Triterpene Glycosides from the Whole Plant of *Anemone hupehensis* var. *japonica* and Their Cytotoxic Activity

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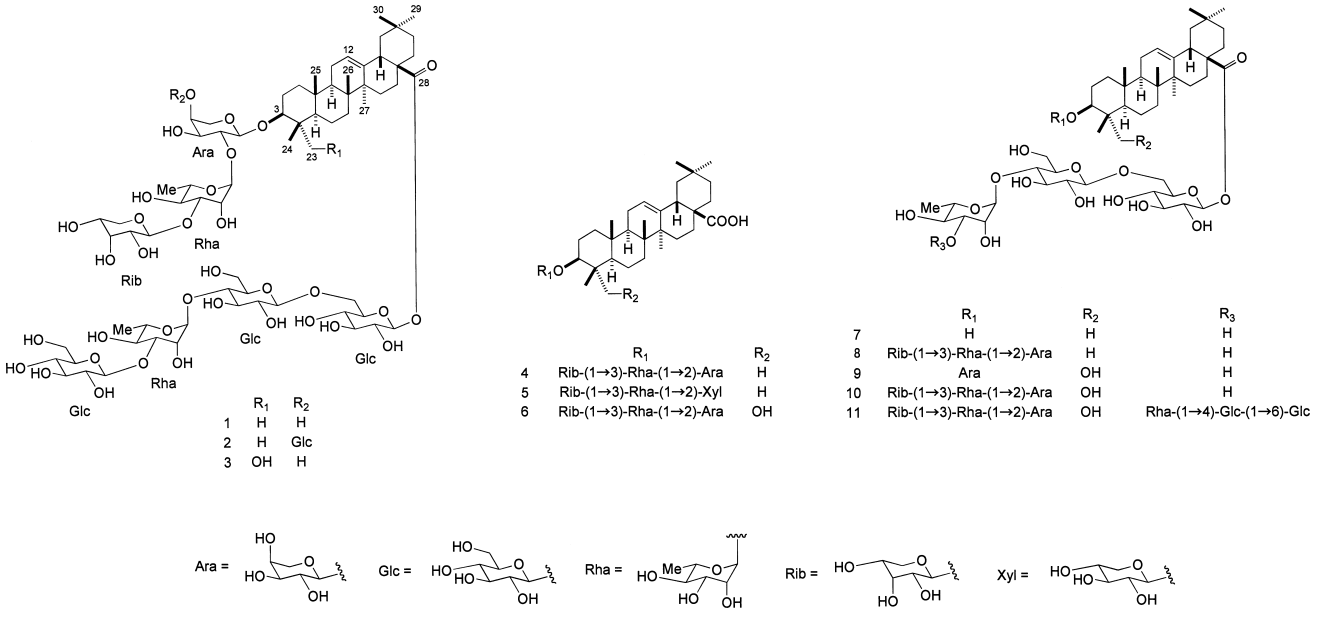
Received August 20, 2009; accepted September 28, 2009; published online September 29, 2009

Three new triterpene glycosides (1—3), together with eight known triterpene glycosides (4—11), were isolated from the whole plant of *Anemone hupehensis* var. *japonica* (Ranunculaceae). The structures of the new compounds were determined on the basis of spectroscopic analysis and the results of hydrolytic cleavage experiments. The isolated compounds were evaluated for their cytotoxic activities against HL-60 human leukemia cells, HSC-2 human oral squamous carcinoma cells, HSC-4 human oral squamous carcinoma cells, and A549 human lung adenocarcinoma cells.

Key words triterpene glycoside; *Anemone hupehensis* var. *japonica*; Ranunculaceae; cytotoxic activity

Anemone hupehensis LEM. var. *japonica* (THUNB.) BOWLES *et* STEARN is a perennial plant belonging to the family Ranunculaceae and is distributed throughout Japan and China.[1)] Its roots have been used in traditional Chinese medicine for the treatment of fever.[2)] Although the triterpene glycosides hupehensis saponin A—G were isolated from *A. hupehensis*,[3)] there have been no reports concerning the secondary metabolites of *A. hupehensis* var. *japonica*. Therefore, in this study, we conducted a phytochemical screening of the whole plant. As a result, three new triterpene glycosides (**1**—**3**) and eight known triterpene glycosides (**4**—**11**) were isolated. Here, we describe the isolation and structural elucidation of the three new triterpene glycosides (**1**—**3**) on the basis of spectroscopic analysis, including various two-dimensional (2D) NMR spectroscopic studies, and the results of hydrolytic cleavage experiments. The cytotoxic activities of the isolated compounds against HL-60 human leukemia cells, A549 human lung adenocarcinoma cells, HSC-2 human oral squamous carcinoma cells, and HSC-4 human oral squamous carcinoma cells are also reported.

