Lupane and Oleanane Triterpenoids from the Cones of *Liquidamber styraciflua*

Yuko Fukuda, Takeshi Yamada, Shun-ich Wada, Kuniyoshi Sakai, Shunyo Matsunaga, and Reiko Tanaka*

Department of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

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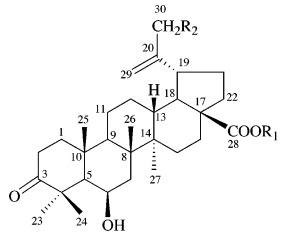
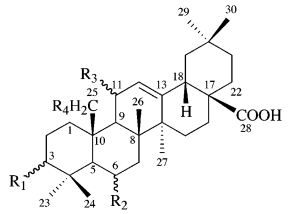
A new lupane- (**1**) and a new oleanane-type (**2**) triterpenoid, together with a known compound, massagenic acid G, were isolated from the cones of *Liquidamber styraciflua*. The structures of **1** and **2** were determined as 6β,30-dihydroxy-3-oxolup-20(29)-en-28-oic acid and 3α-hydroxy-11-oxoolean-12-en-28-oic acid, respectively, on the basis of spectroscopic methods and chemical conversion. Compound **1** and several structural analogues were evaluated for cytotoxicity against the P388 (murine lymphocyte leukemia) and the A549 (human lung cancer) cell lines.

In an earlier report, we described the isolation and structure determination of two new compounds, 25-acetoxy-3α-hydroxyolean-12-en-28-oic acid (**5**) and 3α,25-dihydroxyolean-12-en-28-oic acid, from the cones of *Liquidamber styraciflua* L. (Hamamelidaceae). Their cytotoxicity against a disease-oriented panel of 39 human cancer cell lines and the compounds 3,11-dioxoolean-12-en-28-oic acid (**3**) and 6β-hydroxy-3-oxolup-20(29)-en-28-oic acid (**4**) were also reported.[1] Recently, we described the in vivo two-stage mouse skin carcinogenesis inhibition evaluation of **5** and 3β,25-epoxy-3α-hydroxylup-20(29)-en-28-oic acid (**6**).[2] Further careful examination of the cones of *L. styraciflua* has led to the isolation of two new lupane- and oleanane-type triterpenoids, **1** and **2**, together with the known compound massagenic acid G.

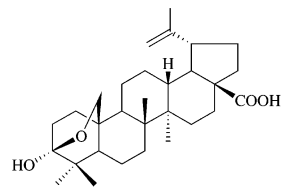
The $CHCl_3$ extract from the cones of *L. styraciflua* was separated by silica gel column chromatography, Sephadex LH-20, and medium-pressure liquid chromatography (MPLC), and two new (**1**, **2**) and one known triterpenoid were obtained. The latter was confirmed as massagenic acid G, which has been isolated from *Melilotus messanensis*, and its physical and spectroscopic data showed good agreement with those already published.[3,4] GC-MS data of 3,11-dioxoolean-12-en-28-oic acid (**3**) were reported already,[5,6] although detailed spectroscopic data were not published so far for the compound. Therefore, we describe the NMR data of **3** in Table 1.

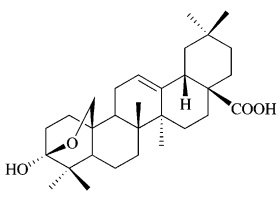




1: R_1 = H, R_3 = OH
1a: R_1 = CH_3, R_3 = OH
4: R_1 = H, R_3 = H

2: R_1 = α-OH, R_2 = H_2, R_3 = O, R_4 = H
3: R_1 = O, R_2 = H_2, R_3 = O, R_4 = H
5: R_1 = α-OH, R_2 = H_2, R_3 = H_2, R_4 = $OCOCH_3$
8: R_1 = O, R_2 = β-OH, R_3 = H_2, R_4 = H





