Triterpene Saponins and Lignans from the Roots of *Pulsatilla chinensis* and Their Cytotoxic Activity against HL-60 Cells

Yoshihiro Mimaki,* Minpei Kuroda, Tomoki Asano, and Yutaka Sashida*

School of Pharmacy, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan

Received April 19, 1999

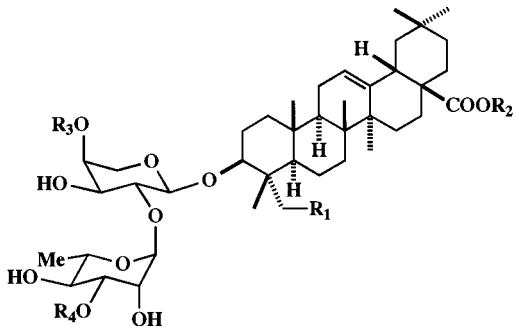
Two new triterpene saponins (**8** and **9**) and seven previously reported triterpene saponins (**1**−**7**) based upon oleanolic acid or hederagenin, along with two known lignans, (+)-pinoresinol (**10**) and *β*-peltatin (**11**), were isolated from a saponin fraction prepared from the MeOH extract of the roots of *Pulsatilla chinensis*. The structures of the new saponins were determined by spectroscopic analysis, including 2D NMR spectroscopic techniques, and the results of hydrolytic cleavage. The isolated compounds and some derivatives were evaluated for their cytotoxic activity against HL-60 human leukemia cells.

Pulsatillae Radix, the roots of *Pulsatilla chinensis* (Bunge) Regel (Ranunculaceae), has been used in traditional Chinese medicine for the treatment of intestinal amebiasis, malaria, vaginal trichomoniasis, and bacterial infections. According to a Chinese herbal dictionary,[1] triterpene saponins are reputed to be mainly responsible for these activities. Recently, a triterpene named pulsatillic acid[2] and three triterpene glycosides, pulsatillosides A−C,[2,3] have been isolated from *P. chinensis*. Pulsatillic acid showed cytotoxic activities against P-388 murine leukemia, Lewis lung carcinoma, and human large-cell lung carcinoma cells.[2] During the course of a systematic survey of the bioactive constituents from higher plants,[4] we have found that a saponin fraction prepared from the MeOH extract of the roots of *P. chinensis* showed cytotoxic activity against HL-60 human promyelocytic leukemia cells. Chromatographic separation of the saponin fraction gave two new triterpene saponins (**8** and **9**) and seven previously reported triterpene saponins (**1**−**7**) based upon oleanolic acid or hederagenin, along with two known lignans (**10** and **11**). In this paper, we report the structural determination of the new saponins on the basis of spectroscopic analysis, including 2D NMR spectroscopic techniques, and the results of hydrolytic cleavage, and also the cytotoxic activity of the isolated compounds and some derivatives against HL-60 cells.

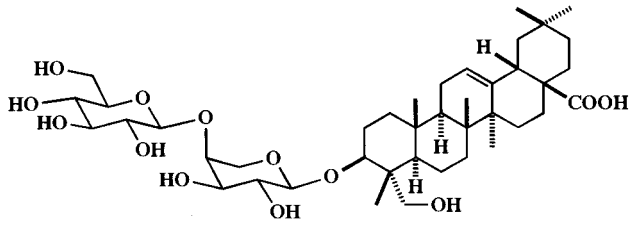
Results and Discussion

The dried roots of *P. chinensis* (3.5 kg) were extracted with hot MeOH. The MeOH extract was passed through a polystyrene resin (Diaion HP-20) column, eluting successively with an H_2O−MeOH gradient (30% MeOH, 60% MeOH, and 80% MeOH), MeOH, EtOH, and EtOAc. The MeOH eluate fraction exhibited cytotoxic activity against HL-60 cells (95.9% cell growth inhibition at a sample concentration of 10 *μ*g/mL; IC_{50} 5.1 *μ*g/mL), and TLC analysis suggested that it contained several saponins. The MeOH fraction was repeatedly subjected to column chromatography on silica gel and octadecylsilanized (ODS) silica gel, as well as preparative TLC, to yield compounds **1** (144 mg), **2** (25 mg), **3** (9.4 mg), **4** (50 mg), **5** (202 mg), **6** (192 mg), **7** (63 mg), **8** (125 mg), **9** (25 mg), **10** (65 mg), and **11** (3.1 mg).

