra, respectively. Their ^{13}C NMR spectra showed that they had in common the presence of carbonyl, oxygen-bearing methine, conjugated acetylene, and other carbons, but the signal of one methoxy carbon (δ 58.70) in the NMR spectrum of **2** was observed. The ^1H NMR spectrum of **3** showed the signals of epoxy protons (δ 2.70 and 2.55), methylene protons between a triple bond and epoxide (δ 2.17 and 1.92), carbonyl carbon conjugated methylene protons (δ 2.06), and other protons. In the ^1H NMR spectrum of **2**, we observed that the methoxy group (δ 2.96) is attached at the C-1, and the other peaks were similar to those of **3**. The connectivity and correlation of proton and carbon atoms were assigned by DEPT, HMQC, and HMBC spectrum. The absolute configuration of **3** was determined to be 9*R* and 10*S* by its asymmetric synthesis (*13*). The synthetic compound was in good accord with an authentic sample **3** in all respects including ^1H and ^{13}C NMR, optical rotation, and TLC in three different solvent systems. On the basis of these data, the structures of **2** and **3** were elucidated as (9*R*,10*S*)-epoxyheptadecan-4,6-diyne-3-one and 1-methoxy-(9*R*,10*S*)-epoxyheptadecan-4,6-diyne-3-one, respectively.

A new compound **1** was obtained as a light yellow oil which showed $[\alpha]_D$ −73°. The UV spectrum of **1** showed a typical absorption band for a diyne chromophore (*12*), and the IR spectrum showed the presence of conjugated triple bonds (2235 cm^{-1}), a carbonyl group (1644 cm^{-1}), and a C−O stretching band (1220 cm^{-1}). It displayed a peak at *m*/*z* 281.1516 in the HRFAB-MS, corresponding to $[M+Na]^+$, indicating a molecular formula of $C_{17}H_{22}O_2$. The ^{13}C NMR spectrum of **1** indicated the presence of 17 carbons. It showed signals similar to those of **2** and **3**, except for the signals of C16 and C17 at δ 139.31 and 115.06 ppm, respectively. The ^1H NMR spectrum of **1** showed the signals of epoxy protons (δ 2.68 and δ 2.51), methylene protons between a triple bond and an epoxide (δ 2.15 and 1.88), carbonyl carbon conjugated methylene protons (δ 2.08), and terminal methyl protons (δ 0.79). The spectrum also

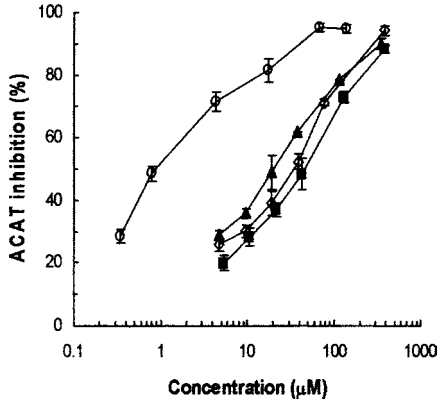


Figure 2. ACAT inhibitory activities of **1** (◇), **2** (■), and **3** (▲). The enzyme reaction was performed at 37 °C for 30 min. Phenylpyropene A (○) was used as a positive control. Data represented the mean ± SE of three individual experiments.

exhibited peaks of olefinic carbon conjugated methylene protons (δ 1.95), and vinyl protons (δ 5.77 and 5.04) were also observed. Also, we were able to observe that the methylene protons located between a triple bond and the epoxide showed the long-range correlations between H-8 and C-4, 5, 6, 7, 9, 10 in the HMBC experiment. The ^1H-^1H COSY spectrum of **1** showed the signal of correlated protons, δ = 2.68, 2.51, 2.15, and 1.88 ppm, and a germinal coupling between δ = 2.15 and 1.88 ppm. The peaks in NMR spectra of **1** were similar to those of **2** and **3** except for the C16−C17 moieties. Optical rotation and ^1H NMR experiments were used to determine the stereochemistry of the epoxy group in **1**. The sign of the optical rotation of **1** was shown to be negative and the ^1H NMR spectrum of **1** displayed H-8 as two doublets-of-doublets at δ_H = 1.88 and 2.15 with *J* values of 6.7, 18.0 and 5.7, 18.0 Hz, respectively. The splitting patterns of the peaks in the ^1H NMR spectrum of **1** coincided with those of **3**. Therefore, the absolute configuration of the epoxy group in **1** is 9*R* and 10*S* and its structure was elucidated as (9*R*,10*S*)-epoxy-16-heptadecene-4, 6-diyne-3-one.

Compounds **1**, **2**, and **3** inhibited ACAT activity in a dose dependent fashion with IC_{50} values of 35 μM (**1**), 47 μM (**2**), and 21 μM (**3**) in an enzyme assay using rat liver microsomes (**Figure 2**). The inhibitory activities of **1**, **2**, and **3** were confirmed by the positive control of phenylpyropene A (*14*) which inhibited ACAT activity with an IC_{50} value of 0.8 μM in the assay system. They showed little different inhibitory activity on ACAT enzyme activity (**Figure 2**), although there is a difference of R_1 group, methyl or methoxy, R_2 group, ethyl or vinyl, and length of chain (**Figure 1**).

Panax ginseng has been used as a medicinal herb in Korea, China, and Japan for hundreds of years, and a number of polyacetylenic compounds were isolated from ginseng root (*15-17*). In fact, dietary ginseng reduced serum total cholesterol and low-density lipoprotein-cholesterol levels in clinical studies (*18-20*). Recently, we reported two novel polyacetylenic compound **2** and **3** from roots of *Panax ginseng* as DGAT inhibitors (*11*). DGAT is considered as a drug target for the specific control of high triglyceride-induced disorders, such as obesity or hypertriglyceridemia (*21*). In this study, we isolated a new polyacetylenic compound **1** with previously reported compounds **2** and **3** as ACAT inhibitor from roots of *Panax ginseng*. Compound **1** also inhibited DGAT enzymes activity with an IC_{50} value of 41 μM (data not shown). Therefore, compounds **1**, **2**, and **3** may be able to provide a new type of lead compounds as lipid-lowering agents through dual inhibitory effect on ACAT and DGAT enzymes, both of which are related lipid storage

and metabolism. In addition, *Panax ginseng* may be used as a food to improve and prevent hyperlipidemia, obesity, and atherosclerosis.

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