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Article

Human Acyl-CoA:Cholesterol Acyltransferase-inhibiting Dammarane Triterpenes from *Rhus chinensis*

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In the process of screening for anti-human acyl-CoA:cholesterol acyltransferase (hACAT) agents from plant resources, we found that the 80% methanolic extract of *Rhus chinensis* (*R. chinensis*) exhibited significant antioxidative and hACAT activities in rats. Two compounds were isolated as active principles from the ethyl acetate soluble fraction of the branches of *R. chinensis*. The structures of compounds 1 and 2 were determined as hydroxydammarenone and semialactone, respectively, by means of MS and NMR spectroscopic analysis. Compound 1 has been isolated from this plant for the first time. Compounds 1 and 2 showed inhibitory activities on both human ACAT1 with IC_{50} values of 12.4, and 79.1 μM and on human ACAT2 with IC_{50} values of 30.5 and 76.9 μM, respectively. These results suggested that *R. chinensis* containing dammarane triterpenes 1 and 2 might be effective in the prevention and treatment of hypercholesterolemia or atherosclerosis via inhibitory effect on hACAT.

Key words: atherosclerosis, hACAT, hydroxydammarenone, hypercholesterolemia, *Rhus chinensis*, semialactone

Acyl-CoA: cholesterol acyltransferase (ACAT) catalyzes the acylation of cholesterol to cholesteryl ester and exists in two isoforms, ACAT1 and ACAT2. ACAT1 is in charge of foam cell formation in macrophages, whereas ACAT2 controls the cholesterol absorption in intestinal mucosal cells [Rudel *et al*., 2001]. Therefore, ACAT inhibition is a useful strategy for treating hypercholesterolemia or atherosclerosis by the effect of lowering plasma cholesterol in humans [Lawrence and Gregory, 2000]. *Rhus chinensis* (*R. chinensis*) is a broad leaf tree, which is widely distributed in Korea, Japan, and China. Its barks and galls have long been traditionally used for the remedies of dysentery and diarrhea. The bark of this tree exhibited antioxidative effects [Lee *et al*., 1993], and *Galla rhois*, which is formed in the leaves of this tree, has been reported on concerning its prophylactic efficacy against recurrent herpes simplex virus type 1 infection [Kurokawa *et al*., 1997], antioxidative activity [Cha *et al*., 2000], antimicrobial activity [Choi *et al*., 2003], anti-diabetic effect by suppressing carbohydrate absorption from intestine [Shim *et al*., 2003], and inhibitory activities on tyrosinase and melanogenesis [Kim *et al*., 1997]. A few of chemical constituents have been reported from this tree. Tannin, flavonoid, and coumarin compounds have been isolated from the stem bark of *R. chinensis* [Chung *et al*., 1999]. Triterpenes have been reported recently from the stem bark of *R. chinensis* [Lee *et al*., 2001], but biological studies on its effect have not been yet reported. Some triterpenes are known to have human ACAT1 and ACAT2 inhibitory activities [Nishimura *et al*., 1999; Lee *et al*., 2006; Liu *et al*., 2007; Lee *et al*., 2009], suggesting that triterpenes in *R. chinensis* might also be useful as ACAT inhibitors. Under

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Table 1. ^{13}C NMR and ^{1}H NMR data of compounds 1 and 2