Compounds **1**−**7** are previously reported triterpene saponins, and their structures were identified as oleanolic acid 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside} (**1**),[5] hederagenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside} (**2**),[6] hederagenin 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside} (**3**),[7] oleanolic acid 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside} (**4**),[8] hederagenin 3-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside} (**5**),[5] oleanolic acid 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside} (**6**),[9] and hederagenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside} (**7**),[9] respectively. Compounds **10** and **11** are known lignans and were identified as (+)-pinoresinol (**10**)[10] and *β*-peltatin (**11**).[11] Although *β*-peltatin is a minor component, it is the first podophyllotoxin derivative isolated from a plant in the family Ranunculaceae.



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	OH	H	H	H
4	H	H	H	β-D-Glc*p*
5	OH	H	H	β-D-Glc*p*
5a	OH	Me	H	β-D-Glc*p*
6	H	H	β-D-Glc*p*	H
7	OH	H	β-D-Glc*p*	H

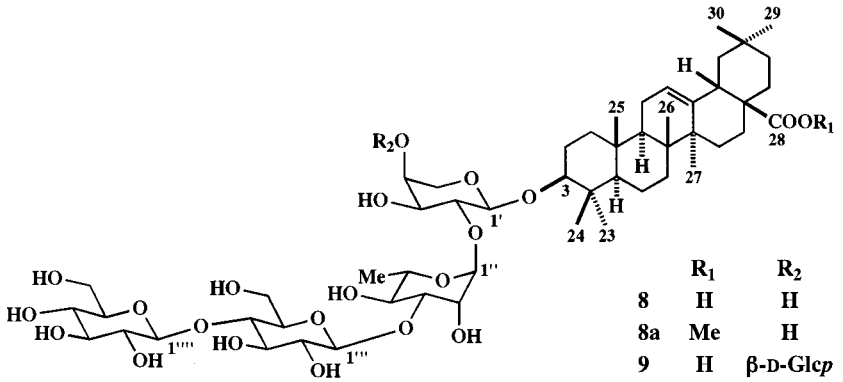


3

* To whom correspondence should be addressed. Tel.: +81 426 76 4577. Fax: +81 426 76 4579. E-mail: mimakiy@ps.toyaku.ac.jp.

10.1021/np9901837 CCC: $18.00
Published on Web 08/05/1999

Chart 1



	R_1	R_2
8	H	H
8a	Me	H
9	H	β-D-Glc*p*

Compound **8** was obtained as an amorphous solid, $[\alpha]_D$ −6.0° ($CHCl_3$−MeOH, 1:1) (Chart 1). The high-resolution FABMS (positive mode) of **8** showed an accurate $[M + Na]^+$ ion peak at *m*/*z* 1081.5565 in accordance with an empirical molecular formula $C_{53}H_{86}O_{21}$, which was supported by the ^{13}C NMR and various DEPT spectral data. The IR spectrum showed a characteristic absorption attributable to a carbonyl group at 1680 cm^{-1}, as well as a broad absorption due to hydroxyl groups near 3400 cm^{-1}. The ^1H NMR spectrum of **8** measured in pyridine-d_5 gave good signal dispersion and exhibited signals for seven tertiary methyl groups at δ 1.31, 1.30, 1.13, 1.01, 0.99, 0.96, and 0.84 (each 3H, s), and an olefinic proton at δ 5.47 (1H, t-like, J = 3.4 Hz), which were characteristic of the oleanolic acid skeleton. Furthermore, four anomeric proton signals were also identified at δ 6.18 (1H, d, J = 0.8 Hz), 5.43 (1H, d, J = 7.9 Hz), 5.19 (1H, d, J = 7.9 Hz), and 4.83 (1H, d, J = 6.1 Hz). Acid hydrolysis of **8** with 1 M hydrochloric acid in dioxane−H_2O (1:1) liberated oleanolic acid as the aglycon, and D-glucose, L-rhamnose, and L-arabinose as the carbohydrate moieties. The identification of the monosaccharides, including their absolute configurations, were carried out by direct HPLC analysis of the hydrolysate using a combination of RI and optical rotary (OR) detectors. The C-28 carbonyl carbon was observed at δ 180.2 in the ^{13}C NMR spectrum of **8**, which suggested that no sugar linkage was formed at C-28 and that a tetraglycoside was attached at C-3 of oleanolic acid in **8**. The exact sequence of the sugars and its linkage position to the aglycon were solved by the concerted use of 2D NMR spectroscopic techniques as previously described.[12] The $^1H-^1H$ COSY and TOCSY experiments allowed the sequential assignment of the proton resonances, the easily distinguished anomeric protons being used as the starting point of analysis. Multiplet patterns and coupling constants, as well as the proton chemical shifts of the glycoside moiety composed of two D-glucopyranoses, one L-rhamnopyranose, and one L-arabinopyranose, were revealed as shown in Table 1. The HMQC spectrum correlated all the proton resonances with those of the corresponding one-bond coupled carbons, leading to the unambiguous assignment of the carbon shifts. Comparison of the carbon chemical shifts thus assigned with those of the reference methyl glycosides,[13] taking into account the known effects of *O*-glycosylation and the results of acid hydrolysis, indicated that **8** contained a terminal β-D-glucopyranosyl, a C-4-substituted β-D-glucopyranosyl, a C-3-substituted α-L-rhamnopyranosyl, and a C-2-substituted α-L-arabinopyranosyl unit. The β-orientations of the anomeric centers of the glucosyl and arabinosyl moieties were supported by the relatively large J values of their anomeric protons. The anomeric orientation of rhamnose could not be deduced from the $^3J_{H-1,H-2}$ value. However, the ^{13}C NMR shifts of C-5 at δ 69.7 indicated the α-anomeric orientation of the rhamnopyranosyl residue in **8**. Finally, the $^3J_{C,H}$ correlation from each anomeric proton across the glycosidic bond to the carbon of another substituted monosaccharide or the aglycon revealed the sugar sequence. In the HMBC spectrum, the anomeric proton signals at δ 5.19 (terminal glucose), 5.43 (substituted glucose), 6.18 (rhamnose), and 4.83 (arabinose) exhibited correlations with the carbon signals at δ 81.1 (C-4 of substituted glucose), 83.5 (C-3 of rhamnose), 75.7 (C-2 of arabinose), and 88.7 (C-3 of aglycon), respectively. Accordingly, the structure of **8** was elucidated as oleanolic acid 3-*O*-{*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside}.

