

Activity-Guided Isolation of Triterpenoid Acyl CoA Cholesteryl Acyl Transferase (ACAT) Inhibitors from *Ilex kudincha*

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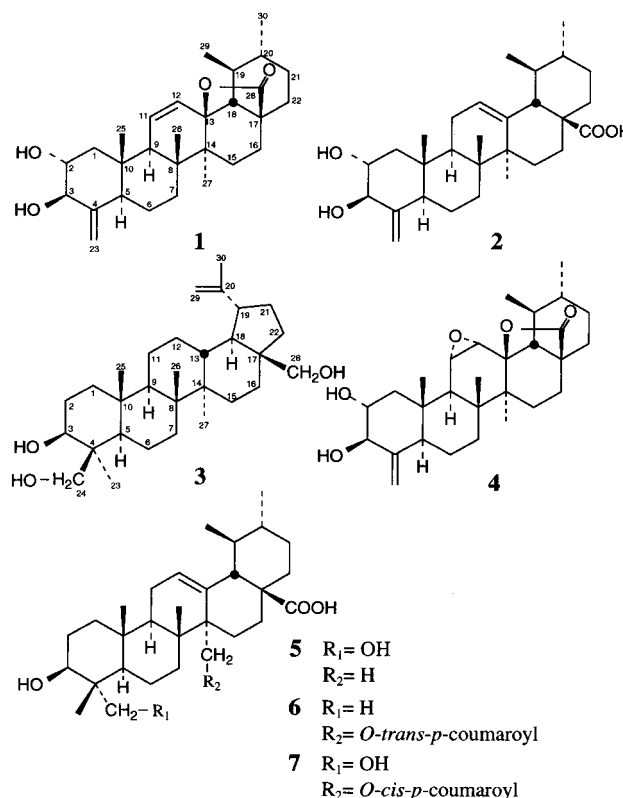
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Activity-guided fractionation of a methanol extract of the leaves of *Ilex kudincha* led to the isolation of seven acyl CoA cholesteryl acyl transferase (ACAT) inhibitory triterpenes. Four of them were identified by spectroscopic methods as ulmoidol (**4**), 23-hydroxyursolic acid (**5**), 27-*trans*-*p*-coumaroyloxyursolic acid (**6**), and 27-*cis*-*p*-coumaroyloxyursolic acid (**7**), and three were new compounds named ilekudinols A–C (**1**–**3**). The structures of these new triterpenoids were elucidated as 2 α ,3 β -dihydroxy-24-nor-urs-4(23),11-dien-28,13 β -olide (**1**), 2 α ,3 β -dihydroxy-24-nor-urs-4(23),12-dien-28-oic acid (**2**), and 3 β ,24,28-trihydroxylupane (**3**). Compounds **1**–**7** showed potent inhibitory activity in the ACAT assay.

An acyl CoA cholesteryl acyl transferase (ACAT) catalyzes the formation of intracellular esterification of cholesterol in various tissues. Inhibitors of ACAT may serve as new types of medicines to treat arteriosclerosis and obesity. In the course of our studies to discover novel ACAT inhibitors from natural sources, we reported recently the isolation and structural elucidation of three monoterpene glycosides from the leaves of *Ligustrum pedunculare* Rehd. (Oleaceae),¹ which is used as a health-giving tea called "Ku-Ding-Cha" in the south and west regions of the People's Republic of China. In the People's Republic of China, the leaves of *Ilex kudincha* C. J. Tseng (Aquifoliaceae) (in Guangxi Province) and *Ilex cornuta* Lindl. ex Paxt. and *Ilex latifolia* Thunb. (in Zhejiang) are also called "Ku-Ding-Cha" and are used in the same manner as *L. pedunculare*.² Also, *I. kudincha* is used as a tea exhibiting an anti-obesity activity in the People's Republic of China.³ The ACAT inhibitory activity of the methanol extract of *I. kudincha* was more potent than that of *L. pedunculare*.⁴ Chen et al. have reported the hypotensive and anti-obesity activities of *I. kudincha*,⁵ while Ouyang et al. reported the presence of twelve ursane-type triterpenoids from this species.^{6–8} Herein we report the isolation of seven ACAT inhibitory triterpenoids (**1**–**7**) from a diethyl ether extract of the leaves of *I. kudincha*, of which **1**–**3** are new compounds.