6 **7**

The molecular formula of compound **1** was assigned as $C_{30}H_{46}O_5$ (M^+; *m*/*z* 486.3335) by HREIMS. The IR spectrum showed hydroxyl groups (ν_{max} 3446 cm^{-1}), a six-membered-ring ketone (ν_{max} 1718 cm^{-1}), a carboxyl group (ν_{max} 3100−2700, 1699 cm^{-1}), a *gem*-dimethyl group (ν_{max} 1377 cm^{-1}), and a terminal methylene (ν_{max} 1645 cm^{-1}). The ^1H and ^{13}C NMR spectra ($CDCl_3$) of **1** (Table 1) exhibited signals for six tertiary methyls, a primary hydroxyl group [δ_H 4.55 (2H, s), 64.6 (t)], 10 methylenes, four methines, a secondary axial hydroxyl group [δ_H 4.65 (1H, brs); δ_C 68.5 (d)], an exocyclic methylene group [δ_H 5.21 (1H, d), 5.55 (1H, d); δ_C 106.1(t), 157.1 (s)], five quaternary carbons, a carboxylic acid [δ_C 178.8 (s)], and a saturated ketone group [δ_C 215.7 (s)]. In the HMBC spectrum (Table S1, Supporting Information) of **1**, correlations were observed from Me-23 (δ_H 1.36) and Me-24 (δ_H 1.66) to C-3 (δ_C 215.7), C-4, and C-5; between H-6α (δ_H 4.65) and C-4, C-5, C-7, C-8, and C-10; and between H_2-29 (δ_H 5.21, 5.55) and C-30 (δ_C 64.6), C-20 (δ_C 157.1), and C-19. Therefore, the secondary and primary hydroxyl groups must be attached at C-6 and C-30. The configuration of the C-6 hydroxyl group was assigned as C-6β axial, because clear NOEs were observed between H-6α and Me-23. On the other hand, NOEs were observed between H-29A (δ_H 5.21) and H-18α and H-19β; H-29B (δ_H 5.55) and H_2-30; and H_2-30 (δ_H 4.55) and H-18α, H-19β, and H-21α, respectively. Methylation with trimethylsilyl-diazomethane gave a methyl ester (**1a**), $C_{31}H_{48}O_3$ (M^+; *m*/*z* 500.3496), δ_H 3.63 (3H, s, COOMe). Finally, the complete structure was determined by synthesizing **1** from 6β-hydroxy-3-oxolup-20(29)-en-28-oic acid (**4**), which was the most abundant triterpene constituent in this cone (Figure S1, Supporting Information). *m*-CPBA oxidation of **4** gave an epoxy derivative (**4a**), and subsequent treatment with NaOEt gave an acetyl derivative (**4b**) and a diol derivative (**4c**). The synthetic compound **4c** was identical with the natural compound **1**. Therefore the structure of **1** was established as summarized in Figure S1, Supporting Information.

Compound **2** was assigned as $C_{30}H_{46}O_4$ (M^+; *m*/*z* 470.3395) by HREIMS. The IR spectrum of **2** showed a hydroxyl (ν_{max} 3436 cm^{-1}), an α,β-unsaturated six-membered-ring ketone (ν_{max} 1655 cm^{-1}), a carboxyl group (ν_{max} 3100−2750, 1700 cm^{-1}), and *gem*-dimethyl groups (ν_{max} 1386 cm^{-1}). The ^1H and ^{13}C NMR spectra of **2** (Table 1) exhibited signals for seven tertiary methyls, nine methylenes, three methines, a hydroxymethine group [δ_H 3.41 (1H, t); δ_C 75.8 (d)], a trisubstituted double bond [δ_H 5.63 (1H, s); δ_C 128.1 (d), 168.3 (s)], six quaternary carbons, a conjugated ketone [δ_C 200.6 (s)], and a carboxylic acid [δ_C 181.7 (s)]. These IR and NMR data were similar to those of 3,11-dioxoolean-12-en-28-oic acid (**3**), which was obtained from *L. styraciflua*, except for the C-3 substituent. In the HMBC spectrum, H-3 (δ_H 3.41) correlated with C-1, C-2, C-4, C-5, C-23, and C-24. The configuration of the C-3 hydroxyl group was established as C-3α axial because the proton signal was observed as a triplet when the coupling constant was 2.7 Hz. NOEs were shown from H-3β (δ_H 3.41) to Me-23 and Me-24 in the NOESY spectrum. Another NOE was observed between H-12 and H-18β; therefore the C/D ring was assigned with

* To whom correspondence should be addressed. Tel and Fax: +81-72-690-1084. E-mail: tanakar@gly.oups.ac.jp.