Table 1. ^1H and ^{13}C NMR Assignments of the Glycoside Moieties of Compounds **8** and **9**[a]

	8			**9**		
position	^1H (ppm)	*J* (Hz)	^{13}C (ppm)	^1H (ppm)	*J* (Hz)	^{13}C (ppm)
1′	4.83 d	6.1	105.2	4.70 d	6.6	105.3
2′	4.52 dd	7.0, 6.1	75.7	4.43 dd	8.5, 6.6	76.1
3′	4.22 m		74.4	4.16 dd	8.5, 3.5	74.3
4′	4.23 m		69.2	4.23 m		69.9
5′a	4.29 dd	10.2, 3.9	65.6	4.40 dd	11.2, 2.8	65.2
5′b	3.79 br d	10.2		3.74 br d	11.2	
1″	6.18 d	0.8	101.6	6.14 d	0.7	101.5
2″	4.93 dd	3.0, 0.8	71.7	4.92 dd	2.9, 0.7	71.5
3″	4.75 dd	9.5, 3.0	83.5	4.74 dd	9.4, 2.9	83.2
4″	4.48 dd	9.5, 9.5	73.0	4.47 dd	9.4, 9.4	72.9
5″	4.62 dq	9.5, 6.1	69.7	4.63 dq	9.4, 6.1	69.7
6″	1.55 d	6.1	18.4	1.56 d	6.1	18.5
1‴	5.43 d	7.9	106.5	5.42 d	7.9	106.4
2‴	4.09 dd	9.1, 7.9	75.5	4.08 dd	9.1, 7.9	75.4
3‴	4.26 dd	9.1, 9.1	76.6	4.25 dd	9.1, 9.1	76.6
4‴	4.36 dd	9.1, 9.1	81.1	4.31 dd	9.1, 9.1	81.2
5‴	3.92 ddd	9.1, 3.6, 2.5	76.6	3.92 ddd	9.1, 3.7, 2.3	76.5
6‴a	4.55 dd	12.0, 3.6	61.8	4.53 dd	12.1, 3.7	61.8
6‴b	4.40 dd	12.0, 2.5		4.42 dd	12.1, 2.3	
1⁗	5.19 d	7.9	105.0	5.15 d	7.9	104.9
2⁗	4.07 dd	9.0, 7.9	74.7	4.05 dd	8.9, 7.9	74.7
3⁗	4.20 dd	9.0, 9.0	78.2	4.18 dd	8.9, 8.9	78.2
4⁗	4.16 dd	9.0, 9.0	71.5	4.15 dd	8.9, 8.9	71.4
5⁗	3.99 ddd	9.0, 5.8, 2.4	78.4	3.98 ddd	8.9, 5.7, 2.5	78.4
6⁗a	4.51 dd	11.8, 2.4	62.4	4.51 dd	11.7, 2.5	62.4
6⁗b	4.27 dd	11.8, 5.8		4.26 dd	11.7, 5.7	
1⁗′				5.11 d	7.8	106.5
2⁗′				4.02 dd	8.9, 7.8	75.4
3⁗′				4.19 dd	8.9, 8.9	78.4
4⁗′				4.21 dd	8.9, 8.9	71.2
5⁗′				3.89 ddd	8.9, 5.1, 2.5	78.7
6⁗′a				4.49 dd	11.9, 2.5	62.5
6⁗′b				4.35 dd	11.9, 5.1	

