Human Acyl-CoA: Cholesterol Acyltransferase Inhibitory Activities of Aliphatic Acid Amides from *Zanthoxylum piperitum* DC.

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Received September 7, 2006; accepted September 27, 2006

Acyl-CoA: cholesterol acyltransferase (ACAT) plays an important role in the esterification of cholesterol with its substrates, cholesterol and fatty acyl coenzyme A, to facilitate both intracellular storage and intercellular transport. ACAT-1 is more involved in macrophage foam cell formation and ACAT-2 plays a critical role in the cholesterol absorption process in intestinal enterocytes. Three aliphatic acid amides, β-sanshool (1), γ-sanshool (2), and hydroxy-β-sanshool (3), were isolated by bioassay-guided fractionation of the ethanolic extracts of *Zanthoxylum piperitum* DC. Compounds 1 and 2 inhibited human ACAT-1 and -2 activities with IC_{50} values of 39.0 and 79.7 μM for 1 and of 12.0 and 82.6 μM for 2, respectively. However, the hACAT-1 and -2 inhibitory activities of compound 3 having hydroxyl group were relatively less than those of compounds 1 and 2. A semi-synthetic compound 4, which has acetyl residue at 2′-OH of compound 3, exhibited the increased hACAT-1 and -2 inhibitory activities with IC_{50} values of 28.1 and 87.5 μM, respectively.

Key words aliphatic acid amide; *Zanthoxylum piperitum* DC.; human acyl-CoA: cholesterol acytransferase (hACAT); atherosclerosis

Cardiovascular diseases are major health problems and the leading cause of death in many industrialized countries.[1,2] Acyl-CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.26) plays an important role in the intracellular esterification of free cholesterol with fatty acyl coenzyme A (CoA) to produce cholesterol esters in a variety of cells and tissues.[3,4] The accumulation of lipid-laden foam cells that are derived from macrophages or smooth muscle cells (SMCs) is a hallmark of the atherosclerotic plaque.[5,6]

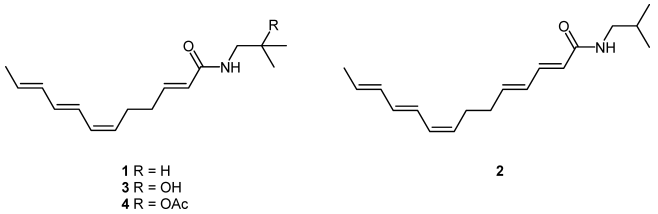
The mammalian ACAT exists as two isoenzymes, ACAT-1 and ACAT-2. ACAT-1 is more involved in macrophage foam cell formation and ACAT-2 plays a critical role in the cholesterol absorption process in intestinal enterocytes.[7] Thus, ACAT inhibitors are being investigated as potent therapeutic agents for the treatment of hypercholesterolemia and atherosclerosis.[8] In the course of a search for ACAT inhibitors from natural sources,[9,10] the ethanolic extracts (100 μg/ml) of stems and pericarps of *Z. piperitum* DC. exhibited hACAT-1 and -2 inhibitory activities with 73% and 21% for stems and 85% and 60% for pericarps, respectively. The extracts of the leaves of *Z. piperitum* have been used as a diuretics and stomachics in traditional Asian medicines.[11] Hashimoto *et al.* reported that aliphatic acid amides isolated from *Z. piperitum* relax the circular muscle of the gastric body, as well as contract the longitudinal muscle of the ileum and distal colon.[12] Subsequent bioactivity-guided fractionation of the ethanolic extracts led to the isolation of three known aliphatic acid amides, β-sanshool (**1**),[13] γ-sanshool (**2**),[14] and hydroxy-β-sanshool (**3**).[13] In this study, we describe the isolation, structure elucidation and determination, and human ACAT (hACAT)-1 and -2 inhibitory activities of the three sanshool derivatives **1**—**3** and semi-synthetic derivative **4**, which has acetyl residue at 2′-OH (Fig. 1).

MATERIALS AND METHODS

General Experimental Methods ^{1}H- and ^{13}C-NMR spectra were obtained on a Varian Unity 300 spectrometer (Varian, U.S.A.) using $CDCl_3$ as solvent. HR-EI-MS was recorded on a JMS-700 (Jeol, Japan). Silica gel (230—400 mesh) for column chromatography and silica gel 60 F_{254} for TLC were supplied by the Merck Korea. [1-^{14}C] Oleoyl-CoA was purchased from Amersham Biosciences Korea. All the reagent grade chemicals were purchased from the Sigma-Aldrich Korea.

