# Two New Monoterpene Glycosides from Ku-Ding-Cha. Inhibitors of Acyl-CoA: Cholesterol Acyltransferase (ACAT)

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Received May 1, 1996; accepted July 29, 1996

**Two new monoterpene glycosides, kudingosides A and B, were isolated from Ku-Ding-Cha (*Ligustrum pedunculare* REHD.), together with a known monoterpene glycoside, (*S*)-lipedoside B-III, and three known phenylethanoid glycosides. Their structures were elucidated by spectroscopic and chemical means. Kudingosides A and B, and (*S*)-lipedoside B-III inhibited acyl-CoA: cholesterol acyltransferase (ACAT) with $IC_{50}$ values of $2.70 \times 10^{-3}$ M, $2.88 \times 10^{-3}$ M, and $2.69 \times 10^{-4}$ M, respectively.**

**Key words** Ku-Ding-Cha; *Ligustrum pedunculare*; monoterpene glycoside; kudingoside; (*S*)-lipedoside B-III; acyl-CoA: cholesterol acyltransferase (ACAT)

Ku-Ding-Cha is drunk as a healthy tea, in the south or west regions of China, such as in the provinces of Sichuan, Yunnan and Guizhou. Chinese people use various kinds of plants in its preparation, varying from region to region; for example, they use plants of Oleaceae in Sichuan, Yunnan and Guizhou, plants of Aquifoliaceae in Zhejiang, and plants of Guttiferae in Guangxi for its preparation.[1] As part of the study of compounds in crude drugs which improve the quality of blood fat, we studied the acyl-CoA: cholesterol acyltransferase (ACAT) inhibiting compounds in Ku-Ding-Cha. ACAT is an enzyme which catalyzes intracellular esterification of cholesterol. An inhibitor of ACAT is anticipated to be a new type of medicine to treat arteriosclerosis.[2] We report here the isolation and structural elucidation of two new monoterpene glycosides of Ku-Ding-Cha, and also the inhibitory effects of Ku-Ding-Cha constituents on ACAT.

## Results and Discussion

**Isolation of Glycosides from Ku-Ding-Cha** The separation of inhibitors of ACAT from Ku-Ding-Cha was carried out as shown in Chart 1. We isolated two new monoterpene glycosides, which we have named kudingosides A (**1**) and B (**2**), together with (*S*)-lipedoside B-III (**3**),[3] osmanthusides B (**4**),[4] D (**5**)[1,5] and isosyringalide 3′-α-L-rhamnopyranoside (**6**).[6]

**Structures of the Monoterpene Glycosides** Kudingoside A (**1**) was obtained as a colorless powder. The FAB-MS of **1** showed its $[M+Na]^+$ ion peak at *m/z* 631 and the elemental analysis data was consistent with the molecular formula $C_{31}H_{44}O_{12}$. Acid hydrolysis[7] of **1** afforded D-glucose and L-rhamnose in the ratio of 1 : 1, as well as *trans-p*-coumaric acid. Enzymatic hydrolysis of **1** afforded geraniol. The $^1$H-NMR spectrum of **1** showed signals of a *trans-p*-coumaroyl group [$\delta$ 6.34 (1H, d, $J$ = 16.0 Hz, H-8′), 6.82 (2H, d, $J$ = 8.5 Hz, H-3′, 5′), 7.46 (2H, d, $J$ = 8.5 Hz, H-2′, 6′), 7.66 (1H, d, $J$ = 16.0 Hz, H-7′)], two olefinic protons [$\delta$ 5.12 (1H, m, H-6), 5.38 (1H, m,

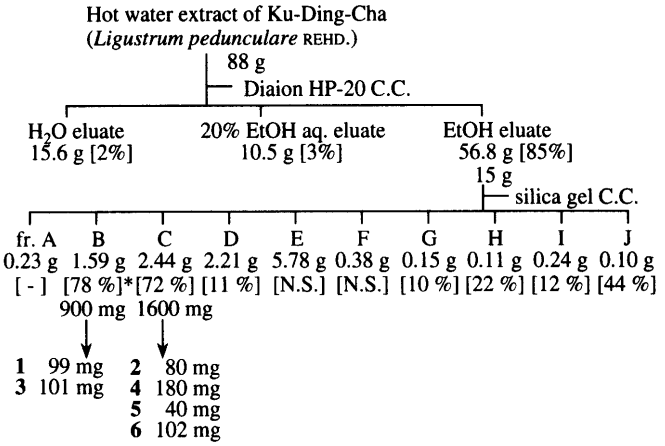


Chart 1. Isolation of Six Glycosides from Ku-Ding-Cha

[ ], inhibitory activity of ACAT, % *vs.* control; a final concentration of test sample: 5.0 mg/ml; *, 1.0 mg/ml; —, insoluble; N.S., no significance.