The leaves of *I. kudincha* were extracted with methanol and the methanol extract was partitioned between water and diethyl ether. The bioactivity of the methanol extract against an ACAT test system was concentrated in the diethyl ether fraction. The diethyl ether fraction was chromatographed on a silica gel column with chloroform–methanol mixtures to give fractions A–P. The biological activity was concentrated in fractions F–P. Combined fractions K and L were rechromatographed by HPLC equipped with an ODS column to give subfractions a–k. The inhibitory activities were observed in subfractions d–i. Subfractions e, f, and i were separated by semipreparative HPLC with an acetonitrile–water–trifluoroacetic acid solvent system to afford compounds **1**–**7** with ACAT



inhibitory activity. Compounds **4**–**7** were identified by comparison of spectral data with reported data as ulmoidol (**4**),⁹ 23-hydroxyursolic acid (**5**),¹⁰ 27-*trans*-*p*-coumaroyloxyursolic acid (**6**),¹¹ and 27-*cis*-*p*-coumaroyloxyursolic acid (**7**).¹¹

Ilekudinol A (**1**) has the elemental composition $\text{C}_{29}\text{H}_{42}\text{O}_4$ as determined by high-resolution FABMS. Its ^1H NMR spectrum suggested the presence of three singlet methyls [δ 0.81, 1.18, 1.21 (each 3H, s)], two doublet methyls [δ 0.86, 0.98 (each 3H, d, $J = 6$ Hz)], two carbinyl protons [δ 4.08 (1H, m), 4.33 (1H, br d, $J = 9$ Hz)] and four olefinic protons [δ 4.97 (1H, br s), 5.65 (1H, dd, $J = 10, 3$ Hz), 5.83 (1H, br s), 6.13 (1H, br d, $J = 10$ Hz)]. In the COSY spectrum the carbinyl proton signal at δ 4.33 was correlated to the carbinyl proton signal at δ 4.08 and the two olefinic proton

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Table 1. ACAT Inhibitory Activity of Compounds 1–7

| compound | IC ₅₀ (10 ⁻⁵ M) |
|----------|---------------------------------------|
| 1 | 15.4 |
| 2 | 4.4 |
| 3 | 32.8 |
| 4 | 46.8 |
| 5 | 6.4 |
| 6 | 7.3 |
| 7 | 19.4 |

signals at δ 4.97 and 5.83, which were both correlated to the carbon signal at δ 104.9 in the HMQC spectrum. The two olefinic proton signals (δ 4.97, 5.83) were correlated to the carbon signals at δ 50.2, 79.4, and 151.6 which were assigned to C-5, C-3, and C-4, respectively, by considering the chemical shifts of those in ulmoidol (**4**).⁹ The two olefinic protons at δ 5.65 and 6.13 were coupled with each other in the COSY spectrum and correlated to the carbon signal at δ 89.4 which was assigned to C-13, by comparing the analogous chemical shifts in ulmoidol (**4**)⁹ and saikosaponin homologues.¹² Based on these data, **1** could be

assigned as 2 α ,3 β -dihydroxy-24-nor-urs-4(23),11-dien-28,13 β -olide. This compound was assumed to be a biosynthetic precursor of compound **4**.