The whole plant of *A. hupehensis* var. *japonica* (3.3 kg) was extracted with hot MeOH. After removal of solvent, the MeOH extract was passed through a porous-polymer polystyrene resin (Diaion HP-20) column successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The MeOH eluted fraction was repeatedly subjected to silica gel and octadecylsilanized (ODS) silica gel column chromatography to afford compounds **1**—**11**. Compounds **4**—**11** were identified by their physical and spectroscopic data as 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid (prosapogenin CP4, **4**),[4)] 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl)oxy]olean-12-en-28-oic acid (huzhangoside A, **5**),[4)] 23-hydroxy-3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid (prosapogenin CP6, **6**),[4)] olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (cussonoside B, **7**),[5)] 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-



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arabinopyranosyl)oxy]olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (huzhangoside B, **8**),[4] 3β-[(α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (**9**),[6] 23-hydroxy-3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (huzhangoside D, **10**),[4] 23-hydroxy 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (hupehensis saponin F, **11**),[7] respectively.

Compound **1** was obtained as an amorphous solid and showed an accurate $[M+Na]^+$ ion at *m/z* 1521.7109 in the high resolution (HR)-electrospray ionization (ESI)-time of flight (TOF)-MS, corresponding to the empirical molecular formula $C_{70}H_{114}O_{34}$. The IR spectrum of **1** was suggestive of a glycoside (3376, 1064 cm^{-1}) and indicated the presence of a carbonyl group (1740 cm^{-1}) in the molecule. The ^{1}H-NMR spectrum of **1** contained signals for seven quaternary methyl groups at δ 1.28, 1.23, 1.13, 1.06, 0.89, 0.87, and 0.85 (each s), an olefinic proton at δ 5.39 (br s), and seven anomeric protons at δ 6.27 (br s), 6.22 (d, J=8.0 Hz), 5.93 (d, J=4.4 Hz), 5.84 (br s), 5.34 (d, J=7.8 Hz), 4.90 (d, J=7.9 Hz), and 4.82 (d, J=6.0 Hz), and the methyl groups of two 6-deoxyhexopyranosyl moieties at δ 1.63 (d, J=6.2 Hz) and 1.52 (d, J=6.1 Hz). Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1 : 1) yielded 3β-hydroxyolean-12-en-28-oic acid (**1a**, oleanolic acid), as well as L-rhamnose, D-ribose, L-arabinose, and D-glucose as the carbohydrate moieties. Identification of the monosaccharides, including their absolute configurations, was carried out by direct HPLC analysis of the hydrolysate. The C-3 oxymethine carbon and C-28 carbonyl carbon were observed at δ 88.7 and 176.5, respectively, in the ^{13}C-NMR of **1**, which suggests that **1** is a 3,28-bisdesmoside of oleanolic acid. In a comparison of the whole ^{13}C-NMR spectrum of **1** with that of 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid β-D-glucopyranosyl ester (clematichinenoside A),[8] the signals due to the aglycone moiety and the triglycoside residue linked to C-3 of the aglycone were observed at almost the same positions for each of the compounds. However, differences were recognized in the tetraglycoside moiety attached to C-28 of the aglycone. The exact structures of the sugar moieties and their linkage positions to the aglycone were solved by detailed analysis of the one-dimensional (1D) totally correlated spectroscopy (TOCSY) and 2D NMR spectra. The ^{1}H-NMR subspectra of individual monosaccharide units were obtained by using selective irradiation of easily identifiable anomeric proton signals, as well as non-overlapping proton signals in a series of 1D TOCSY experiments.[9—11] Subsequent analysis of the ^{1}H–^{1}H shift correlation spectroscopy (COSY) spectrum resulted in the sequential assignment of all the proton resonances due to the seven glycosyl units, including identification of their multiplet patterns and coupling constants (Table 1). The ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) and ^{1}H-detected heteronuclear single-quantum coherence (HSQC)-TOCSY spectra correlated the proton resonances with those of the corresponding one-bond coupled carbons, leading to unambiguous assignments of the carbon shifts. Comparison of the carbon chemical shifts thus assigned with those of reference methyl glycosides,[12,13] taking into account the known effects of *O*-glycosylation, indicated that **1** contains a C-2 substituted α-L-arabinopyranosyl moiety (Ara), two C-3 substituted α-L-rhamnopyranosyl moieties (Rha, Rha′), a terminal β-D-ribopyranosyl moiety (Rib), a C-6 substituted β-D-glucopyranosyl moiety (Glc), a C-4 substituted β-D-glucopyranosyl moiety (Glc′), and a terminal β-D-glucopyranosyl moiety (Glc″). In the ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum of **1**, long-range correlations were observed between the anomeric proton (H-1) of Rib at δ_H 5.93 and C-3 of Rha at δ_C 81.2, H-1 of Rha at δ_H 6.27 and C-2 of Ara at δ_C 75.3, H-1 of Ara at δ_H 4.82 and C-3 of the aglycone at δ_C 88.7, H-1 of Glc″ at δ_H 5.34 and C-3 of Rha′ at δ_C 83.8, H-1 of Rha′ at δ_H 5.84 and C-4 of Glc′ at δ_C 77.2, H-1 of Glc′ at δ_H 4.90 and C-6 of Glc at δ_C 69.3, and between H-1 of Glc at δ_H 6.22 and C-28 of the aglycone at δ_C 176.5 (Fig. 1). Accordingly, the structure of **1** was formulated as 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid *O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester.