[a] Spectra were measured in pyridine-d_5.

Compound **9** was obtained as an amorphous solid, $[\alpha]_D$ −5.0° ($CHCl_3$−MeOH, 1:1). It gave an accurate $[M + Na]^+$

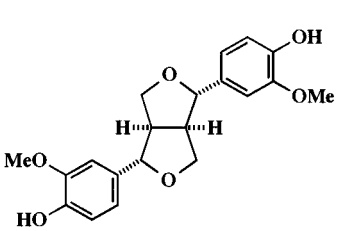
Table 2. Cytotoxic Activity of Compounds **1**−**5**, **5a**, **6**−**8**, **8a**, **9**−**11**, Oleanolic Acid, and Etoposide Against HL-60 Cells[a]

compound	IC_{50} (μg/mL)
1	4.4
2	7.1
3	>10
4	3.6
5	7.8
5a	8.0
6	2.3
7	3.8
8	2.6
8a	7.8
9	2.7
10	>10
11	0.0052
oleanolic acid	>50
etoposide[b]	0.3

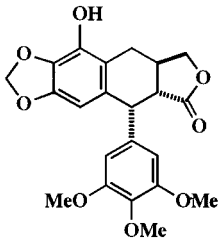
[a] The cells were continuously treated with each sample for 72 h, and the cell growth was evaluated using modified MTT reduction assay. Data are mean values of three experiments performed in triplicate. [b] Clinically used anticancer agent.

ion peak at *m*/*z* 1243.6179 in the HRFABMS, appropriate for a molecular formula of $C_{59}H_{96}O_{26}$. The ^{1}H NMR spectrum contained five anomeric proton signals at δ 6.14 (1H, d, J = 0.7 Hz), 5.42 (1H, d, J = 7.9 Hz), 5.15 (1H, d, J = 7.9 Hz), 5.11 (1H, d, J = 7.8 Hz), and 4.70 (1H, d, J = 6.6 Hz), as well as seven tertiary methyl proton signals due to the aglycon moiety at δ 1.30 (3H × 2, s), 1.13, 1.01, 0.98, 0.96, and 0.83 (each 3H, s). Acid hydrolysis of **9** with 1 M hydrochloric acid in dioxane−H_2O (1:1) gave oleanolic acid, D-glucose, L-rhamnose, and L-arabinose. On comparison of the whole ^{13}C NMR spectrum of **9** with that of **8**, a set of additional six signals corresponding a terminal β-D-glucopyranosyl group appeared at δ 106.5 (CH), 75.4 (CH), 78.4 (CH), 71.2 (CH), 78.7 (CH), and 62.5 (CH_2), and the signal due to C-4 of the arabinosyl moiety was displaced downfield by 10.7 ppm and observed at δ 79.9, suggesting that the C-4 hydroxyl group of the arabinosyl moiety was the glycosylated position to which the additional D-glucose was linked. This was confirmed by a long-range correlation from the anomeric proton of the glucosyl at δ 5.11 to the arabinose C-4 oxymethine carbon at δ 79.9. The structure of **9** was formulated as oleanolic acid 3-*O*-{*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside}.