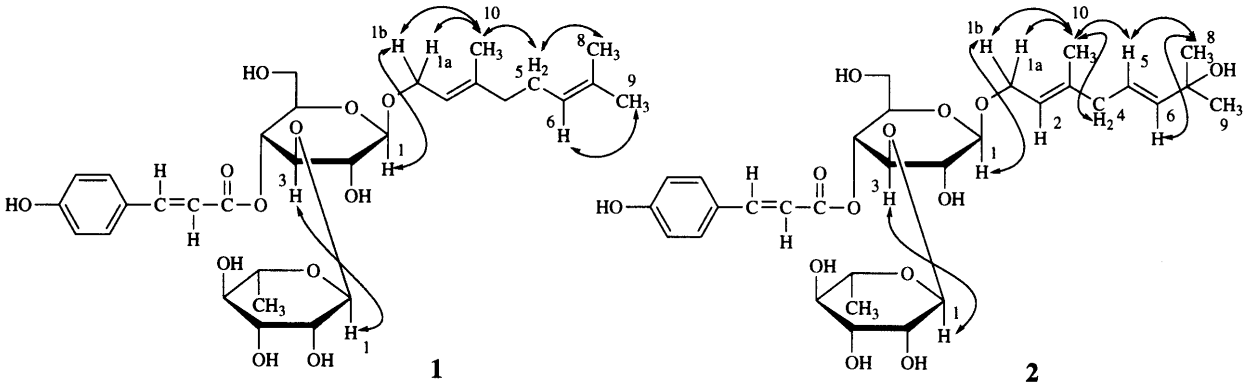

Fig. 1. NOE Experiments of Kudingosides A (**1**) and B (**2**)

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H-2)], and four methyl groups [$\delta$ 1.09 (3H, d, $J$=6.0 Hz), 1.62 (3H, s) , 1.69 (6H, s)]. The $^{13}$C-NMR signals of **1** were similar to those of **3**, except for carbon signals of an aglycone moiety, and the spectrum showed the presence of a geraniol group [$\delta$ 16.5 (C-10), 17.8 (C-8), 25.9 (C-9), 27.5 (C-5), 40.6 (C-4), 66.5 (C-1), 121.4 (C-2), 125.1 (C-6), 132.5 (C-7), 142.1 (C-3)]. The nuclear Overhauser effect (NOE) difference spectrum of **1** (Fig. 1) showed that the glucosyl group is joined to the C-1 of the aglycone, and the rhamnosyl group is joined to the C-3 of glucosyl moiety. From these results, the structure of kudingoside A was concluded to be geranyl $\alpha$-L-rhamnopyranosyl-(1→3)-(4-*O*-*trans*-*p*-coumaroyl)-$\beta$-D-glucopyranoside (**1**).