Ilekudinol B (**2**) was shown to have the molecular formula C₂₉H₄₄O₄ by HRFABMS, indicating eight degrees of unsaturation. The ¹H NMR spectrum was similar to that of ilekudinol A (**1**), showing three singlet methyls at δ 0.82, 1.08, and 1.21 (each 3H, s), two doublet methyls at δ 0.97 and 1.00 (each 3H, d, J = 6 Hz), two carbinyis at δ 4.01 (1H, m) and 4.32 (1H, br d, J = 9 Hz), three olefinic proton signals at δ 4.92, 5.48, and 5.80 (each 1H, br s), and a signal at δ 5.48 (1H, br s), and indicated the presence of a trisubstituted double bond. In the ¹³C NMR spectrum, the carbon signals due to the A- and B-rings were almost superimposable on those of ilekudinol A (**1**) and those due to the C-, D-, and E-rings were almost superimposable on those of 6 β -hydroxyasiatic acid (2 α ,3 β ,6 β ,23-tetrahydroxyurs-12-en-28-oic acid).¹³ The EIMS fragment m/z 248 was characteristic peak due to a *retro*-Diels–Alder fragmentation of Δ^{12} -ursene-type triterpenoid.¹⁴ From the

Table 2. ¹H and ¹³C NMR Spectral Data of Ilekudinols A–C (**1**–**3**) and Ulmoidol (**4**) in Pyridine-*d*₅ at 35 °C^a