The cytotoxic activities of the isolated compounds (**1**−**11**), the C-28 methyl esters (**5a**, **8a**) prepared by the treatment of **5** and **8** with CH_2N_2, and oleanolic acid were evaluated against HL-60 human promyelocytic leukemia cells. The saponins, except for **3**, which has no substituent at C-2 of the arabinosyl moiety attached to the aglycon, exhibited moderate cytotoxic activity with IC_{50} values ranging from 2.3 to 7.8 μg/mL, when compared with etoposide used as a positive control (IC_{50} 0.3 μg/mL). There was no significant difference of the activity between the saponins based upon oleanolic acid and hederagenin as the aglycon. Methyl ester formation at the C-28 carboxyl group of the active saponins gave no influence on the activity. Oleanolic acid itself was not cytotoxic. These results suggested that the glycoside moiety attached to C-3 of the aglycon is essential for the appearance of the cytotoxic activity among the saponins evaluated. β-Peltatin (**11**) was very cytotoxic to HL-60 cells with an IC_{50} value of 0.0052 μg/mL, in accordance with expectations, while another lignan, (+)-pinoresinol (**10**), showed no activity.



10



11

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a JASCO A-100 spectrophotometer. NMR spectra were recorded on a Bruker DPX-400 (400 MHz for ^{1}H NMR) or on a Bruker DRX-500 (500 MHz for ^{1}H NMR) spectrometer. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. MS were recorded on a VG AutoSpec E mass spectrometer. Diaion HP-20 (Mitsubishi-Kasei, Tokyo, Japan), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and ODS silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 or 0.5 mm thick, Merck, Darmstadt, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh), a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and Rheodyne injection port with a 20 μL sample loop. A Kaseisorb NH_2-60-5 column (4.6 mm i.d. × 250 mm, 5 μm, Tokyo-Kasei, Tokyo, Japan) was employed for HPLC analysis. The following materials and reagents were used for cell culture and assay of cytostatic activity: microplate reader, Inter Med Immuno-Mini NJ-2300 (Tokyo, Japan); 96-well flat-bottom plate, Iwaki Glass (Chiba, Japan); HL-60 cells, ICN Biomedicals (Costa Mesa, CA); RPMI 1640 medium, GIBCO BRL (Rockville, MD); MTT, Sigma (St. Louis, MO). All other chemicals used were of biochemical reagent grade.

Plant Material. *P. chinensis* was purchased from a wholesale firm in Uchida-Wakanyaku, Tokyo, Japan. It was identified by Prof. Zhongzheng Zhao, Hong Kong Babtist University, Kowloon Tang, Hong Kong. A voucher of the plant is on file in our laboratory.

Extraction and Isolation. The plant material (dry weight, 3.5 kg) was extracted with hot MeOH. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (702 g) was passed through a Diaion HP-20 column eluting with increased amounts of MeOH in H_2O (30% MeOH, 60% MeOH, and 80% MeOH), MeOH, EtOH, and EtOAc. The MeOH eluate fraction exhibited considerable cytotoxic activity against HL-60 cells (95.9% cell growth inhibition at the sample concentration of 10 μg/mL; IC_{50} 5.1 μg/mL), while the other fractions were far less active (30% MeOH eluate fraction, 3.8% inhibition at 10 μg/mL; 60% MeOH, 9.2% inhibition; 80% MeOH, 8.1% inhibition; EtOH, 47.0% inhibition; EtOAc, 4.8% inhibition). Column chromatography of the MeOH eluate portion on silica gel and elution with a stepwise gradient mixture of $CHCl_3$−MeOH (19:1; 9:1; 2:1; 1:1), and finally with MeOH alone, gave six fractions (I−VI). Fraction II was chromatographed on silica gel eluting with $CHCl_3$−Me_2CO (6:1) and ODS silica gel with MeCN−H_2O (2:3) to give **10** (65 mg) and **11** with a few impurities. Compound **11** was completely refined by preparative TLC developing with $CHCl_3$−EtOAc (5:1) to furnish **11** (3.1 mg) as a pure compound. Fraction III was subjected to column chromatography on silica gel eluting with $CHCl_3$−MeOH−H_2O (40:10:1) and ODS silica gel with MeOH−H_2O (4:1) to yield **1** (144 mg), **2** (25 mg), and **3** (9.4 mg). Fraction IV was subjected to a silica gel column eluting with $CHCl_3$−MeOH−H_2O (20:10:1) and an ODS silica

gel column with MeOH−H_2O (4:1) and MeCN−H_2O (1:1) to give **4** (50 mg), **5** (202 mg), **6** (192 mg), and **7** (63 mg). Fraction V was separated by silica gel column chromatography eluting with $CHCl_3$−MeOH−H_2O (20:10:1) and ODS silica gel column chromatography with MeCN−H_2O (2:3) to give **8** (125 mg) and **9** (25 mg).