No.	1			2		
	^{13}C NMR		^{1}H NMR	^{13}C NMR		^{1}H NMR
	δ ppm		δ ppm (*J* in Hz)	δ ppm		δ ppm (*J* in Hz)
1	40.14	CH_2	1.40 m, 1.88 m	30.02	CH_2	1.96 m
2	34.38	CH_2	2.43 m	35.53	CH_2	1.13 m, 2.17 m
3	218.12	C	-	98.16	C	-
4	47.67	C	-	35.45	C	-
5	55.56	CH	1.32 m	49.86	CH	1.20 dd (3.5, 12.7)
6	19.94	CH_2	1.44 m	19.80	CH_2	1.45 m
7	34.80	CH_2	1.29 s, 1.52 m	33.06	CH_2	1.13 m, 1.45 m
8	40.52	C	-	39.63	C	-
9	50.24	CH	1.40 m	45.33	CH	1.43 d (2.1)
10	37.08	C	-	40.43	C	-
11	22.31	CH_2	1.46 m, 1.70 m	23.08	CH_2	1.64 m
12	27.80	CH_2	1.24 m, 1.80 m	25.28	CH_2	1.61 m
13	42.62	CH	1.63 m	44.95	CH	2.43 m
14	50.49	C	-	49.31	C	-
15	31.43	CH_2	1.07 m, 1.43 m	33.08	CH_2	1.13 m, 1.45 m
16	25.08	CH_2	1.46 m	29.45	CH_2	1.66 m, 2.17 m
17	50.02	CH	1.70 m	39.99	CH	2.96 dd (9.1, 9.8)
18	15.50	CH_3	0.96 s	15.39	CH_3	0.88 s
19	16.33	CH_3	0.90 s	67.98	CH_2	3.72 d (8.8), 4.24 dd (2.3, 8.8)
20	75.53	C	-	149.18	C	-
21	25.75	CH_3	1.11 s	113.44	CH_2	5.23 s, 5.27 s
22	40.73	CH_2	1.43 m	80.76	CH	4.75 dd (3.3, 12.5)
23	22.85	CH_2	2.01 m	28.97	CH_2	2.35 m, 2.53 m
24	124.83	CH	5.08 m	139.18	CH	6.61d (6.2)
25	131.73	C	-	128.34	C	-
26	18.03	CH_3	1.59 s	166.03	C	-
27	26.05	CH_3	1.65 s	17.00	CH_3	1.92 s
28	26.98	CH_3	1.04 s	26.73	CH_3	1.02 s
29	21.30	CH_3	1.00 s	18.44	CH_3	0.98 s
30	16.64	CH_3	0.85 s	16.51	CH_3	0.89 s

this background, we isolated triterpenes, having inhibitory activities against hACAT1 and hACAT2, from the branches of *R. chinensis*.

R. chinensis was collected from mountain Yeogi in Suwon, South Korea, in June 2003. A voucher specimen (MPS000017) has been deposited at the Herbarium of Department of Herbal Crop Research, National Institute of Horticultural & Herbal Science, RDA, South Korea. The branches of *R. chinensis* were separated, dried at room temperature, and powdered. Column chromatography was performed using silica gel (silica gel 60, 70~230 mesh, Merck, Germany). Thin layer chromatography (TLC) was performed on Kieselgel 60 F254 plate (silica gel, 0.25 mm, Merck, Germany). Spots were detected using UV lamp and 10% H_2SO_4 reagent. Mass spectra were measured on a JEOL JMS-AX505WA (Jeol, Tokyo, Japan) and nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-LA400 FT-NMR spectrometer (Jeol, Tokyo, Japan) and a Brucker AVANCE-600 NMR spectrometer (Brucker, Rheinstetten, Germany). The powdered branches of *R. chinensis* (6 kg) were extracted with 80% MeOH (20 L×5) at room temperature. The concentrated MeOH extracts (365 g) were suspended in H_2O (2 L) and partitioned with EtOAc (2 L×5) and then *n*-BuOH (2 L×5), successively. The EtOAc extract (60 g out of total 165 g) was applied to a silica gel column (7×30 cm, 63-200 μm, Merck, Germany) using a step gradient of *n*-hexane-EtOAc (15:1, 3 L→ 1:20, 3 L), followed by EtOAc-MeOH (20:1, 3 L→5:1, 3 L). Forty nine fractions (No. 1~49) were obtained and compounds **1** (110 mg) and **2** (240 mg) were isolated from fraction No. 9 and No. 29, respectively.

