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Acyl-CoA: Cholesterol Acyltransferase Inhibitors from *Ilex macropoda*

Kyung-Ran Im[1], Tae-Sook Jeong[1], Byoung-Mog Kwon[1], Nam-In Baek[2], Sung-Hoon Kim[3], and Dae Keun Kim

College of Pharmacy, Woosuk University, Samrye 565-701, Korea, [1]Korea Research Institute of Bioscience and Biotechnology, Taejeon 305-333, Korea, [2]Graduate School of Biotechnology & Plant Metabolism Research Center, Kyunghee University, Suwon 449-701, Korea, and [3]Graduate School of East-West Medical Science, Kyunghee University, Suwon 449-701, Korea

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Twigs from *Ilex macropoda* were extracted with MeOH, and the concentrated extracts were partitioned with CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O. Repeated column chromatography of the CH_2Cl_2 fraction ultimately resulted in the isolation of two compounds, *via* activity-guided fractionation, using ACAT inhibitory activity measurements. According to the physico-chemical data, the chemical structures of these isolated compounds were identified as lupeol (**1**) and betulin (**2**). Compounds **1** and **2** were shown to inhibit the activity of hACAT-1 and hACAT-2 in a dose-dependent manner, and compounds **1** and **2** inhibited hACAT-1 with IC_{50} values of 48 and 83 μM, respectively.

Key words: *Ilex macropoda*, ACAT inhibitory activity, Lupeol, Betulin

INTRODUCTION

Acyl-coenzyme A:cholesterol transferase (ACAT), also referred to as sterol *o*-acyltransferase (SOAT; EC2.3.1.26), is responsible for the esterification of cholesterol with fatty acids (Goodman *et al.*, 1964). In mammals, this enzyme can be found in two isoforms, (Anderson *et al.*, 1998; Coses *et al.*, 1998) ACAT-1 and ACAT-2, and these forms exhibit different tissue distribution and membrane topology (Joyce *et al.*, 2000). ACAT-1 performs a critical role in the formation of macrophage foam cells, whereas ACAT-2 modulates cholesterol absorption in intestinal mucosal cells (Rudel *et al.*, 2001). The inhibition of ACAT activity has been previously associated with a decrease in plasma cholesterol levels *via* the suppression of cholesterol absorption, and *via* a diminution of the assembly and secretion of apolipoprotein B-containing lipoproteins, such as very low density lipoprotein (Leon *et al.*, 2005). Therefore, ACAT is an inhibition target for the treatment of both hypercholesterolemia and atherosclerosis (Brown *et al.*, 1975).

In order to locate novel ACAT inhibitors, a host of natural resources have been screened, most notably medicinal plants and other such sources. It was determine that a total methanolic extract of the twigs of *Ilex macropoda* (Aquifoliaceae) exerted an ACAT-inhibitory effect. The anti-ACAT activity evidenced by this methanolic extract was determined to be concentrated primarily in the CH_2Cl_2-soluble fraction. Therefore, we were ultimately able to isolate two triterpenoid compounds from the CH_2Cl_2-soluble fraction.

In this paper, we describe the ACAT-1 and ACAT-2 inhibitory effects exerted by two compounds, both of which were isolated from twig samples of *Ilex macropoda*.

Correspondence to: Dae Keun Kim, College of Pharmacy, Woosuk University, Samrye 565-701, Korea
E-mail : dkkim@woosuk.ac.kr

MATERIALS AND METHODS

General procedures

^1H- and ^{13}C-NMR spectra were obtained using a JEOL JMN-EX 400 spectrometer. TLC was conducted on Merck precoated silica gel F_{254} plates, using Kiesel gel 60 (230-400 mesh, Merck) as the silica gel. Sephadex LH-20 was employed for column chromatography (Pharmacia, 25-100 μm). All other solvents used in this study were analytical grade, and were used without any further purification. A 1450 Microbeta Trilux scintillation counter, manufactured by Qallac Oy (Turku, Finland) was used to conduct the ACAT activity assay. The [1-^{14}C] oleoyl-CoA (56.0 mCi/

mmol) used in this study was obtained from Amersham Biosciences Korea, Ltd. The KH_2PO_4, dithiothreitol, and bovine serum albumin (fatty acid free) were all acquired from Sigma-Aldrich Korea, Ltd. All reagent-grade chemicals were purchased from Sigma-Aldrich Korea, Ltd.