Compound 8: amorphous solid; $[\alpha]^{27}_D$ −6.0° (*c* 0.10, $CHCl_3$−MeOH, 1:1); IR (KBr) ν_{max} 3400 (OH), 2930 (CH), 1680 (C=O) cm^{-1}; ^1H NMR (C_5D_5N) δ 5.47 (1H, t-like, J = 3.4 Hz, H-12), 3.29 (1H, dd, J = 11.7, 4.4 Hz, H-3), 1.31 (3H, s, Me-27), 1.30 (3H, s, Me-23), 1.13 (3H, s, Me-24), 1.01 (3H, s, Me-30), 0.99 (3H, s, Me-26), 0.96 (3H, s, Me-29), 0.84 (3H, s, Me-25); signals for the tetraglycoside moiety, see Table 1; ^{13}C NMR (C_5D_5N) δ 38.9 (C-1), 26.6 (C-2), 88.7 (C-3), 39.6 (C-4), 56.0 (C-5), 18.5 (C-6), 33.2 (C-7), 39.7 (C-8), 48.0 (C-9), 37.0 (C-10), 23.7 (C-11), 122.5 (C-12), 144.8 (C-13), 42.1 (C-14), 28.3 (C-15), 23.7 (C-16), 46.6 (C-17), 42.0 (C-18), 46.4 (C-19), 30.9 (C-20), 34.2 (C-21), 33.1 (C-22), 28.2 (C-23), 17.1 (C-24), 15.5 (C-25), 17.3 (C-26), 26.2 (C-27), 180.2 (C-28), 33.3 (C-29), and 23.7 (C-30); signals for the tetraglycoside moiety, see Table 1; HRFABMS (positive mode) *m*/*z* 1081.5565 $[M + Na]^+$ (calcd for $C_{53}H_{86}O_{21}$-Na, 1081.5559).

Acid Hydrolysis of 8. A solution of **8** (10 mg) in 1 M HCl (dioxane−H_2O, 1:1, 2 mL) was heated at 80 °C for 4 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and chromatographed over silica gel using a discontinuous gradient of $CHCl_3$−MeOH (9:1 to 1:1) to give oleanolic acid (3.2 mg) and a sugar fraction (3.7 mg). The sugar fraction was dissolved in H_2O and passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA), which was then analyzed by HPLC under the following conditions: solvent, MeCN−H_2O (3:1); flow rate, 0.6 mL/min; detection, RI and OR. The identification of D-glucose, L-arabinose, and L-rhamnose present in the sugar fraction was carried out by the comparison of their retention times and polarities with those of authentic samples: t_R (min) 10.66 (L-rhamnose, negative polarity); 13.57 (L-arabinose, positive polarity); 16.61 (D-glucose, positive polarity).

Compound 9: amorphous solid; $[\alpha]^{27}_D$ −5.0° (*c* 0.10, $CHCl_3$−MeOH, 1:1); IR (KBr) ν_{max} 3400 (OH), 2930 (CH), 1680 (C=O) cm^{-1}; ^1H NMR (C_5D_5N) δ 5.47 (1H, t-like, J = 3.0 Hz, H-12), 3.23 (1H, dd, J = 11.7, 4.2 Hz, H-3), 1.30 (3H × 2, s, Me-23 and Me-27), 1.13 (3H, s, Me-24), 1.01 (3H, s, Me-30), 0.98 (3H, s, Me-26), 0.96 (3H, s, Me-29), 0.83 (3H, s, Me-25); signals for the pentaglycoside moiety, see Table 1; ^{13}C NMR (C_5D_5N) δ 38.8 (C-1), 26.6 (C-2), 88.6 (C-3), 39.5 (C-4), 56.0 (C-5), 18.5 (C-6), 33.1 (C-7), 39.7 (C-8), 48.0 (C-9), 37.0 (C-10), 23.6 (C-11), 122.5 (C-12), 144.8 (C-13), 42.1 (C-14), 28.3 (C-15), 23.7 (C-16), 46.6 (C-17), 41.9 (C-18), 46.4 (C-19), 30.9 (C-20), 34.2 (C-21), 33.1 (C-22), 28.1 (C-23), 17.1 (C-24), 15.5 (C-25), 17.3 (C-26), 26.1 (C-27), 180.2 (C-28), 33.2 (C-29), and 23.7 (C-30); signals for the pentaglycoside moiety, see Table 1; HRFABMS (positive mode) *m*/*z* 1243.6179 $[M + Na]^+$ (calcd for $C_{59}H_{96}O_{26}$-Na, 1243.6088).