Kudingoside B (**2**) was obtained as a colorless powder. The FAB-MS of **2** showed its $[M+Na]^+$ ion peak at $m/z$ 647 and the elemental analysis data was consistent with the molecular formula $C_{31}H_{44}O_{13}$. Acid hydrolysis of **2** afforded D-glucose and L-rhamnose in the ratio of 1 : 1, as well as *trans*-*p*-coumaric acid. The $^1$H-NMR spectrum of **2** showed signals of a *trans*-*p*-coumaroyl group [$\delta$ 6.33 (1H, d, $J$=16.3 Hz, H-8′), 6.81 (2H, d, $J$=8.5 Hz, H-3′, 5′), 7.46 (2H, d, $J$=8.5 Hz, H-2′, 6′), 7.66 (1H, d, $J$=16.3 Hz, H-7′)], three olefinic protons [$\delta$ 5.43 (1H, m, H-2), 5.61 (1H, dd, $J$=16.0, 6.0 Hz, H-5), 5.64 (1H, d, $J$=16.0 Hz, H-6)], and four methyl groups [$\delta$ 1.09 (3H, d, $J$=6.3 Hz), 1.28 (6H, s), 1.69 (3H, s)]. The $^{13}$C-NMR signals of **2** were similar to those of **1** except for carbon signals of an aglycone moiety. Enzymatic hydrolysis of **2** afforded (2*E*,5*E*)-7-hydroxy-3,7-dimethyl-2,5-octadienol (**2a**) as an aglycone. On the other hand, a photo-oxidation reaction of geraniol by a mercury lamp also afforded **2a**.[8] The NOE difference spectrum of **2** (Fig. 1) showed that glucose is joined to the C-1 of the aglycone, and rhamnose is joined to the C-3 of the glucosyl moiety. Therefore, the structure of kudingoside B was concluded to be (2*E*,5*E*)-7-hydroxy-3,7-dimethyl-2,5-octadienyl *O*-($\alpha$-L-rhamnopyranosyl)-(1→3)-(4-*O*-*trans*-*p*-coumaroyl)-$\beta$-D-glucopyranoside (**2**).

Compound **3** was identified as lipedoside B-III (**3**) by comparison of spectral data with reported data, and the absolute configuration at the C-3 of **3** was determined as *S* because it showed good coincidence in the chemical shifts in the aglycone moiety, particularly in the C-4 on the $^{13}$C-NMR spectra of capsianoside A[9] (geranyllinaloyl glucoside). It was reported[10] that the $^{13}$C-NMR spectral data in the C-4 of linaloyl glucosides differ by 1.2 ppm in *R* and *S*.

**Inhibitory Effects of Ku-Ding-Cha Constituents on ACAT** The inhibitory effects of three monoterpene glycosides, **1**—**3**, and three phenylethanoid glycosides, **4**—**6**, isolated from Ku-Ding-Cha, on ACAT were examined, and the results are summarized in Table 1. Among the tested compounds, monoterpene glycosides **1**—**3** showed inhibition, with $IC_{50}$ values of $2.69\times10^{-4}$—$2.88\times10^{-3}$ M, although the inhibitory activity of all phenylethanoid glycosides, **4**—**6**, on ACAT was not strong.

## Experimental

**General Procedure** UV spectra were recorded on a Hitachi U-2000 spectrophotometer. $^1$H- and $^{13}$C-NMR spectra were obtained using a JEOL JNM $\alpha$-400 NMR ($^1$H, 400 MHz; $^{13}$C, 100 MHz) or JEOL JNM GX-270 FT NMR spectrometer ($^1$H, 270 MHz; $^{13}$C, 68 MHz). Chemical shifts were given in $\delta$ ppm with tetramethylsilane as an internal standard. FAB-MS were recorded on a JEOL JMS SX102 spectrometer. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter. Gas chromatography (GC) was run on a Hitachi G-3000 gas chromatograph. Analytical TLC was conducted on Kieselgel 60 $F_{254}$ plates (0.2 mm thick).

**Material** Ku-Ding-Cha (leaves of *Ligustrum pedunculare* REHD.) was purchased in Chengdu City, Sichuan Province, China in 1993. The original plant of Ku-Ding-Cha was identified by Prof. Liu, Chengdu Institute of

Table 1. Inhibitory Effects of Ku-Ding-Cha Constituents on ACAT

| Compound | $IC_{50}$ (M) |
|---|---|
| Monoterpene glycoside | |
| Kudingoside A (**1**) | $2.70\times10^{-3}$ |
| Kudingoside B (**2**) | $2.88\times10^{-3}$ |
| Lipedoside B-III (**3**) | $2.69\times10^{-4}$ |
| Phenylethanoid glycoside | |
| Osmanthuside B (**4**) | $>1.00\times10^{-2}$ |
| Osmanthuside D (**5**) | $>1.00\times10^{-2}$ |
| Isosyringalide 3′-$\alpha$-L-rhamnopyranoside (**6**) | $>1.00\times10^{-2}$ |