Plant materials

The *I. macropoda* twigs were collected and air-dried during October of 2003, in Wanju, Jeonbuk, Korea. A voucher specimen was also deposited in the herbarium of the College of Pharmacy, Woosuk University (WSU-03-017).

Extraction and isolation

The air-dried plant materials (600 g) were extracted three times with MeOH at room temperature. The resultant MeOH extract (120 g) was then suspended in water, and fractionated with equal volumes of CH_2Cl_2, ethyl acetate, and *n*-BuOH, in succession. Each of the fractions was evaporated *in vacuo,* yielding the residues of the CH_2Cl_2 soluble fraction (25 g), ethyl acetate soluble fraction (7 g) and *n*-BuOH soluble fraction (45 g). The CH_2Cl_2 soluble fraction was chromatographed over a silica gel column using $CHCl_3$-EtOAc (7:1), resulting in the production of five subfractions (MC1-MC5). The MC2 subfraction was then applied to silica gel (*n*-hexane-EtOAc, 5:1) and purified with Sephadex LH-20 (MeOH), yielding compound **1** (20 mg). The MC4 subfraction was then chromatographed on a silica gel column with *n*-hexane-EtOAc (3:1), and purified with a silica gel column ($CHCl_3$:CH_2Cl_2, 1:3) to yield compound **2** (45 mg).

Lupeol (1)

White powder (MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.69 and 4.55 (each 1H, m, H-29), 3.18 (1H, dd, *J*=10.2, 4.2, H-3), 1.68 (3H, s, H-30), 1.04 (3H, s, H-26), 0.98 (3H, s, H-23), 0.96 (3H, s, H-27), 0.85, 0.80 and 0.78 (each 3H, s, H-25, 28, 24); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 150.8 (C-20), 109.3 (C-29), 78.9 (C-3), 55.2 (C-5), 50.3 (C-9), 48.3 (C-18), 47.9 (C-19), 42.9 (C-17), 42.7 (C-14), 40.7 (C-8), 39.9 (C-22), 38.8 (C-4), 38.7 (C-1), 38.0 (C-13), 37.1 (C-10), 35.5 (C-16), 34.2 (C-7), 29.8 (C-21), 27.9 (C-23), 27.4 (C-2), 27.3 (C-15), 25.0 (C-12), 20.9 (C-11), 19.2 (C-30), 18.2 (C-6), 18.0 (C-28), 16.1 (C-25), 15.9 (C-26), 15.3 (C-24), 14.5 (C-27).

Betulin (2)

White powder (MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.68 and 4.58 (each 1H, m, H-29), 3.79 and 3.33 (each 1H, d, *J*=11.0, H-28), 3.20 (1H, dd, *J*=10.1, 4.7, H-3), 1.65, 0.98, 0.97, 0.96, 0.82 and 0.76 (each 3H, s, H-30, 27, 26, 23, 25, 24); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 150.3 (C-20), 109.5 (C-29), 78.9 (C-3), 60.4 (C-28), 55.2 (C-5), 50.3 (C-9), 48.7 (C-18), 47.7 (C-17), 47.7 (C-19), 42.7 (C-14), 40.8 (C-8), 38.8 (C-4), 38.6 (C-1), 37.2 (C-13), 37.1 (C-10), 34.2 (C-7), 33.9 (C-22), 29.7 (C-21), 29.1 (C-16), 27.9 (C-23), 27.3 (C-2), 27.0 (C-15), 25.1 (C-12), 20.8 (C-11), 19.0 (C-30), 18.3 (C-6), 16.1 (C-25), 15.9 (C-26), 15.3 (C-24), 14.7 (C-27).

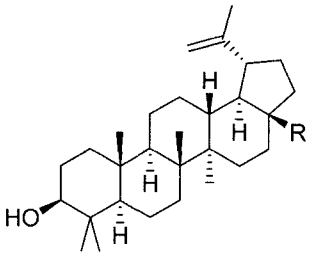
ACAT activity assay

Microsomal fractions of Hi5 cells harboring baculovirally-expressed hACAT-1 or hACAT-2, and rat liver microsomes were used as enzyme sources. The activities of hACAT-1 and hACAT-2 were determined *via* the method developed by Brecher and Chan (Brecher *et al.*, 1980) with some slight modifications (Jeong *et al.*, 1995; Lee *et al.*, 2001).