Acid Hydrolysis of 9. Compound **9** (6.2 mg) was subjected to acid hydrolysis as described for **8** to give oleanolic acid (1.7 mg) and a sugar fraction (2.1 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **8** showed the presence of D-glucose, L-arabinose, and L-rhamnose.

Methylation of 5. Compound **5** (20 mg) was dissolved in MeOH (3 mL) and cooled at 0 °C. A large excess of CH_2N_2 in Et_2O was added to the sample solution. After leaving for 12 h at room temperature, the reaction mixture was chromatographed on silica gel eluting with $CHCl_3$−MeOH−H_2O (40:10:1) to furnish the methyl ester (**5a**) (4.6 mg) of **5**.

Compound 5a: amorphous solid; IR (KBr) ν_{max} 3420 (OH), 2925 (CH), 1720 (C=O) cm^{-1}; ^1H NMR (C_5D_5N) δ 6.27 (1H, d, J = 0.6 Hz, H-1″), 5.51 (1H, d, J = 7.8 Hz, H-1‴), 5.37 (1H, t-like, J = 3.3 Hz, H-12), 5.03 (1H, d, J = 6.8 Hz, H-1′), 4.35 and 3.94 (each 1H, ABq, J = 10.7 Hz, H_2-23), 4.28 (1H, overlapping, H-3), 3.71 (3H, s, OMe), 1.56 (3H, d, J = 6.1 Hz, Me-6″), 1.17, 1.15, 0.97, 0.93, 0.89, and 0.85 (each 3H, s, tertiary methyls); ^{13}C NMR (C_5D_5N) δ 39.0 (C-1), 26.4 (C-2), 81.2 (C-3), 43.6 (C-4), 47.6 (C-5), 18.1 (C-6), 32.8 (C-7), 39.7 (C-8), 48.0 (C-9), 36.8 (C-10), 23.8 (C-11), 122.9 (C-12), 144.1 (C-13), 41.9 (C-14), 28.1 (C-15), 23.4 (C-16), 46.9 (C-17), 41.8 (C-18), 46.0 (C-19), 30.8 (C-20), 33.9 (C-21), 32.7 (C-22), 64.0 (C-23), 14.2 (C-24), 16.1 (C-25), 17.2 (C-26), 26.1 (C-27), 178.0 (C-28), 33.1 (C-29), 23.6 (C-30), 51.6 (OMe), 105.0 (C-1′), 75.5 (C-2′), 75.1 (C-3′), 69.8 (C-4′), 66.5 (C-5′), 101.5 (C-1″), 71.6 (C-2″), 83.0 (C-3″), 73.0 (C-4″), 69.7 (C-5″), 18.5 (C-6″), 106.9 (C-1‴), 75.9 (C-2‴), 78.5 (C-3‴), 71.8 (C-4‴), 78.6 (C-5‴), and 62.6 (C-6‴); FABMS (positive mode) *m*/*z* 949 $[M + Na]^+$.

Methylation of 8. Compound **8** (25 mg) was converted to the methyl ester (**8a**) (10.5 mg) by the same procedure as described for **5**.