Compound **2** was found to have a molecular formula of $C_{76}H_{124}O_{39}$ as determined by HR-ESI-TOF-MS analysis (*m/z* 1661.7778 $[M+H]^+$). The deduced molecular weight of **2** was higher than that of **1**, with the difference corresponding to a $C_6H_{10}O_5$ unit. The ^{1}H-NMR spectrum of **2** contained signals for eight anomeric protons at δ 6.32 (br s), 6.26 (d, J=8.1 Hz), 5.98 (d, J=4.2 Hz), 5.88 (br s), 5.38 (d, J=7.8 Hz), 5.14 (d, J=7.8 Hz), 4.90 (d, J=7.9 Hz), 4.72 (d, J=6.8 Hz). On the basis of the spectral properties of **2** and the results of acid hydrolysis experiments, in which **2** yielded **1a**, L-rhamnose, D-ribose, L-arabinose, and D-glucose, **2** was shown to be a triterpene glycoside closely related to **1**; however, the

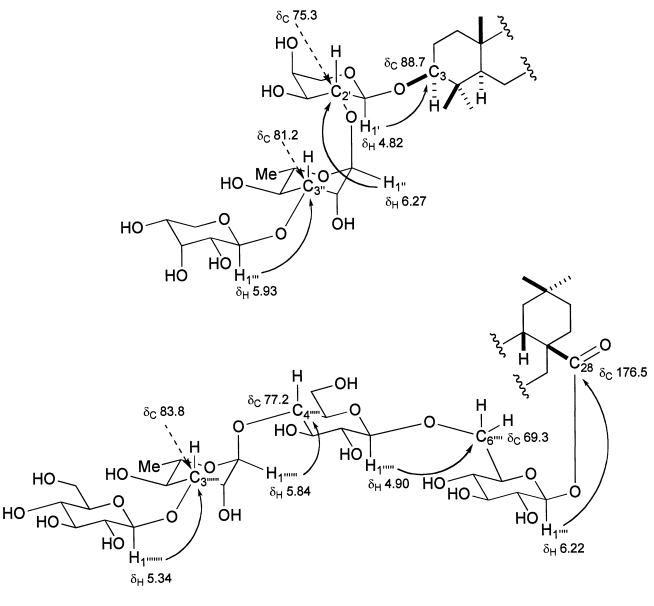

Fig. 1. HMBC Correlations of the Glycoside Moieties of **1**

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shift Assignments of the Sugar Moieties for Compounds **1**—**3**[a)]