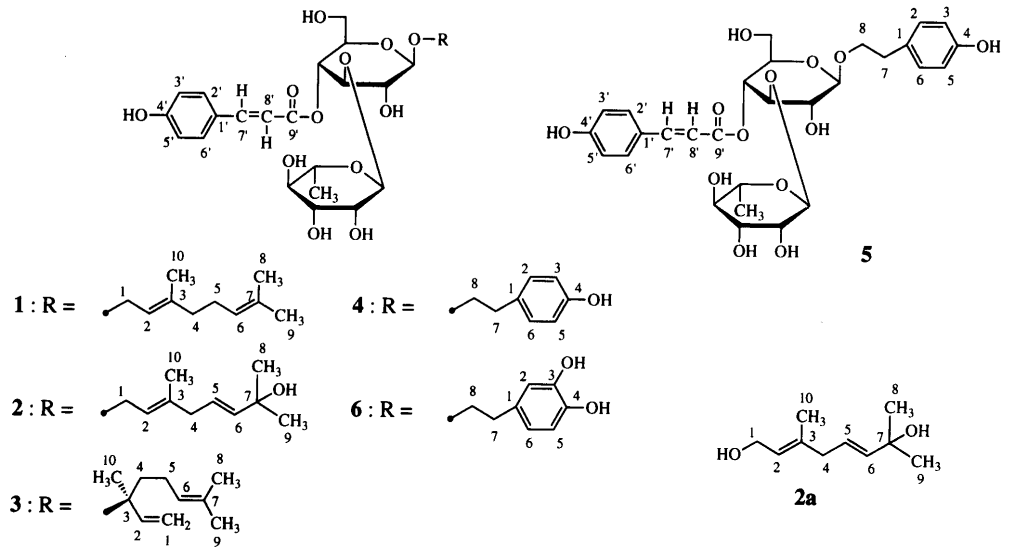


Chart 2

Biology, Academia Sinica, China.

**Extraction and Isolation** Ku-Ding-Cha (leaves of *Ligustrum pedunculare* REHD.) (300 g) was extracted with boiling water (3 l) under reflux. The extract was passed through a porous polymer gel, Diaion HP-20 (Mitsubishi Chemical Co.) column, and the adsorbed material was eluted successively with $H_2O$ and 20% EtOH aq. and EtOH. These eluates were concentrated *in vacuo* to give brown residues (yield, 15.6 g, 10.5 g, 56.8 g, respectively). The EtOH eluate (15.0 g) was separated by column chromatography on silica gel with $CHCl_3$–MeOH, increasing the MeOH content to afford fr. A—J. Fraction B (900 mg) was separated by preparative HPLC [Waters $\mu$-Bondasphere C-18 (5 $\mu$) 100 Å, 19 × 150 mm; MeOH–$H_2O$ (32 : 68)] and gave **1** (99 mg) and **3** (101 mg). Fraction C (1600 mg) was purified by preparative HPLC [$\mu$-Bondasphere; $CH_3CN$–$H_2O$ (40 : 60)], to give **2** (80 mg), **4** (180 mg), **5** (40 mg) and **6** (102 mg).