Plant Material Stems of *Z. piperitum* were collected at Geochang on December, 2004 and dried pericarps from ripe fruits of *Z. piperitum* were purchased from Geochang Market, Korea. A voucher specimen (027—089) has been deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Extraction and Isolation The dried stems of *Z. piperitum* (1 kg) were cut into the small pieces and extracted with 95% EtOH (2 l) for 3 d at room temperature. The concentrated ethanolic extracts (34 g) were suspended in H_2O and successively partitioned with *n*-hexane, $CHCl_3$, and EtOAc. Then, the EtOAc-extracted residue (1.3 g) was shown the highest hACAT-1 and -2 inhibitory activities with 90% and 78% inhibition at 100 μg/ml, respectively and chromatographed on silica gel (ϕ5×15 cm) with a step gradient of *n*-hexane/EtOAc (from 1 : 1 to 0 : 1, v/v). The eluents were pooled four fractions on the basis of TLC analysis. Among them, the active fraction (Fr. 1-1, 310 mg) was separated by a silica gel column (ϕ5×15 cm) eluting with *n*-hexane/EtOAc (5 : 1, v/v) to yield 8 fractions. The active sixth fraction (Fr.



1 R = H
3 R = OH
4 R = OAc

2

Fig. 1. Structures of Aliphatic Acid Amides, β-Sanshool (**1**), γ-Sanshool (**2**), Hydroxy-β-sanshool (**3**), and β-Sanshool Acetate (**4**)

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1-1-6, 77 mg) was subjected to sephadex LH-20 column (Sigma, ϕ1.5×50 cm) chromatography with 100% MeOH and further chromatographed on silica gel (ϕ3×5 cm) with *n*-hexane/EtOAc (2 : 1, v/v) to afford compound **1** (29 mg). The *n*-hexane-extracted residue (8.6 g) was also shown the inhibitory activity against hACAT-1 and -2 (87% and 50% inhibition at 100 μg/ml, respectively) and subjected to a silica gel column (ϕ5×15 cm) eluting with *n*-hexane/EtOAc (2 : 1, 1 : 1, v/v) and EtOAc/MeOH (1 : 1, v/v) to give three pooled fractions based on TLC analysis. Among them, the active fraction (Fr. 1-2, 1.4 g) was separated by a silica gel column (ϕ5×15 cm) eluting with *n*-hexane/EtOAc (5 : 1, v/v) to give 5 fractions. The active second fraction (Fr. 1-2-2, 76 mg) was separated by a sephadex LH-20 column (ϕ1.5×50 cm) with MeOH and further chromatographed on silica gel (ϕ3×5 cm) with *n*-hexane/EtOAc (5 : 1, v/v) to afford compound **2** (39 mg).

The dried pericarps (500 g) were extracted with 95% EtOH (2 l) for 3 d at room temperature. The concentrated ethanolic extracts (67 g) were suspended in distilled water and successively partitioned with *n*-hexane, $CHCl_3$, and EtOAc. The $CHCl_3$-extracted residue (9.9 g) was shown the inhibitory activity against hACAT-1 and -2 (88% and 67% inhibition at 100 μg/ml, respectively) and chromatographed on silica gel (ϕ5×15 cm) with a step gradient of *n*-hexane/EtOAc (from 1 : 1 to 0 : 1, v/v) to give three pooled fractions based on TLC analysis. The active first fraction (Fr. 1-1, 5.3 g) contained the upper isolated compounds **1** and **2**. The active third fraction (Fr. 1-3, 2.1 g) was further purified by a silica gel column (ϕ5×10 cm) with *n*-hexane/EtOAc (1 : 1, v/v) to obtain compound **3** (1.3 g).

The structures of compounds **1**—**3** were easily identified by comparison of ^{1}H- and ^{13}C-NMR spectroscopic data with those reported in the literature.[13,14]

Compound **1** (β-Sanshool, $C_{16}H_{25}NO$): Colorless oil, HR-EI-MS ($[M]^+$ at m/z=247.1936, Calcd 247.1936).

Compound **2** (γ-Sanshool $C_{18}H_{27}NO$): Colorless oil, HR-EI-MS ($[M]^+$ at m/z=273.2093, Calcd 273.2093).