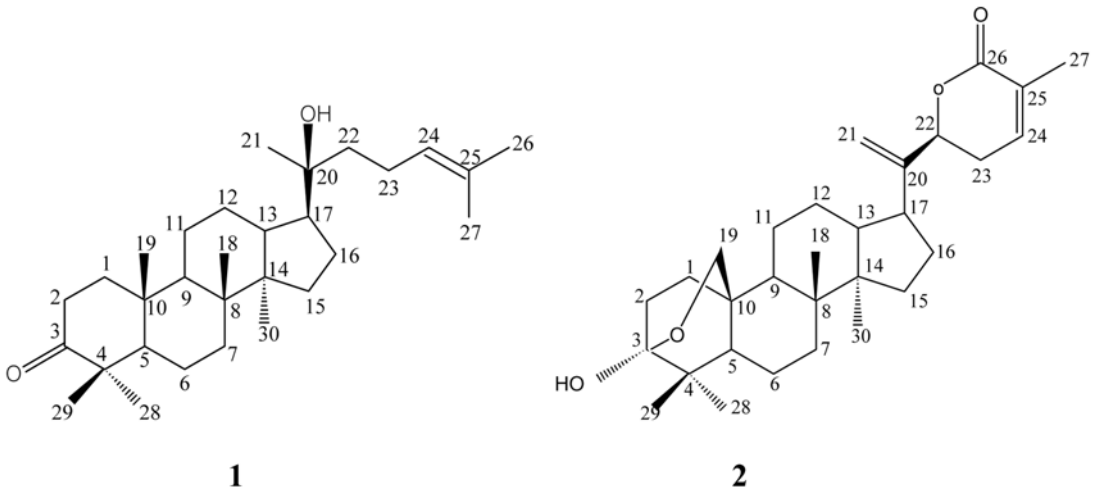

Fig. 1. The structures of compounds 1 and 2.

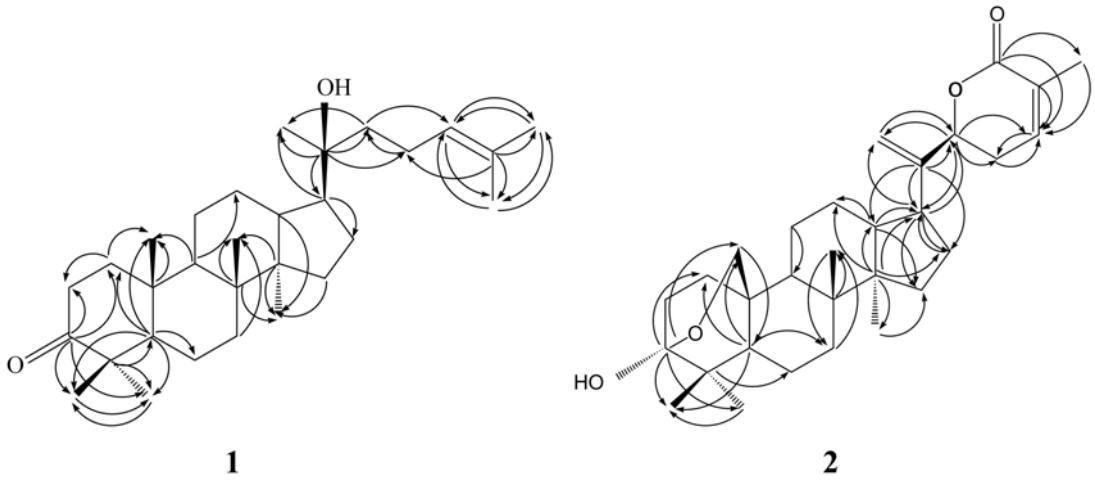

Fig. 2. HMBC connectivity of compounds 1 and 2. Connectivity direction: CH.

Compound 1: Colorless needles; CI-MS (positive ion mode): *m/z* 443 $[M+H]^+$; ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) and ^1H NMR (400 MHz, $CDCl_3$, δ ppm): Table 1; HMBC (400 MHz $CDCl_3$, δ ppm): Fig. 2.