Compound 8a: amorphous solid; IR (KBr) ν_{max} 3425 (OH), 2930 (CH), 1725 (C=O) cm^{-1}; ^1H NMR (C_5D_5N) δ 6.23 (1H, br s, H-1″), 5.46 (1H, d, J = 7.9 Hz, H-1‴), 5.38 (1H, t-like, J = 3.3 Hz, H-12), 5.22 (1H, d, J = 7.9 Hz, H-1⁗), 4.84 (1H, d, J = 6.1 Hz, H-1′), 3.72 (3H, s, OMe), 3.30 (1H, dd, J = 11.7, 4.2 Hz, H-3), 1.57 (3H, d, J = 6.1 Hz, Me-6″), 1.32, 1.25, 1.18, 0.94, 0.93, 0.88, and 0.84 (each 3H, s, tertiary methyls); ^{13}C NMR (C_5D_5N) δ 38.9 (C-1), 26.6 (C-2), 88.6 (C-3), 39.6 (C-4), 55.9 (C-5), 18.5 (C-6), 33.0 (C-7), 39.6 (C-8), 47.9 (C-9), 37.0 (C-10), 23.6 (C-11), 123.0 (C-12), 144.1 (C-13), 41.9 (C-14), 28.1 (C-15), 23.4 (C-16), 46.9 (C-17), 41.8 (C-18), 46.0 (C-19), 30.8 (C-20), 33.9 (C-21), 32.8 (C-22), 28.2 (C-23), 17.1 (C-24), 15.6 (C-25), 17.2 (C-26), 26.1 (C-27), 178.0 (C-28), 33.1 (C-29), 23.7 (C-30), 51.6 (OMe), 105.3 (C-1′), 75.6 (C-2′), 74.5 (C-3′), 69.3 (C-4′), 65.7 (C-5′), 101.6 (C-1″), 71.8 (C-2″), 83.6 (C-3″), 73.1 (C-4″), 69.7 (C-5″), 18.5 (C-6″), 106.6 (C-1‴), 75.5 (C-2‴), 76.6 (C-3‴), 81.1 (C-4‴), 76.6 (C-5‴), 61.9 (C-6‴), 105.0 (C-1⁗), 74.7 (C-2⁗), 78.3 (C-3⁗), 71.5 (C-4⁗), 78.5 (C-5⁗), and 62.4 (C-6⁗); FABMS (positive mode) *m*/*z* 1095 $[M + Na]^+$.

Cell Culture Assay. HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The leukemia cells were washed and resuspended in the above medium to 3 × 10^4 cells/mL, and 196 μL of this cell suspension was placed in each well of a 96-well flat-bottom plate. The cells were incubated in 5% CO_2/air for 24 h at 37 °C. After incubation, 4 μL of EtOH−H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.0001−50 μg/mL and 4 μL of EtOH−H_2O (1:1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[14] Briefly, after termination of the cell culture, 10 μL of 5 mg/mL MTT in phosphate-buffered saline was added to every well and the plate was further reincubated in 5% CO_2/air for 4 h at 37 °C. The plate was then centrifuged at 1500*g* for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μL of the supernatant was removed from every well, and 175 μL of DMSO was added to dissolve the MTT formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader at 550 nm. A dose−response curve was plotted for the fractions and compounds which showed more than 50% of cell growth inhibition at a sample concentration of 10 μg/mL, and the concentration giving 50% inhibition (IC_{50}) was calculated.

Acknowledgment. We are grateful to Dr. Y. Shida, Tokyo University of Pharmacy and Life Science, for the measurements of the mass spectra.

References and Notes

(1) *Dictionary of Chinese Medicinal Materials*; Shanghai Scientific and Technological Press: Shanghai, 1977; Vol. 1, pp 704−706.
(2) Ye, W. C.; Ji, N. N.; Zhao, S. X.; Liu, J. H.; Ye, T.; McKervey, M. A.; Stevenson, P. *Phytochemistry* **1996**, *42*, 799−802.
(3) Ye, W.; He, A.; Zhao, S.; Che, C. T. *J. Nat. Prod.* **1998**, *61*, 658−659.
(4) Mimaki, Y.; Kuroda, M.; Fukasawa, T.; Sashida, Y. *J. Nat. Prod.* **1999**, *62*, 194−197.
(5) Saito, S.; Sumita, S.; Tamura, N.; Nagamura, Y.; Nishida, K.; Ito, M.; Ishiguro, I. *Chem. Pharm. Bull.* **1990**, *38*, 411−414.
(6) Kang, S. S. *Arch. Pharm. Res.* **1989**, *12*, 42−47.
(7) Joshi, B. S.; Moore, K. M.; Pelletier, S. W.; Puar, M. S.; Pramanik, B. N. *J. Nat. Prod.* **1992**, *55*, 1468−1476.

(8) Choi, J. S.; Woo, W. S. *Planta Med.* **1987**, *53*, 62−65.
(9) Ekabo, O. A.; Farnsworth, N. R.; Henderson, T. O.; Mao, G.; Mukherjee, R. *J. Nat. Prod.* **1996**, *59*, 431−435.
(10) Nishibe, S.; Chiba, M.; Hisada, S. *Yakugaku Zasshi* **1977**, *97*, 1134−1137.
(11) Broomhead, A. J.; Dewick, P. M. *Phytochemistry* **1990**, *29*, 3839−3844.
(12) Mimaki, Y.; Sashida, Y.; Nikaido, T.; Ohmoto, T. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 458−462.
(13) Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307−3330.
(14) Sargent, J. M.; Taylor, C. G. *Br. J. Cancer* **1989**, *60*, 206−210.

NP9901837