

## Human ACAT-1 and ACAT-2 Inhibitory Activities of Pentacyclic Triterpenes from the Leaves of *Lycopus lucidus* TURCZ.

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**Acyl-CoA: cholesterol acyltransferase (ACAT) catalyzes the acylation of cholesterol to cholesteryl ester with long chain fatty acids and ACAT inhibition is a useful strategy for treating hypercholesterolemia or atherosclerosis. Pentacyclic triterpenes, ursolic acid (1), oleanolic acid (2), and betulinic acid (3) were isolated from the methanol extracts of the leaves of *Lycopus lucidus* TURCZ. by bioassay-guided fractionation. The structures of compounds 1—3 were elucidated by their spectroscopic data analysis. Among them, betulinic acid (3) exhibited more potent human ACAT-1 and ACAT-2 inhibitory activities with IC<sub>50</sub> values of 16.2±0.6 and 28.8±1.3 μM, respectively.**

**Key words** *Lycopus lucidus*; acyl-CoA: cholesterol acyltransferase (ACAT); pentacyclic triterpene; atherosclerosis

Acyl-CoA: cholesterol acyltransferase (ACAT, E.C.2.3.1.26) has found to be present as two isoforms, ACAT-1 and ACAT-2, with different intracellular localizations, membrane topology in mammalian species, and metabolic function for each enzyme.<sup>1–3</sup> ACAT-1 is responsible for foam cell formation in macrophages, whereas ACAT-2 is in charge of the cholesterol absorption process in intestinal mucosal cells.<sup>4</sup> To maximize the therapeutic effect, we have focused on developing the specific inhibitor against human ACAT-1 (hACAT-1) or human ACAT-2 (hACAT-2) from the plant resources. Recently, we reported that the sesquieolignan, saucerneol B, and dineolignans, manassantin A and B, were isolated from the root of *S. chinensis* and showed specificity of inhibitory activity against hACAT-1 or hACAT-2.<sup>5</sup> Also, the derivatives of saucerneol B were synthesized to develop more potent ACAT inhibitor.<sup>6</sup> In connection with our studies on the cholesterol-lowering and anti-atherosclerotic agents, we found that methanol extracts of the leaves of *Lycopus lucidus* TURCZ. exhibited significant hACAT-1 and hACAT-2 inhibitory activities.

The Labiatae plant, *L. lucidus* TURCZ. is a perennial herb and widely distributed in China and Korea. The leaves of this plant are used in folk medicine as a tonic, cardiotonic, wound-healing, and pain relieving agent. Several chemical constituents such as flavonoids, triterpenes, and tannins were isolated from the leaves of *L. lucidus*.<sup>7</sup> Among them, a number of triterpenes are known to have a variety of biological activities, such as antineoplastic activity against several human cancer cell line,<sup>8</sup> inhibitory effects on NO production and iNOS induction in LPS-activated mouse peritoneal macrophages,<sup>9</sup> and anti-inflammatory activity.<sup>10</sup> In this study, we describe the isolation, characterization of pentacyclic triterpenes 1—3, and hACAT-1 and hACAT-2 inhibitory activities of compounds 1—3 and a commercial pentacyclic triterpene 4, betulin (Fig. 1).

### MATERIALS AND METHODS

**Plant Material** The dried leaves of *L. lucidus* are collected at Guchang Korea. A voucher specimen (CA04-062) was deposited in the Korea Plant Extract Bank, Korea Re-

search Institute of Bioscience and Biotechnology (KRIBB).

**Extraction and Isolation** The dried leaves of *L. lucidus* (1.0 kg) were ground and extracted with 100% MeOH (10 l) by maceration at room temperature for 3 weeks. Filtration and concentration gave the resultant yellow oil extract (117 g), which was suspended in H<sub>2</sub>O and then partitioned with *n*-hexane, CHCl<sub>3</sub>, and EtOAc sequentially. Then, CHCl<sub>3</sub>-extractable residue (11 g) was shown hACAT-1 and -2 inhibitory activities with 72% and 70% inhibition at 100 μg/ml, respectively, which was chromatographed on a silica gel (Merck, 200—300 mesh, 1 kg), eluting a step gradient of CHCl<sub>3</sub>/MeOH (from 100:1 to 0:1) to give nine fractions. The active fraction (CHCl<sub>3</sub>-MeOH, 50:1, 3.5 g) was rechromatographed on silica gel column (500 g) by eluting with *n*-hexane/EtOAc gradient (from 30:1 to 0:1) to yield fourteen fractions. The active fifth fraction (*n*-hexane/EtOAc, 10:1, 450 mg) was applied to silica gel column chromatography (ϕ3×15 cm) by eluting with CHCl<sub>3</sub>/MeOH (100:1) to give compound 1 (30 mg) as a white powder. Among fourteen fractions at the second silica gel column, the active sixth fraction (*n*-hexane/EtOAc, 10:1, 1.6 g) were applied to C18 reverse-phase column chromatography (Lichroprep RP-18, 40—63 μm, ϕ3×20 cm, Merck) with MeOH/H<sub>2</sub>O gradient. The active fraction (MeOH/H<sub>2</sub>O, 30:1—50:1, 200 mg) was applied to silica gel column chromatography (50 g) by eluting with CHCl<sub>3</sub>/MeOH gradient (from 100:1 to 0:1) to give compound 2 (8 mg) as a white powder. Among ten fractions at the first silica gel column, the active fractions (CHCl<sub>3</sub>/MeOH, 20:1, 1.49 g) were