Compound **3** (Hydroxy-β-sanshool, $C_{16}H_{25}NO_2$): Colorless oil, HR-EI-MS ($[M]^+$ at m/z=263.1885, Calcd 263.1885).

Acetylation of Compound 3 Compound **3** (10 mg) was dissolved in acetic anhydride (2 ml) and stirred with a catalytic amount of *p*-TsOH for 1 h at 60 °C. An aliquot of water (20 ml) was slowly added to the reaction mixture. The above solution was extracted with EtOAc (30 ml×2). The combined extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give the residue. The crude product was purified by a silica gel column with *n*-hexane/EtOAc (2 : 1, v/v) to give compound **4**, β-sanshool acetate (6.2 mg, yield 54%).

ACAT Activity Assay The rate of incorporation of oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or hACAT-2 according to the method described.[10] Microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 or hACAT-2 were used as the sources of enzymes.[15] In brief, the reaction mixture, containing 32 μg microsomal protein, 0.1 M potassium-phosphate buffer (pH 7.4, 2 mM dithiothreitol), 6.7 mg/ml BSA (fatty acid free), 0.40 μg/ml cholesterol, and test sample in a total volume of 92 μl, was preincubated for 20 min at 37 °C. The reaction was initiated by the addition of 8 μl of [1-^{14}C] oleoyl-CoA solution (56.0 μCi/μmol, final conc. 10 μM, Amersham Biosciences). After 25 min 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 0.4 ml of 0.1 M potassium-phosphate buffer (pH 7.4, 2 mM dithiothreitol) was then added to the terminated reaction mixture. The above solution was mixed and allowed to phase separation under gravity for 2 min. Cholesteryl oleate was recovered in the upper heptane phase (total volume 0.9—1.0 ml). The radioactivity in 100 μl of the upper phase was measured in scintillation vial with 3 ml of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (1450 Microbeta Trilux Wallac Oy, Turku, Finland) to detect the radioactivity of the cholesteryl [1-^{14}C] oleate. Background values were obtained by preparing heat inactivated microsomes. Some of the radioactivity recovered in the upper heptane phase was due to enzymatic incorporation of radioactive fatty acid into other products rather than cholesteryl oleate. For determination of the accurate IC_{50} value of tested compounds, the partitioned heptane phase were separated by TLC (Merck, silica gel $60F_{254}$) using a solvent system of *n*-hexane–diethyl ether–acetic acid (90 : 10 : 1) to verify inhibition of ACAT and the radioactivity of the cholesteryl ester region was measured. The ACAT inhibitory activities of the compounds were confirmed by the positive control with oleic acid anilide, which inhibited hACAT-1 and hACAT-2 with IC_{50} values of 0.14 and 0.17 μM, respectively.[15]

RESULTS AND DISCUSSION

Zanthoxylum piperitum DC. is widely utilized in Korea and Japan as a spice for its pleasant flavor. Pungency and actions on sensory nerves of sanshool derivatives have been reported in relation to transient receptor potential vanilloid type 1 (TRPV1) inhibition.[16] Sanshool derivatives have been reported on stimulation of digestive tract movements.[12] In this study, the compounds **1**—**3** were first time evaluated the inhibitory activities of hACAT-1 and -2.[15] β-Sanshool (**1**) and γ-sanshool (**2**) inhibited hACAT-1 with IC_{50} values of 39.0 and 12.0 μM, respectively, whereas they showed relatively weak inhibitory activity on hACAT-2 with IC_{50} values of 79.7 and 82.6 μM, respectively (Fig. 2). However, hydroxy-β-sanshool (**3**) showed relatively weak inhibitory activity on hACAT-1 and -2 with IC_{50} values of 154.2 and 263.0 μM, respectively (Fig. 2). Human ACAT inhibitory activities of compounds **1**—**3** were displayed by the different chain length of unsaturated alkyl group and their lipophilicity. Namely, compound **2** having five unsaturated alkyl chain exhibited more potent hACAT-1 inhibitory activity than that of compound **1** involving four unsaturated alkyl chain, whereas compounds **1** and **2** showed similar inhibitory activities on hACAT-2 regardless of the number of double bond. However, compound **3** having hydroxyl group extremely reduced hACAT-1 and -2 inhibitory activities. Then, the hydroxyl moiety of compound **3** was protected with acetic anhydride to give compound **4**. The semi-synthetic compound **4**, which has acetyl residue at 2′-OH, exhibited the increased hACAT-1 and -2 inhibitory activities with IC_{50} values of 28.1 and 87.5 μM, respectively, compared to compound **3**. In previous study, the unsaturated fatty acid amides, 9(*Z*)-octadece-