Compound 2: White powder; EI-MS (positive ion mode): *m/z* 468 $[M]^+$; ^{13}C NMR (150 MHz, $CDCl_3$, δ ppm) and ^1H NMR (600 MHz, $CDCl_3$, δ ppm): Table 1; HMBC (600 MHz $CDCl_3$, δ ppm): Fig. 2.

Microsomal fractions of Hi5 cells containing baculovirally expressed hACAT1 or hACAT2 were used as the sources of enzymes [Cho *et al.,* 2003]. The rate of incorporation of oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT1 or hACAT2. The activities of hACAT1 and hACAT2 were measured using the methods of Brecher and Chan [1980] and Cho *et al*. [2003] with slight modification. The reaction mixture, containing 10 μL of cholesterol solution (30 mg/mL protein), 10 μL of 0.5 M KH_2PO_4 buffer (0.5 M KH_2PO_4, 10 mM dithiothreitol, pH 7.4), 5 μL of 0.6 mM bovine serum albumin (essentially fatty acid free), 10 μL of microsomes fraction (8 mg/mL protein), 10 μL of test sample, and 40 μL of distilled water, was preincubated for 30 min at 37°C. The reaction was initiated by the addition of 10 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 30 min of incubation at 37°C, 500 μL of isopropanol:heptane (4:1, v/v), 300 μL of heptanes, and 200 μL of 0.1 M KH_2PO_4 (pH 7.4) were subsequently added to the reaction mixture. The reaction mixture was mixed intensively and allowed to phase separate under gravity for 2 min. Cholesteryl oleate was recovered in the upper heptanes. Two hundred μL of the upper phase was added in scintillation vial with 4 mL of scintillation cocktail (Lipoluma, Lumac Co. USA) and then radioactivity of the cholesteryl [1-^{14}C] oleate was measured using oleic acid anilide as a positive control [Jeong *et al*., 1995].

The molecular formula of compound 1 was determined as $C_{30}H_{50}O_2$ by ^{13}C NMR spectrum and CI-MS, which

exhibited a molecular ion peak at *m/z* 443 $[M+H]^+$. In the ^1H NMR spectrum, eight singlet methyl protons were showed at δ 0.85 (s, H-30), 0.90 (s, H-19), 0.96 (s, H-18), 1.00 (s, H-29), 1.04 (s, H-28), 1.11 (s, H-21), 1.59 (s, H-26), and 1.65 (s, H-27) (Table 1), indicating the skeleton of dammarane triterpene. One olefinic methine proton signal was detected at δ 5.08 (m, H-24), which was confirmed by the correlative signals with the neighboring carbons at δ 18.03 (C-26) and 26.05 (C-27) in the heteronuclear multiple bond correlation (HMBC) spectrum. The methine carbon signal at δ 131.73 (C-25) also showed correlation signals with proton signals at δ 1.59 (s, H-26), 1.65 (s, H-27), and 2.01 (m, H-23). There were a total of 30 carbon signals, including eight tertiary methyl groups, ten methylene groups, and five methine groups including one olefinic methine group (δ 124.83), and seven quaternary signals including one hydroxylated carbon (δ 75.53) and carbonyl group (δ 218.12); Each were confirmed by distortionless enhancement by polarization transfer (DEPT) and ^{13}C NMR spectra. One oxymethine group at δ 75.53 (C-20) showed HMBC connectivity with methyl protons at δ 1.11 (s, H-21) and methylene protons at δ 2.01 (m, H-23). In addition, other HMBC connectivity such as C-22/H-21, C-22/H-24, C-20/H-17, and C-17/H-21 were also shown. Carbonyl carbon (δ 218.12) was assigned to C-3 since HMBC connectivity with methyl protons at δ 1.00 (s, H-29) and 1.04 (s, H-28) was detected. On the basis of ^1H, ^{13}C NMR, DEPT, heteronuclear multiple quantum coherence (HMQC), and HMBC data, compound 1 was finally identified as 20-hydroxy-24-dammaren-3-one (hydroxydammarenone), which has been isolated from this plant for the first time. This compound has been isolated from *Myroxylon balsamum* (Peru balsam), but its extensive biological activities have not been reported yet.