| | 1 | | | 2 | | | 3 | | | 4 | | |
|------------|------------------------|-----------------|----------------------------------|------------------------|-----------------|----------------------------------|----------------------------|-----------------|-------------|------------------------|-----------------|-------------------|
| | ¹ H | ¹³ C | HMBC (C) | ¹ H | ¹³ C | HMBC (C) | ¹ H | ¹³ C | HMBC (C) | ¹ H | ¹³ C | HMBC (C) |
| 1 α | 1.43 ^b | | | 1.46 ^b | | | 0.98 ^b | | | 1.32 m | | |
| 1 β | 2.50 dd (12.5, 5) | 47.5 | 2, 3, 5, 6, 9, 10, 25 | 2.31 dd (12.5, 5) | 48.3 | 2, 3, 5, 10, 25 | 1.70 ^b | 39.1 | | 2.58 dd (13, 5) | 46.3 | |
| 2 | 4.08 m | 73.6 | 1, 3 | 4.01 m | 73.5 | | | 28.7 | | 4.06 m | 73.2 | |
| 3 | 4.33 br d (9) | 79.4 | 2, 4, 23 | 4.32 br d (9) | 79.5 | 2, 4, 23 | 3.65 ^b | 80.2 | 23, 24 | 4.31 br d (9) | 78.8 | 2, 4, 23 |
| 4 | | 151.6 | | | 150.4 | | | 43.3 | | | 148.2 | |
| 5 | | 50.2 | | | 50.7 | | 0.94 ^b | 56.5 | | 1.67 m | 49.6 | |
| 6 | | 20.9 | | | 21.4 | | | 19.1 | | | 20.1 | |
| 7 | | 30.0 | | | 32.0 | | | 34.9 | | | 29.9 | |
| 8 | | 42.1 | | | 39.5 | | | 41.3 | | | 41.5 | |
| 9 | 2.26 br s | 51.0 | 1, 10, 11, 25, 26 | | 45.5 | | | 50.9 | | | 49.2 | |
| 10 | | 38.2 | | | 38.8 | | | 37.3 | | | 38.0 | |
| 11 | 6.13 br d (10) | 133.6 | 8, 9, 13 | | 23.8 | | | 21.4 | | 3.31 m | 54.7 | 9, 10, 12 |
| 12 | 5.65 dd (10, 3) | 129.4 | 9, 13, 18 | 5.48 br s | 124.0 | 14 | | 25.8 | | 3.08 d (3) | 56.2 | 11, 13, 14, 18 |
| 13 | | 89.4 | | | 139.6 | | | 37.7 | | | 89.0 | |
| 14 | | 42.4 | | | 42.8 | | | 43.0 | | | 41.5 | |
| 15 | | 25.9 | | | 28.6 | | | 27.6 | | | 26.8 | |
| 16 | 2.16 m | 23.2 | 15, 17, 28 | | 24.7 | | | 30.1 | | | 22.7 | |
| 17 | | 45.2 | | | 48.2 | | | 48.4 | | | 45.1 | |
| 18 | 1.65 ^b | 60.6 | 12, 13, 14, 16, 17, 19, 28 | 2.63 br d (11) | 53.9 | 12, 13, 16, 17, 20, 28, 29 | 1.70 dd (11.5, 11.5) | 49.2 | 13, 17, 19 | 1.77 ^b | 60.6 | 13 |
| 19 | 1.80 ^b | 40.4 | | | 39.5 | | | 48.6 | | | 40.2 | |
| 20 | | 38.3 | | | 40.2 | | | 151.3 | | | 37.5 | |
| 21 | | 31.0 | | | 31.1 | | | 30.5 | | | 30.5 | |
| 22 | | 32.0 | | | 25.0 | | | 35.1 | | | 31.4 | |
| 23 | 4.97 br s 5.83 br s | 104.9 | 3, 4, 5 3, 4, 5 | 4.92 br s 5.80 br s | 104.7 | 3, 4, 5 3, 4, 5 | 1.54 s | 23.6 | 3, 4, 5, 24 | 4.95 br s 5.83 br s | 105.3 | 3, 4, 5 3, 5 |
| 24 | | | | | | | 3.69 br d (10.5) | 64.5 | 3, 4, 23 | | | |
| 25 | 0.81 s | 16.4 | 1, 5, 9, 10 | 0.82 s | 15.3 | 1, 5, 9, 10 | 4.49 br d (10.5) | | 3, 4, 23 | | | |
| 26 | 1.21 s | 16.1 | 7, 8, 9 | 1.08 s | 17.5 | 7, 8, 9 | 0.85 s | 16.8 | 1, 5, 9, 10 | 0.90 s | 15.6 | 1, 5, 9, 10 |
| 27 | 1.18 s | 19.1 | 13, 14, 15 | 1.21 s | 23.9 | 8, 14, 15 | 0.98 s | 16.1 | 7 | 1.15 s | 20.0 | 7, 8, 9, 14 |
| 28 | | 179.3 | | | 179.9 | | 1.05 s | 15.0 | 13, 14, 15 | 1.17 s | 1.17 | 13, 14, 15 |
| 29 | 0.98 d (6) | 18.0 | 18, 19, 20 | 1.00 d (6) | 17.6 | 18, 19, 20 | 3.66 br d (11) | 59.5 | 16, 22 | | 179.1 | |
| 30 | 0.86 d (6) | 19.1 | 19, 20, 21 | 0.97 d (6) | 21.4 | 19, 20 | 4.08 br d (11) | | 16, 22 | | | |
| | | | | | | | 4.74 m | 109.9 | 19, 30 | 1.32 d (6) | 17.2 | 18, 19, 20 |
| | | | | | | | 4.89 br s | | 19, 30 | | | |
| | | | | | | | 1.78 s | 19.3 | 19, 20, 29 | 0.91 d (6) | 19.5 | 19, 20, 21 |

^a Coupling constants (Hz) are in parentheses; assignments are based upon COSY, HMQC, and HMBC experiments. ^b Overlapping with other signals.

spectral data obtained and the analysis of its COSY, HMQC and HMBC spectra, the structure of ilekudinol B was determined as 2 α ,3 β -dihydroxy-24-nor-urs-4(23),12-dien-28-oic acid (**2**).