**Kudingoside A (1)** An amorphous powder, $[\alpha]_D^{26}$ −96.9° ($c$ = 1.13, MeOH). *Anal.* Calcd for $C_{31}H_{44}O_{12} \cdot 3/2H_2O$: C, 58.57; H, 7.45. Found: C, 58.26; H, 7.51. FAB-MS *m/z*: 631 ($[M+Na]^+$). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 229 (4.10), 315 (4.38). $^1$H-NMR ($CD_3OD$, 400 MHz) $\delta$: 1.09 (3H, d, $J$ = 6.0 Hz, rha H-6), 1.62 (3H, br s, H-9), 1.69 (6H, br s, H-8, 10), 2.06 (3H, m, H-4, 5a), 2.13 (1H, m, H-5b), 3.29 (1H, t, $J$ = 9.5 Hz, rha-4), 3.40 (1H, dd, $J$ = 9.0, 8.0 Hz, glc-2), 3.53—3.62 (3H, m, glc-5, 6), 3.57 (2H, m, rha-3, 5), 3.82 (1H, t, $J$ = 9.0 Hz, glc-3), 3.92 (1H, dd, $J$ = 3.0, 1.5 Hz, rha-2), 4.27 (1H, dd, $J$ = 12.0, 7.5 Hz, H-1b), 4.35 (1H, dd, $J$ = 12.0, 5.5 Hz, H-1a), 4.37 (1H, d, $J$ = 8.0 Hz, glc-1), 4.92 (1H, t, $J$ = 9.5 Hz, glc-4), 5.12 (1H, m, H-6), 5.38 (1H, m, H-2), 5.20 (1H, d, $J$ = 1.5 Hz, rha-1), 6.34 (1H, d, $J$ = 16.0 Hz, H-8′), 6.82 (2H, d, $J$ = 8.5 Hz, H-3′, 5′), 7.46 (2H, d, $J$ = 8.5 Hz, H-2′, 6′), 7.66 (1H, d, $J$ = 16.0 Hz, H-7). $^{13}$C-NMR ($CD_3OD$, 100 MHz) $\delta$: 16.5 (C-10), 17.8 (C-8), 18.4 (rha-6), 25.9 (C-9), 27.5 (C-5), 40.6 (C-4), 62.4 (rha-6), 66.5 (C-1), 70.4 (rha-5), 70.7 (glu-4), 72.1 (rha-3), 72.3 (rha-2), 73.8 (rha-4), 76.1 (glc-2, 5), 81.6 (glc-3), 102.6, (glc-1), 102.9 (rha-1), 114.8 (C-8′), 116.9 (C-3′, 5′), 121.4 (C-2), 125.1 (C-6), 127.1 (C-1′), 131.3 (C-2′, 6′), 132.5 (C-7), 142.1 (C-3), 147.6 (C-7′), 161.4 (C-4′), 168.3 (C-9′).

**Kudingoside B (2)** An amorphous powder, $[\alpha]_D^{26}$ −93.5° ($c$ = 2.60, MeOH). *Anal.* Calcd for $C_{31}H_{44}O_{13} \cdot 3/2H_2O$: C, 57.13; H, 7.27. Found: C, 56.89; H, 7.40. FAB-MS *m/z*: 647 ($[M+Na]^+$). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 199 (4.36), 229 (4.08), 315 (4.36). $^1$H-NMR ($CD_3OD$, 400 MHz) $\delta$: 1.09 (3H, d, $J$ = 6.3 Hz, rha-6), 1.28 (6H, s, H-8, 9), 1.69 (3H, s, H-10), 2.75 (2H, br d, $J$ = 5.4 Hz, H-4), 3.29 (1H, t, $J$ = 9.4 Hz, rha-4), 3.52 (1H, m, glc-5), 3.40 (1H, dd, $J$ = 9.0, 8.0 Hz, glc-2), 3.56—3.62 (3H, m, glc-6, rha-5), 3.58 (1H, dd, $J$ = 9.4, 3.1 Hz, rha-3), 3.82 (1H, t, $J$ = 9.0 Hz, glc-3), 3.92 (1H, dd, $J$ = 3.1, 1.6 Hz, rha-2), 4.27 (1H, br dd, $J$ = 12.0, 7.5 Hz, H-1b), 4.37 (1H, d, $J$ = 8.0 Hz, glc-1), 4.38 (1H, br dd, $J$ = 12.0, 6.5 Hz, H-1a), 4.92 (1H, t, $J$ = 9.0 Hz, glc-4), 5.20 (1H, d, $J$ = 1.6 Hz, rha-1), 5.43 (1H, m, H-2), 5.61 (1H, dd, $J$ = 16.0, 6.0 Hz, H-5), 5.64 (1H, d, $J$ = 16.0 Hz, H-6), 6.33 (1H, d, $J$ = 16.3 Hz, H-8′), 6.81 (2H, d, $J$ = 8.5 Hz, H-3′, 5′), 7.46 (2H, d, $J$ = 8.5 Hz, H-2′, 6′), 7.66 (1H, d, $J$ = 16.3 Hz, H-7′). $^{13}$C-NMR ($CD_3OD$, 100 MHz) $\delta$: 16.6 (C-10), 18.4 (rha-6), 30.0 (C-8, 9), 43.2 (C-4), 62.4 (glc-6), 66.6 (C-1), 70.4 (rha-5), 70.7 (glc-4), 71.1 (C-7), 72.1 (rha-3), 72.3 (rha-2), 73.8 (rha-4), 76.1 (glc-2, 5), 81.6 (glc-3), 102.8 (rha-1), 102.9 (glc-1), 114.8 (C-8′), 116.9 (C-3′, 5′), 122.1 (C-2), 125.3 (C-5), 127.0 (C-1′), 131.3 (C-2′, 6′), 141.0 (C-3), 141.2 (C-6), 147.6 (C-7′), 161.6 (C-4′), 168.3 (C-9′).