Compound 2 exhibited molecular ion peak at 468 $[M]^+$ in the positive ion mode EI-MS. Combining its ^{13}C NMR data, the molecular formula was determined as $C_{30}H_{44}O_4$. In ^1H NMR spectrum, five methyl signals characteristic to the dammarane triterpene were observed at δ 0.88 (s, H-18), 0.89 (s, H-30), 0.98 (s, H-29), 1.02 (s, H-28), and 1.92 (s, H-27) (Table 1). In down field, one olefinic signal was appeared at δ 6.61 (d, *J*=6.2 Hz, H-24). The proton signals were assigned according to correlative signals with carbonyl carbon at δ 166.03 (C-26), methyl at δ 17.00 (C-27), and oxymethine at δ 80.76 (C-22). Two proton signals at δ 5.23 (s, H-21a) and 5.27 (s, H-21b) attached to C-21, showed HMBC connectivity with the oxymethine carbon signal at δ 80.76 (C-22) and with the methine carbon signal at δ 39.99 (C-17). One oxymethylene proton signal at δ 4.24 (dd, *J*=2.3, 8.8 Hz, H-19b) and 3.72 (d, *J*=8.8 Hz, H-19a) showed HMBC connectivity with quaternary carbon at δ 98.16 (C-3) and methane carbon at δ 49.86 (C-5), indicating the presence of an epoxy group. The oxygenated quaternary carbon signal was determined as C-3 from the HMBC connectivity with two methyl proton signals δ 1.02 (s, H-28) and 0.98 (s, H-29). The configuration of carbon of C-22 was determined to be in the "*S*" form due to the axial direction of the proton of H-2 from its coupling constant (dd, *J*= 3.3, 12.5 Hz). All carbons were assigned on the basis of ^1H, ^{13}C NMR, DEPT, HMQC, and HMBC spectra data, which were exactly corresponding to those of 3-hydroxy-3,19-epoxydammar-20,24-dien-22,26-olide (semialactone) [Lee e*t al*., 2001].

Table 2. hACAT inhibitory activity of compounds 1 and 2 (IC_{50}: μM)

Compounds	1	2
hACAT1 inhibition	12.4	79.1
hACAT2 inhibition	30.5	76.9

Oleic acid anilide (300 nM), which was used as a positive control, showed 57 and 65% inhibition rate against hACAT1 and hACAT2, respectively.

Compound 1 exhibited relatively higher inhibitory activity with inhibition values of 91 and 84% against hACAT1 and hACAT2, respectively, at 50 mg/mL, whereas 100 mg/mL of compound 2 showed a 77 and 87% inhibition rate against hACAT1 and hACAT2, respectively. Positive control (300 nM oleic acid anilide) showed 57 and 65% inhibition rate against hACAT1 and hACAT2, respectively. The IC_{50} values of 1 and 2 were 12.4 and 79.1 μM against hACAT and 30.5 and 76.9 μM against hACAT2, respectively (Table 2). It was remarkable that compound 1 showed higher inhibitory effects than betulinic acid, which had been reported as a potent hACAT1 inhibitor that (IC_{50} value, 16.2 μM) [Lee *et al*., 2006]. The hACAT inhibitory activities of hydroxydammarenone (1) and semialactone (2) have been reported for the first time in this work.

On the other hand, it has been reported that the concentration level of plasma cholesteryl ester is related directly with β-amyloid (Aβ), which has been well known as a neurotoxic protein in brain causing Alzheimer's disease (AD) [Wahrle *et al*., 2002], and hACAT inhibitors recently has been suggested to be useful for the treatment of AD by controlling indirectly Aβ biosynthesis [Puglielli *et al.*, 2001; Runz *et al*., 2002; Hua *et al*., 2003; Puglielli *et al.*, 2003; Hutter-Paier *et al.*, 2004].

Our results conclusively suggested that hydroxydammarenone and semialactone from *R. chinensis* might be useful for the prevention and treatment of hypercholesterolemia, atherosclerosis, and dementia by inhibiting hACAT.

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