A molecular formula of C₃₀H₅₀O₃ was determined by HREIMS (*m/z* 458.3719) for ilekudinol C (**3**). The ¹H NMR spectrum, with five singlet methyls at δ 0.85, 0.98, 1.05, 1.54, and 1.78 (each 3H, s), a carbinyll at δ 3.65 (overlapped), two sets of oxymethylenes at δ 3.66 (1H, br d, *J* = 11 Hz), 4.08 (1H, br d, *J* = 11 Hz), 3.69 (1H, br d, *J* = 10.5 Hz), and 4.49 (1H, br d, *J* = 10.5 Hz), and a pair of exomethylene proton signals at δ 4.74 (1H, m), 4.89 (1H, br s), suggested a lupane skeleton for this compound.¹⁵ The oxymethylene proton signals at δ 3.69 and 4.49 were correlated to the carbon signals at δ 64.5 in the HMQC spectrum and to the carbon signals at δ 23.6, 43.3, 56.5, and 80.2, which were assigned to C-23, C-4, C-5, and C-3, respectively, by comparing the chemical shifts of those of 3 β ,24-dihydroxylupane¹⁶ in the HMBC spectrum. These data led us to assign the structure of ilekudinol C as 3 β ,24,28-trihydroxylupane.

Compounds **1**–**7** were isolated as inhibitory active components against ACAT of the methanol extract of *I. kudincha* and the IC₅₀ data are listed in Table 1. These compounds are a new type of inhibitors against ACAT. The inhibitory activity of compounds **2**, **5**, and **6** are much higher than those of lignans (IC₅₀ 25–207 mM) isolated from *Schisandra chinensis* (Turcz.) Baill., *Machilus thunbergii* Sieb. et Zucc., *Magnolia denudata* Desr.,¹⁷ and *Magnolia ovata* Thunb.¹⁸ and polyacetylenes (IC₅₀ 42–86 mM) from *Panax ginseng* C. A. May.¹⁹ as ACAT inhibitors. Compounds **2**, **5**, and **6** had higher inhibitory activity than those of compounds **1**, **3**, **4**, and **7**. It is possible that a free carboxylic group was a function to be more inhibitory active against ACAT.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 polarimeter. ¹H and ¹³C NMR spectra were run on a JEOL α -400 NMR spectrometer (400 MHz for ¹H and 100 MHz for ¹³C) at 35 °C in pyridine-*d*₅ with tetramethylsilane as internal standard. High-resolution FAB (in a positive mode) and EI mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. Radioactivities were measured on a Aloka liquid scintillation system LSC-700. HPLC was carried out on a JASCO System 800 instrument. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography. Precoated silica gel Kieselgel 60 F₂₅₄ plates (0.25 mm thick) were used for TLC, and the spots were detected by spraying with 50% H₂SO₄, followed by heating.

Materials. Reagents for the bioassay were purchased from the following companies: (±)-dithiothreitol (DTT), Wako Pure Chemical Industries, Ltd., Osaka, Japan; bovine serum albumin (BSA), ICN Biomedicals, Inc., Aurora, OH; [1-¹⁴C]-oleoyl CoA, Du Pont NEN Products, Boston, MA.

Plant Material. The leaves of *I. kudincha* C. J. Tseng were collected at Daxin Prefecture, Guangxi Province, People's Republic of China, in December 1996. The plant material was authenticated by Prof. Zhaoguang Liu, Chengdu Institute of Biology, Academia Sinica, Chengdu, People's Republic of China, and a voucher specimen (No. 970401) has been deposited in the Herbarium, School of Pharmaceutical Sciences, University of Shizuoka.

Extraction and Isolation. Air-dried and powdered leaves of *I. kudincha* (1 kg) were extracted twice with methanol for 1 h. The methanolic extract (inhibitory activity 44% at 1 mg/mL) was concentrated under reduced pressure, and the residue was suspended in water and extracted with diethyl ether three times. The diethyl ether extract (inhibitory activity 67% at 1 mg/mL) (46 g) was chromatographed on a silica gel column (9