The reaction mixture, which contained 4 μL of microsomes (8 mg/mL protein) and 20 μL of 0.5 M potassium phosphate buffer (pH 7.4) with 10 mM dithiothreitol, 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL of cholesterol on acetone (20 μg/mL, added last), 41 μL of water and 10 μL of test sample in a total volume of 92 μL, was preincubated for 20 min at 37°C, with brief vortexing and sonication. The reaction was initiated *via* the addition of 8 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 25 minutes of incubation at 37°C, the reaction was halted *via* the addition of 0.1 mL isopropanol-heptane (4:1; v/v). A mixture of 0.6 mL heptane and 0.4 mL of 0.1 M potassium phosphate buffer (pH 7.4), also containing 2 mM dithiothreitol, was then subsequently added. This solution was mixed and allowed to phase-separate under gravity for 2 min. Cholesterol oleate was recovered from the upper heptane phase (total volume 0.9-1.0 mL). The radioactivity in 100 μL of the upper phase was then measured in a liquid scintillation vial containing 3 mL of scintillation cocktail (Lipoluma, Lumac Co.), using a liquid scintillation counter (1450 Microbeta Trilux Wallac Oy, Turku, Finland). The background values were determined *via* the preparation of heat-inactivated or normal insect cell lysate microsomes; the background value normally fell within 200-250 cpm, at 8000 cpm for the ACAT reaction. ACAT activity was expressed as a defined unit: cholesteryl oleate pmol/min/mg protein.

RESULTS AND DISCUSSION

Twigs of *I. macropoda* were extracted with MeOH. The extracts were then sequentially partitioned with CH_2Cl_2, ethyl acetate, *n*-BuOH, and water. Activity-guided fractionation for ACAT-1-inhibitory ability, using repeated silica gel and Sephadex LH-20 column chromatography of the CH_2Cl_2 fraction, resulted in the isolation of two lupane-type triterpenoid compounds, lupeol (**1**, Ahmad *et al.*, 1994; Ito *et al.*, 1978), and betulin (**2**, Patra *et al.*, 1988; Siddiqui *et al.*, 1988). The structures (Fig. 1) of these



1 R = CH_3
2 R = CH_2OH

Fig. 1. Structures of **1** and **2**

Table I. hACAT-1 and hACAT-2 inhibitory activities of compound **1** and **2** from *I. macropoda*

Compounds	Concentration (μM)	hACAT-1	hACAT-2
1	100	72.3 ± 1.3%	48.2 ± 1.0%
	50	51.1 ± 0.8%	28.2 ± 0.6%
2	100	52.4 ± 1.2%	6.6 ± 0.3%
	50	45.6 ± 1.0%	1.3 ± 0.1%
Positive control (oleic acid anilide, nM)	300	42.0 ± 1.1%	45.2 ± 1.2%

The data are presented as the mean ± standard deviation of three replications.
*The value denotes the concentration of each compound.

compounds were elucidated *via* comparisons of the UV, IR, and NMR data with those in the relevant literature.

In service of the goal of developing a useful hypercholesterolemic or anti-atherogenic agent, compounds **1** and **2** were evaluated with regard to their possible ACAT inhibitory abilities. As shown in Table I, the inhibitory activities of compounds **1** and **2** were verified by the oleic acid anilide positive control (Roth *et al.*, 1992; Kim *et al.*, 1994), which inhibited both hACAT-1 and hACAT-2, with inhibitory values of 42.0 and 45.2% at a 300 nM concentration. Compounds **1** and **2** were shown to inhibit hACAT-1 activity dose-dependently. Compounds **1** and **2** both evidenced hACAT-1 inhibitory activity, with values of 51.1 and 45.6%, respectively, at 50 μM concentration. The concentration of compounds **1** and **2** required for an IC_{50} value were 48 (compound **1**) and 83 μM (compound **2**) (hACAT-1). Compounds **1** and **2** evidenced activities less potent than those of the positive control, oleic acid anilide.

In this study, we have demonstrated that the two lupane-type triterpenoids isolated from *I. macropoda*, lupeol and betulin, exert an inhibitory effect against ACAT activity. Previously, we reported the isolation of ursane- and oleanane-type triterpenoid compounds (Kim *et al.*, 2005). Although these triterpenoid compounds isolated from those plants evidenced relatively minor inhibitory activity as compared to the positive control, the relative paucity of naturally occurring ACAT inhibitors renders this discovery of somewhat more important than might be expected. In conclusion, the findings of this study suggest that the methanolic extract of *I. macropoda*, as well as its isolated compounds, lupeol and betulin, may prove useful in the treatment of hypercholesterolemia and atherosclerosis.

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