**(*S*)-Lipedoside B-III (3)** An amorphous powder, UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 199 (4.34), 229 (4.07), 315 (4.37). $^1$H-NMR ($CD_3OD$, 270 MHz) $\delta$: 1.08 (3H, d, $J$ = 6.0 Hz, rha H-6), 1.38 (3H, s, H-10), 1.59 (3H, s, H-9), 1.60 (2H, m, H-4), 1.66 (3H, s, H-8), 2.02 (2H, m, H-5), 3.25—3.61 (8H), 3.78 (1H, m, glc H-3), 3.92 (1H, m, rha H-2), 4.43 (1H, d, $J$ = 7.9 Hz, glc H-1), 4.90 (1H, t, $J$ = 9.3 Hz, glc H-4), 5.10 (1H, m, H-6), 5.18—5.28 (3H), 5.93 (1H, dd, $J$ = 17.8, 10.9 Hz, H-2), 6.33 (1H, d, $J$ = 16.0 Hz, H-8′), 6.81 (2H, d, $J$ = 8.5 Hz, H-3′, 5′), 7.46 (2H, d, $J$ = 8.5 Hz, H-2′, 6′), 7.65 (1H, d, $J$ = 16.0 Hz, H-7′). $^{13}$C-NMR ($CD_3OD$, 68 MHz) $\delta$: 17.7 (C-9), 18.4 (C-6), 23.2 (C-10), 23.6 (C-5), 25.8 (C-8), 42.6 (C-4), 62.4 (glc-6), 70.3 (rha-5), 70.7 (glc-4), 72.0 (rha-3), 72.3 (rha-2), 73.8 (rha-4), 75.7 (glc-2), 76.2 (glc-5), 81.6 (glc-3), 81.9 (C-3), 99.4 (glc-1), 103.0 (rha-1), 114.8 (C-8′), 115.5 (C-1), 116.8 (C-3′, 5′), 125.6 (C-6), 127.0 (C-1′), 131.3 (C-2′, 6′), 132.2 (C-7), 144.3 (C-2), 147.5 (C-7′), 161.4 (C-4′), 168.3 (C-9).

**Acid Hydrolysis of 1 and 2** Each glycoside (2 mg) was heated in 5% $H_2SO_4$ (0.05 ml) at 100 °C for 1 h. The reaction mixture was diluted with water and extracted with ethyl acetate 3 times. The ethyl acetate layer was concentrated and subjected to HPLC to reveal a peak due to *trans-p*-coumaric acid. HPLC conditions: column, Develosil ODS-7 4.6 mm × 25 cm; solvent, 22.5% $CH_3CN$ aq. + 0.05% trifluoroacetic acid (THF); flow rate, 1.0 ml/min; UV nm; 315; $t_R$, 7.6 min. The water layer was passed through an Amberlite IRA-60E column. The water eluate was concentrated and the residue was reacted with D-cysteine (0.05 mg) in water (0.03 ml) and pyridine (0.015 ml) at 60 °C for 1 h with stirring. After evaporation of the solvent, pyridine (0.015 ml), hexamethyl disilazane (0.015 ml) and trimethyl silylchloride (0.015 ml) were added to the residue. The reaction mixture was heated at 60 °C for 30 min, then centrifuged, and the supernatant was applied to GC. GC conditions: column, Supelco SPB™-1, 0.25 mm × 27 m; column temperature, 230 °C; carrier gas, $N_2$; $t_R$, D-glucose 18.52 min, L-glucose 18.00 min, D-rhamnose 12.47 min, L-rhamnose 12.64 min. D-Glucose and L-rhamnose were detected from **1** and **2** at the ratio of 1 : 1. The ratio of each sugar was determined by its peak area.