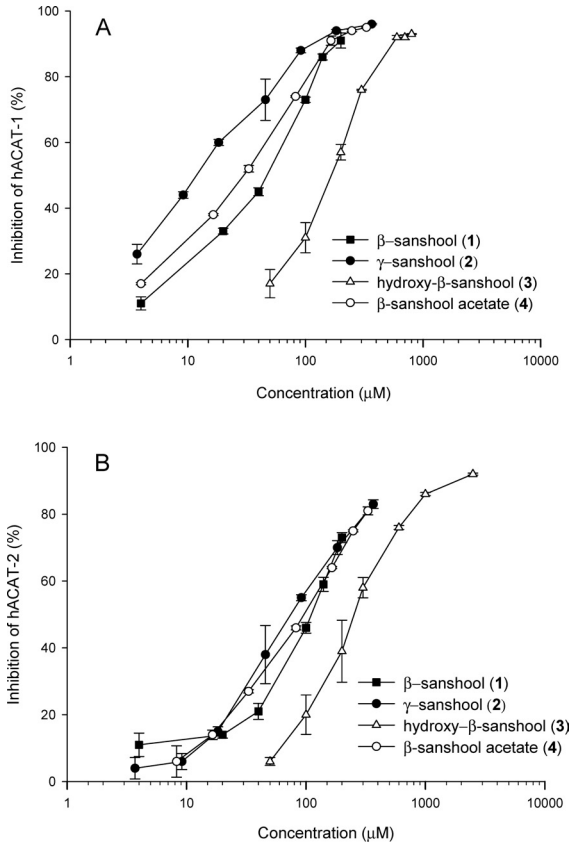

Fig. 2. Effect of Compounds **1**—**4** on the Activities of hACAT-1 (A) and hACAT-2 (B)

In vitro ACAT inhibitory activity was measured using expressed hACAT-1 and -2 and carried out as described in Materials and Methods. Data are shown as mean±S.D. of triplicate experiments.

namide and 9(*Z*), 12(*Z*)-octadecadienamide inhibited rat liver microsomal ACAT, hACAT-1, and hACAT-2, but their unsaturated fatty acid, oleic acid and linoleic acid not inhibited hACAT-1 and -2.[17] Human ACAT-1 is a homotetrameric integral membrane protein[18] and may be located within the plane(s) of the endoplasmic reticulum (ER) membrane, such that the enzyme biosynthesizes cholesteryl esters within the ER membrane.[19] Human ACAT-2 active site, H434 (the equivalent of H460 in hACAT-1) may also be located within the plane of the membrane lipid bilayer.[20] From the previous results, betulinic acid exhibited more potent hACAT-1 and -2 inhibitory activities than ursolic acid and oleanolic acid.[10] Manassantin A preferentially inhibited hACAT-2 than hACAT-1 and manassantin B dominantly inhibited hACAT-1,[9] whereas pyripyropene A inhibited only hACAT-2.[9,15] The selective inhibition of ACAT-1 and -2 provides evidence for uniqueness in structure and function of these two enzymes.[21] Also, it may be not only influenced at substrate binding site but also at lipophilicity of inhibitors that are through ER membrane. Therefore, these results may be rationalized that more preferential inhibitory activity of compounds **1**—**4** on hACAT-1 may be due to substrate binding effect between various unsaturated alkyl chains and the enzyme.

In conclusion, we isolated sanshool derivatives **1**—**3** from *Z. piperitum* and evaluated their inhibitory activities on hACAT-1 and -2. On the extension of this study, we synthesized a compound **4**, which has acetyl residue at 2′-OH, to improve biological activity of compound **3**. Furthermore, *in vivo* hypocholesterolemic and anti-atherosclerotic effects of the ethanolic extracts of *Z. piperitum* in high cholesterol-fed mice are under investigation.

Acknowledgements This work was supported by grants from National Research Laboratory Program of the Ministry of Science and Technology and KRIBB Research Initiative Program, Korea. Yong-Dae Park and Dr. Woo Song Lee contributed equally to this work.

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