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Table 1. NMR Data for Compounds **1**,[a] **2**, and **3**[b] (125 and 500 MHz)[c]

	1[a]		**2**[b]		**3**[b]	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1α	42.5 t	1.26 m	33.4 t	1.35 m	39.7 t	1.44 m
1β		1.90 ddd (12.8,6.2,2.7)		2.57 dt (13.5,3.0)		2.96 m
2α	34.8 t	2.35 ddd (14.7,4.4,2.5)	25.4 t	1.51 m	34.2 t	2.37 ddd (15.8,7.0,4.2)
2β		2.92 m		2.01 ddd (3.0,4.1,14.2)		2.60 ddd (15.8,10.8,7.0)
3β	215.7 s		75.8 d	3.41 t (2.7)	217.2 s	
4	49.4 s		37.5 s		47.7 s	
5α	56.9 d	1.26 m	48.4 d	1.22 m	55.3 d	1.30 m
6α	68.5 d	4.65 brs (w/2 = 9.0)	17.3 t	1.45 m	18.7 t	1.50 m
6β				1.31 m		1.50 m
7α	42.4 t	1.68 m	32.8 t	1.63 m	32.2 t	1.64 m
7β		1.84 m		1.34 m		1.40 m
8	40.5 s		45.2 s		44.8 s	
9α	51.2 d	1.47 dd (8.2,5.5)	61.6 d	2.44 s	61.0 d	2.42 s
10	37.2 s		37.4 s		36.8 s	
11			200.6 s		199.6 s	
11α	21.6 s	1.40 m				
11β						
12α	27.3 t	1.29 m	128.1 d	5.63 s	127.9 d	5.67 s
12β		1.83 m				
13β	37.2 d	2.89 ddd (12.6,12.6,3.5)	168.3 s		168.8 s	
14	43.0 s		43.5 s		43.6 s	
15α	30.4 t	1.30 m	27.7 t	1.26 m	27.8 t	1.29 m
15β		1.97 m		1.71 m		1.73 m
16α	32.8 t	1.58 m	22.7 t	2.05 m	22.6 t	2.07 ddd (13.5,5.1,3.3)
16β		2.63 dt (12.6,3.2)		1.73 m		1.76 m
17	56.6 s		45.9 s		46	
18α	50.4 d	2.04 dd (11.2,11.2)				
18β			41.4 d	2.97 dd (13.2,4.3)	41.4 d	3.00 dd (12.0,5.2)
19α			44.1 t	1.63 m	44.1 t	1.63 m
19β	43.5 d	3.55 ddd (11.4,11.4,4.1)		1.22 m		1.22 m
20	157.1 s		30.7 s		30.7 s	
21α	33.1 t	1.69 m	33.6 t	1.39 m	33.6 t	1.38 m
21β		2.42 m		1.28 m		1.30 m
22α	37.5 t	1.70 m	31.6 t	1.68 m	31.5 t	1.66 m
22β		2.26 m		1.78 m		1.78 m
23	25.2 q	1.36 s	28.5 q	0.95 s	26.5 q	1.09 s
24	23.9 q	1.66 s	22.3 q	0.84 s	21.3 q	1.04 s
25	17.0 q	1.58 s	16.1 q	1.12 s	15.6 q	1.23 s
26	17.2 q	1.69 s	19.2 q	0.93 s	18.6 q	0.96 s
27	15.1 q	1.06 s	23.8 q	1.38 s	23.5 q	1.37 s
28	178.8 s		181.7 s		183.0 s	
29A	106.1 t	5.21 d (1.8)	32.8 q	0.94 s	32.8 q	0.94 s
29B		5.55 d (1.8)				
30	64.6 t	4.55 t (1.4)	23.4 q	0.94 s	23.4 q	0.95 s

[a] Pyridine-d_5. [b] $CDCl_3$. [c] Assignments confirmed by decoupling, H/H COSY, NOESY, HMQC, and HMBC spectra. *J* values are given in Hz.

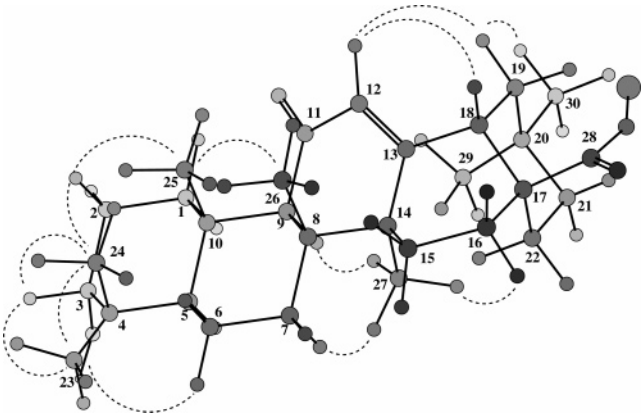

Figure 1. Selected NOESY correlations of **2**.

the *cis* configuration (Figure 1). Accordingly, compound **2** was established as 3α-hydroxy-11-oxoolean-12-en-28-oic acid, which is a new natural product.