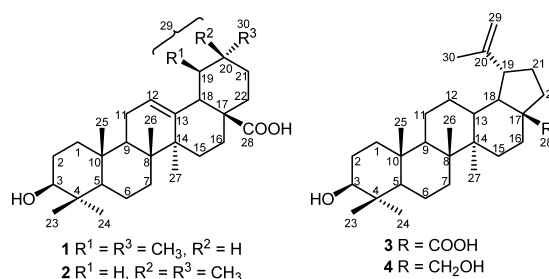


Fig. 1. Structures of Pentacyclic Triterpenes 1—3 Isolated from the Leaves of *L. lucidus* TURCZ. and a Commercial Triterpene 4, Betulin

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applied to C18 reverse-phase column chromatography ( $\phi 1.5 \times 20$  cm) with MeOH/H<sub>2</sub>O gradient. Finally, the active fraction (MeOH/H<sub>2</sub>O, 20:1—50:1, 140 mg) was applied to silica gel column chromatography (20 g) by eluting with *n*-hexane/EtOAc (from 10:1 to 0:1) to give compound **3** (30 mg) as a white powder.

The structures of compounds **1—3** were easily identified by comparison of <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopic data with those of the corresponding authentic samples or literature values.<sup>11–13</sup>

Compound **1** (Ursolic Acid, C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>): EI-MS ( $[M]^+$  at  $m/z=456$ ) and HR-EI-MS ( $[M]^+$  at  $m/z=456.3603$ , Calcd 456.3603),  $[\alpha]_D^{25} +59.8^\circ$  ( $c=1.0$ , EtOH) {lit.  $[\alpha]_D^{25} +63^\circ$  ( $c=1.0$ , EtOH)}.<sup>11</sup>

Compound **2** (Oleanolic Acid, C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>): HR-EI-MS ( $[M]^+$  at  $m/z=456.3608$ , Calcd 456.3603),  $[\alpha]_D^{25} +68^\circ$  ( $c=1.0$ , CHCl<sub>3</sub>) {lit.  $[\alpha]_D^{25} +64.6^\circ$  ( $c=0.27$ , CHCl<sub>3</sub>)}.<sup>12</sup>

Compound **3** (Betulinic Acid, C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>): The EI-MS ( $[M]^+$  at  $m/z=456$ ) and HR-EI-MS ( $[M]^+$  at  $m/z=456.3603$ , Calcd 456.3603),  $[\alpha]_D^{25} +8.0^\circ$  ( $c=1.0$ , pyridine) {lit.  $[\alpha]_D^{25} +12.4^\circ$  ( $c=0.61$ , pyridine)}.<sup>12</sup>

**ACAT Activity Assay** The rate of incorporation of oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or hACAT-2. Microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 or hACAT-2 were used as the sources of enzymes.<sup>14</sup> The activity of the hACAT-1 and hACAT-2 was measured according to the method described previously.<sup>14</sup> In brief, the reaction mixture, containing 32  $\mu$ g microsomal protein, 0.1 M potassium-phosphate buffer (pH 7.4, 2 mM dithiothreitol), 6.7 mg/ml BSA (fatty acid free), 0.40  $\mu$ g/ml cholesterol, and test sample in a total volume of 92  $\mu$ l, was preincubated for 20 min at 37 °C. The reaction was initiated by the addition of 8  $\mu$ l of [<sup>1-14</sup>C] oleoyl-CoA solution (56.0  $\mu$ Ci/ $\mu$ mol, final conc. 10  $\mu$ M, 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  $\mu$ l 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 [<sup>1-14</sup>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 (16.7 $\pm$ 0.3% for hACAT-1 and 14.1 $\pm$ 0.7% for hACAT-2) rather than cholesteryl oleate. For determination of the accurate IC<sub>50</sub> value of tested compounds, the partitioned heptane phase were separated by TLC (Merck, silica gel 60F<sub>254</sub>) 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 as described previously.<sup>15</sup>