**Enzymatic Hydrolysis of 1** Compound **1** (2 mg) and naringinase (10 mg) in a citrate buffer (2 ml, pH 4) were stirred overnight at room temp. The reaction mixture was extracted with ethyl acetate 3 times. The ethyl acetate layer was concentrated and subjected to TLC to reveal a spot due to geraniol. TLC conditions: solvent, $CHCl_3$–MeOH (95 : 5); *Rf*, 0.67.

**Photo-Oxidation of Geraniol** Geraniol was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. A mixture of geraniol (2.0 g), Rose Bengal (100 mg) and MeOH (100 ml) was irradiated using a mercury lamp (250 W) for 30 min, and then the solvent was removed by evaporation. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (100 : 0→99 : 5), to give **2a** (12 mg). $^1$H-NMR ($CDCl_3$) $\delta$: 1.32 (6H, s, H-8, 9), 1.66 (3H, s, H-10), 2.72 (2H, d, $J$ = 6.0 Hz, H-4), 4.17 (2H, d, $J$ = 7.2 Hz, H-1), 5.43 (1H, m, H-2), 5.62 (1H, dd, $J$ = 16.0, 6.0 Hz, H-5), 5.64 (1H, d, $J$ = 16.0 Hz, H-6). $^{13}$C-NMR ($CDCl_3$) $\delta$: 16.3 (C-10), 29.8 (C-8, 9), 42.1 (C-4), 59.4 (C-1), 70.7 (C-7), 124.1/124.3 (C-2, 5), 138.4 (C-3), 140.0 (C-6).

**Enzymatic Hydrolysis of 2** Compound **2** (2 mg) and naringinase (10 mg) in a citrate buffer (2 ml, pH 4) were stirred overnight at room temp. The reaction mixture was extracted 3 times with ethyl acetate. The ethyl acetate layer was concentrated and subjected to TLC to reveal a spot due to **2a**. TLC conditions: solvent, $CHCl_3$–MeOH (95 : 5); *Rf*, 0.39.

**Estimation of Inhibitory Effects of Ku-Ding-Cha Glycosides on ACAT** Reagents other than Ku-Ding-Cha constituents were purchased from the following companies: (±)-dithiothreitol (DTT), Wako Pure Chemical Industries Ltd., Osaka, Japan; bovine serum albumin (BSA), ICN Biomedicals Inc., Ohio, U.S.A.; [1-$^{14}$C]oleoyl CoA, Du Pont NEN Products, Boston, U.S.A. The inhibitory activity of each sample was determined by the reported method[11] with a slight modification. A microsomal protein was prepared from the livers of rats. A mixture consisting of microsomal protein solution [17.3 mg/ml in 10 mM HEPES buffer (pH 7.4)] (8 $\mu$l), 100 mM DTT (0.8 $\mu$l), 400 $\mu$M BSA (4 $\mu$l), a tested sample solution [4 $\mu$l; in water–dimethylsulfoxide (9 : 1, v/v)], 1.5 M phosphate buffer (pH 7.4) (8 $\mu$l), 100 $\mu$M [1-$^{14}$C]oleoyl-CoA (11.1 MBq/mmol) (5 $\mu$l), and water (15.2 $\mu$l), was incubated for 5 min at 30 °C. The enzyme reaction was stopped by adding MeOH (200 $\mu$l), and lipids were extracted with *n*-hexane. Cholesteryl oleate formation was quantified by TLC. TLC conditions: plate, Kieselgel 60 $F_{254}$ plates (0.25 mm thick); solvent, petroleum ether–diethyl ether–acetic acid (85 : 15 : 3); *Rf*, 0.65. The inhibitory activity (%) was calculated using the following equation, and was given as the mean value of five experiments.

$$\text{inhibitory activity (\%)} = (1 - B/A) \times 100$$

where $A$ is the ACAT activity in the absence of the tested sample, and $B$ is that in the presence of the samples.

**Acknowledgement** We thank the staff of the Analytical Laboratory of the University of Shizuoka for elemental analysis and measurement of MS.

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