Compounds **1**, **4**, **4b**, and **5**−**8** were tested for in vitro cytotoxity against the P388 (murine lymphocyte leukemia) and the A549 (human lung cancer) cell lines using the MTT method. Compounds **2** and **3** were not assayed because they were obtained only in small amounts. The ED_{50} values are listed in Table S2, Supporting Information. Compounds **5**−**8** were found to show weak activity against the P388 and A549 tumor cell lines. Compounds **4** and **8** are skeletal isomers, and the activity of **8** was slightly more potent than that of **4**.

Experimental Section

General Experimental Procedures. Melting points were measured with a Yanagimoto micro-melting point apparatus without correction. Optical rotations were determined with a JASCO DIP-1000 digital polarimeter. IR spectra were recorded using a Perkin-Elmer 1720X FTIR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. $CDCl_3$ and pyridine-d_5 were used as the solvent and Me_4Si (TMS) as the internal standard. EIMS were recorded on a Hitachi 4000H double-focusing mass spectrometer (70 eV). Column chromatography was carried out over silica gel (70−230 mesh, Merck), and medium-pressure liquid chromatography (MPLC) was carried out with silica gel (230−400 mesh, Merck) and LH-20. HPLC was run on a JASCO PU-1586 instrument equipped with a differential refractometer (RI 1531). Fractions obtained from column chromatography were monitored by TLC (silica gel 60 F_{254}, Merck). Preparative TLC was carried out on Merck silica gel F_{254} plates (20 × 20 cm, 0.5 mm thick).

Extraction and Isolation. The extraction and preliminary silica gel column chromatography of the $CHCl_3$ extract of the cones of *L. styraciflua* have been reported,[1] with separation into nine main fractions (A−I). Isolation of 25-acetoxy-3α-hydroxyolean-12-en-28-oic acid (**5**), 3α,25-dihydroxyolean-12-en-28-oic acid, 3,11-dioxoolean-12-en-28-oic acid (**3**), and 6β-hydroxy-3-oxolup-20(29)-en-28-oic acid (**4**) by residues E, G, and H was also reported.[1] Repeated column chromatography of the filtrate of **5** (residue G) on silica gel (1 kg) eluting with $CHCl_3$−EtOAc (5:1) afforded a crystalline solid (fractions 33−47, 575.0 mg), which was recrystallized from MeOH−$CHCl_3$ to give compound **8** (212.0 mg). Further elution with the same solvent gave an amorphous gum, which was subjecteded to LH-20 using $CHCl_3$−MeOH (1:1) and

recrystallized from MeOH−$CHCl_3$ to give compound **6** (43.8 mg); further elution with the same solvent afforded crystalline solids (fractions 73−77, 8.9 mg, and fractions 136−150, 526.6 mg). The former was recrystallized from MeOH−$CHCl_3$ to give compound **1** (12.1 mg), and the latter was subjected to LH-20 using $CHCl_3$−MeOH (1:1) and recrystallized from MeOH−$CHCl_3$ to give compound **2** (2.7 mg). Repeated column chromatography of residue H on MPLC (300 g), eluting with $CHCl_3$−EtOAc (5:1), afforded two crystalline solids (fractions 51−53, 547 mg, and fractions 73−77, 34.4 mg), which were recrystallized from MeOH−$CHCl_3$, respectively, to give compound **7** (185.0 mg) and massagenic acid G (221. mg).

Compound 1: colorless prisms; mp 109−110 °C (from MeOH−$CHCl_3$); $[\alpha]_D^{27}$ −23.8 (*c* 0.10, $CHCl_3$); IR (KBr) ν_{max} 3446 (OH), 3100−2700 and 1699 (COOH), 2929, 2870, 1718 (C═O), 1645 (C═CH_2), 1458, 1377, 1364, 1180, 1051, 967, 927, 898, 758 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 486 (1) $[M]^+$, 468 (7) $[M - H_2O]^+$, 422 (3), 285 (3), 232 (21), 219 (26), 217 (31), 205 (42), 203 (100), 187 (68), 185 (63), 175 (49), 148 (47), 133 (54); HREIMS *m*/*z* $C_{30}H_{46}O_5$ (M^+; *m*/*z* 486.3335, requires 486.3342).