× 22 cm) using chloroform–methanol mixtures as eluting solvents to give fractions A–P. Combined fractions K (inhibitory activity 74% at 1 mg/mL) and L (inhibitory activity 82% at 1 mg/mL) (4.48 g), eluted with CHCl₃–MeOH (97:3) were subjected to preparative HPLC on an ODS column (5 × 100 cm) using acetonitrile–water (58:42) to yield subfractions a–k. Subfraction e (inhibitory activity 75% at 0.2 mg/mL) (96 mg) was subjected to semipreparative HPLC [column: YMC SH-843-5 C4 2 × 25 cm, CH₃CN–H₂O (42.5:57.5) + 0.05% TFA] to afford **1** (17 mg), **2** (11 mg) and **4** (66 mg). Subfraction f (inhibitory activity 77% at 0.2 mg/mL) (34 mg) was subjected to semipreparative HPLC [column: YMC SH-843-5 C4 2 × 25 cm, CH₃CN–H₂O (45:55) + 0.05% TFA] to afford **5** (7.5 mg). Finally, subfraction i (inhibitory activity 85% at 0.2 mg/mL) (118 mg) was subjected to semipreparative HPLC [column: Develosil PhA-5 2 × 25 cm, CH₃CN–H₂O (52.5:47.5) + 0.05% TFA] to afford **3** (13 mg), **4** (10 mg), **6** (13 mg) and **7** (22 mg). The known compounds (**4**–**7**) were identified by comparison of ¹H and ¹³C NMR data with reported data.^{9–11}

Ilekudinol A (1): amorphous powder, [α]_D²⁵ +64.0° (*c* 1.68, MeOH); IR (CHCl₃) ν_{\max} 3420, 3010, 1750, 1045 cm⁻¹; ¹H and ¹³C NMR, see Table 2; EIMS *m/z* 454 [M]⁺, 435 [M – H₂O]⁺, 410 [M – CO₂]⁺, 390, 375; HRFABMS *m/z* 455.3211 [matrix, dithiothreitol- α -thioglycerol (1:2)], calcd for C₂₉H₄₂O₄ + H, 455.3161.

Ilekudinol B (2): amorphous powder, [α]_D²⁵ +75.5° (*c* 1.12, MeOH); IR (CHCl₃) ν_{\max} 3450, 3015, 1690, 1040 cm⁻¹; ¹H and ¹³C NMR, see Table 2; EIMS *m/z* 456 [M]⁺, 438 [M – H₂O]⁺, 248 [C₁₆H₂₄O₂]⁺; HRFABMS *m/z* 479.3095 [matrix, dithiothreitol- α -thioglycerol (1:2)], calcd for C₂₉H₄₄O₄ + Na, 479.3137.

Ilekudinol C (3): amorphous powder, [α]_D²⁵ +16.6° (*c* 1.34, MeOH); IR (CHCl₃) ν_{\max} 3430, 3010, 2930, 1045 cm⁻¹; ¹H and ¹³C NMR, see Table 2; EIMS *m/z* 458 [M]⁺, 440 [M – H₂O]⁺, 427 [M – CH₂OH]⁺, 409 [M – H₂O – CH₂OH]⁺; HREIMS *m/z* 458.3719, calcd for C₃₀H₅₀O₃, 458.3760.

Estimation of Inhibitory Activities of the Fractions and Triterpenes from *I. kudincha* against Acyl CoA Cholesteryl Acyl Transferase (ACAT). The inhibitory activity of each sample was determined by a reported method²⁰ with slight modifications. The 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 mL), 100 mM DTT (0.8 mL), 400 mM BSA (4 mL), a test sample solution [4 mL; in water–dimethyl sulfoxide (9:1, by volume)], 1.5 M phosphate buffer (pH 7.4) (8 mL), 100 mM [1-¹⁴C]oleoyl-CoA (11.1 MBq/mmol) (5 mL), and water (15.2 mL), was incubated for 5 min at 30 °C. The enzyme reaction was stopped by adding methanol (200 mL), and lipids were extracted with *n*-hexane. Cholesteryl oleate formation was quantified by the radioactivity of the zone after TLC. TLC conditions: plate, Kieselgel 60 F₂₅₄ plates (0.25 mm thick); solvent, petroleum ether–diethyl ether–acetic acid (85:15:3); *R_f* 0.65. The inhibitory activity (%) [(1 – B/A) × 100] was calculated and was given as the mean value of five experiments, where A is the ACAT activity in the absence of the test sample, and B is that in the presence of the samples.

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