## RESULTS AND DISCUSSION

Pentacyclic triterpenes **1—4** have reported to exhibit various biological activities,<sup>11</sup> however, their potential as inhibitor of hACAT-1 or -2 was first time evaluated. The catalytic activity of formation of cholesteryl esters from cholesterol and long-chain fatty acyl-coenzyme A was determined using expressed hACAT-1 or -2 from Hi5 cells as described above. Oleic acid anilide was used as the reference ACAT inhibitor.<sup>14</sup> Ursolic acid (**1**) and oleanolic acid (**2**) inhibited hACAT-1 with IC<sub>50</sub> values of 58.8 $\pm$ 0.1 and 40.7 $\pm$ 1.3  $\mu$ M, respectively, whereas compounds **1** and **2** showed weak hACAT-2 inhibitory activity with 34.1 $\pm$ 4.7% at 100  $\mu$ M and 74.1 $\pm$ 4.4  $\mu$ M, respectively. Betulinic acid (**3**) exhibited more potent hACAT-1 and hACAT-2 inhibitory activities with IC<sub>50</sub> values of 16.2 $\pm$ 0.6 and 28.8 $\pm$ 1.3  $\mu$ M, respectively. However, betulin (**4**) dominantly inhibited hACAT-1 not hACAT-2; the IC<sub>50</sub> for hACAT-1 was 74.4 $\pm$ 0.1  $\mu$ M while hACAT-2 was inhibited only 8.0 $\pm$ 3.4% at 300  $\mu$ M (Fig. 2). All compounds **1—4** are shown to be more selective hACAT-1 inhibitors than hACAT-2. According to recent results, manassantin A showed more specific inhibitory activity against hACAT-2 compared to hACAT-1, whereas manassantin B dominantly inhibited hACAT-1.<sup>5</sup> On the other hand, pyripyropene A inhibited only hACAT-2.<sup>14,16</sup> Previously, Lada *et al.* described that some regions of either enzyme were on opposite sides of the membrane, suggesting uniqueness of function for ACAT-

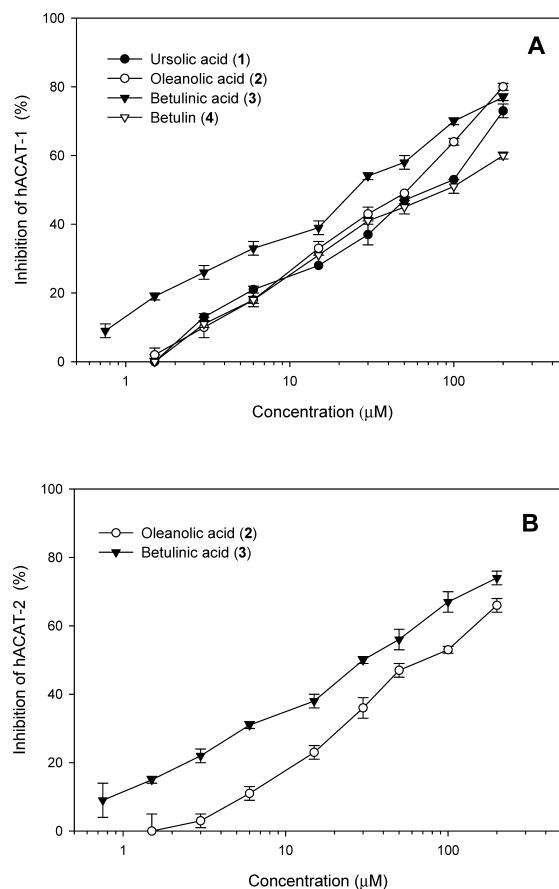


Fig. 2. Effect of Compounds **1—4** on the Activities of hACAT-1 (A) and hACAT-2 (B)

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

1 and ACAT-2.<sup>3)</sup> The cellular localization of ACAT-1 and ACAT-2 also was unique.<sup>4)</sup> The results in the present study again suggest uniqueness of function for ACAT-1 and ACAT-2.

Tabas *et al.* have reported that rabbit and human liver contain a pentacyclic triterpene ester with ACAT inhibitory activity.<sup>17)</sup> They have suggested the hypothesis that the triterpenoid moiety of the rabbit ACAT inhibitor arises from dietary absorption of a plant triterpene. In this study, we reported that pentacyclic triterpenes **1**—**3** isolated from an edible plant, the leaves of *L. lucidus* TURCZ. exhibited hACAT-1 and hACAT-2 inhibitory activities.

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