Methyl 6*β*,30-dihydroxy-3-oxolup-20(29)-en-28-oate (1a). A MeOH (1 mL) and C_6H_6 (1 mL) solution of compound **1** (10.8 mg) was added to a trimethylsilyldiazomethane 2.0 M solution in *n*-hexane ($TMSCHN_2$) (0.5 mL) and left for 5 h at room temperature. Evaporation of the solvent under reduced pressure afforded a residue, which was purified by preparative TLC ($CHCl_3$−MeOH, 25:1) to afford compound **1a** (8.6 mg): colorless prisms; mp 110−113 °C (from MeOH−$CHCl_3$); $[\alpha]_D^{21}$ −39.5 (*c* 0.39, $CHCl_3$); IR (KBr) ν_{max} 2947, 2869, 1723 (COOMe), 1700 (C═O), 1649, 1456, 1375, 1314, 1189, 1171, 1144, 1055, 895 cm^{-1}; HREIMS *m*/*z* $C_{31}H_{48}O_5$ (M^+; *m*/*z* 500.3496, requires 500.3499).

Synthesis of Compound 1 from 6*β*-Hydroxy-3-oxolup-20(29)-en-28-oic acid (4). To a solution of 6*β*-hydroxy-3-oxolup-20(29)-en-28-oic acid (**4**) (517.5 mg, 1.1 mmol) in CH_2Cl_2 (3.0 mL) was added *m*-CPBA (228.4 mg, 1.1 mmol), and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with 1 M K_2CO_3 and extracted with CH_2Cl_2. The CH_2Cl_2 layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was purified by HPLC using MeOH−$CHCl_3$ (80:20) as an eluent to afford 20,29-epoxy-6*β*-hydroxy-3-oxolupan-28-oic acid (**4a**) (507.6 mg) as a colorless powder. To a solution of **4a** (388.8 mg, 0.8 mmol) in EtOH (10.0 mL) was added sodium ethoxide (157.8 mg, 2.3 mmol), and the reaction mixture was stirred at room temperature for 72 h. The mixture was neutralized with AcOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was chromatographed over a silica gel column with a $CHCl_3$−EtOAc−MeOH gradient as the eluent. The EtOAc−MeOH (100:1) eluate (167.2 mg) was further purified by HPLC using MeOH−H_2O (70:30) as the eluent to afford **4b** (37.8 mg) and **4c** (50.5 mg) as colorless powders. Compound **4c** was identical with natural compound **1**.

Compound 2: colorless crystals; mp 169−170 °C (MeOH−$CHCl_3$); $[\alpha]_D^{21}$ +76.2 (*c* 0.15, $CHCl_3$); IR (KBr) ν_{max} 3436 (OH), 3100−2750 and 1700 (COOH), 2927, 2857, 1655 (C═C−C═O), 1386, 1364, 1181, 803, 753 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 470 (33) $[M]^+$, 452 (5) $[M - H_2O]^+$, 424 (8), 303 (73), 263 (12), 262 (100), 257 (56), 248 (15), 235 (16), 217 (44), 203 (19), 193 (13), 189 (59), 187 (16), 175 (80), 161 (19), 135 (28), 119 (37), 105 (24); HREIMS *m*/*z* $C_{30}H_{46}O_4$ (M^+; *m*/*z* 470.3395, requires 470.3393].

Assay for Cytotoxicity. A549 and P388 cells were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. The cytotoxicity against cancer cells was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[7] The cells (1×10^4 cells/well) were cultured in 96-well culture plates with the compounds for 72 h. Stock MTT solution (5 mg/mL) was added to each well (10 μL/well), and the plates were incubated at 37 °C for another 4 h. Acidic SDS solution (50 μL of 0.02 N HCl in 20% SDS solution) was added to each well and mixed thoroughly. The absorbance was read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 620 nm.

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Supporting Information Available: This information (Figure S1, Table S1, and Table S2) is available free of charge via the Internet at http://pubs.acs.org/jnp.

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