1				**2**				**3**			
Position	^{1}H	*J* (Hz)	^{13}C	Position	^{1}H	*J* (Hz)	^{13}C	Position	^{1}H	*J* (Hz)	^{13}C
Ara 1′	4.82 d	6.0	105.2	Ara 1′	4.72 d	6.8	105.3	Ara 1′	5.06 d	6.6	105.0
2′	4.57 dd	8.7, 6.0	75.3	2′	4.51 dd	8.2, 6.8	75.8	2′	4.59 dd	8.1, 6.6	75.2
3′	4.24 dd	8.7, 4.7	74.7	3′	4.24 br d	8.2	75.0	3′	4.02 dd	8.1, 4.4	75.4
4′	4.21 m		69.4	4′	4.23 m		80.4	4′	4.12 m		69.8
5′ a	4.29 br d	11.0	65.7	5′ a	4.38 br d	10.7	65.5	5′ a	4.25 dd	9.7, 2.9	66.4
b	3.80 br d	11.0		b	3.75 br d	10.7		b	3.67 br d	10.9	
Rha 1″	6.27 br s		101.4	Rha 1″	6.32 br s		101.4	Rha 1″	6.36 br s		101.3
2″	4.89 br d	3.7	72.0	2″	4.93 br d	2.8	72.0	2″	4.90 br d	2.9	72.0
3″	4.72 dd	9.7, 3.7	81.2	3″	4.75 dd	9.6, 2.8	81.1	3″	4.77 dd	9.6, 2.9	81.2
4″	4.42 dd	9.7, 9.7	72.8	4″	4.46 dd	9.6, 9.6	72.9	4″	4.43 dd	9.6, 9.6	72.9
5″	4.61 dq	9.7, 6.1	69.8	5″	4.66 dq	9.6, 6.1	69.9	5″	4.71 dq	9.6, 6.2	69.8
6″	1.52 d	6.1	18.4	6″	1.58 d	6.1	18.6	6″	1.55 d	6.2	18.4
Rib 1‴	5.93 d	4.4	104.6	Rib 1‴	5.98 d	4.2	104.8	Rib 1‴	5.96 d	4.4	104.7
2‴	4.29 m		72.8	2‴	4.33 m		72.8	2‴	4.31 m		72.8
3‴	4.51 m		70.2	3‴	4.51 m		70.3	3‴	4.51 m		70.3
4‴	4.17 m		68.9	4‴	4.18 m		68.8	4‴	4.16 m		68.9
5‴ a	4.34 br d	8.5	65.3	5‴ (2H)	4.18 m		65.3	5‴ a	4.31 br d	10.8	65.3
b	4.16 br d	8.5						b	4.14 br d	10.8	
Glc 1⁗	6.22 d	8.0	95.6	Glc 1⁗	5.14 d	7.8	106.8	Glc 1⁗	6.25 d	8.1	95.6
2⁗	4.09 dd	8.9, 8.0	73.8	2⁗	4.03 dd	8.6, 7.8	75.5	2⁗	4.08 dd	9.2, 8.1	73.9
3⁗	4.19 dd	8.9, 8.9	78.7	3⁗	4.17 dd	8.6, 8.6	78.8	3⁗	4.20 dd	9.2, 9.2	78.7
4⁗	4.32 dd	8.9, 8.5	70.8	4⁗	4.24 dd	8.6, 8.6	71.3	4⁗	4.32 dd	9.2, 8.5	70.8
5⁗	4.06 m		77.9	5⁗	3.89 m		78.8	5⁗	4.02 m		77.9
6⁗ a	4.63 br d	11.3	69.3	6⁗ a	4.49 br d	11.5	62.6	6⁗ a	4.65 br d	11.1	69.3
b	4.28 br d	11.3		b	4.37 br d	11.5		b	4.30 br d	11.1	
Glc′ 1⁗′	4.90 d	7.9	105.0	Glc′ 1⁗′	6.26 d	8.1	95.7	Glc′ 1⁗′	4.93 d	7.9	104.7
2⁗′	3.89 dd	8.7, 7.9	75.3	2⁗′	4.10 dd	8.7, 8.1	73.9	2⁗′	3.91 dd	8.5, 7.9	75.4
3⁗′	4.10 dd	9.4, 8.7	76.4	3⁗′	4.19 dd	8.7, 8.7	78.6	3⁗′	4.13 dd	9.3, 8.5	76.4
4⁗′	4.40 dd	9.4, 9.4	77.2	4⁗′	4.33 dd	8.7, 8.7	70.9	4⁗′	4.43 dd	9.3, 9.3	77.2
5⁗′	3.53 m		77.0	5⁗′	4.08 m		77.9	5⁗′	3.56 m		77.1
6⁗′ a	4.14 br d	2.5	61.2	6⁗′ a	4.64 br d	11.1	69.3	6⁗′ a	4.15 br d	11.9	61.2
b	4.00 br d	12.5		b	4.29 br d	11.1		b	4.03 br d	11.9	
Rha′ 1⁗′	5.84 br s		102.4	Glc″ 1⁗′	4.90 d	7.9	105.1	Rha′ 1⁗′	5.87 br s		102.4
2⁗′	4.90 br d	2.8	71.9	2⁗′	3.90 dd	8.9, 8.5	75.4	2⁗′	4.90 br d	2.6	71.9
3⁗′	4.63 dd	9.3, 2.8	83.8	3⁗′	4.11 dd	9.0, 8.9	76.5	3⁗′	4.65 dd	9.3, 2.6	83.9
4⁗′	4.50 dd	9.3, 9.3	73.0	4⁗′	4.43 dd	9.0, 9.0	77.3	4⁗′	4.43 dd	9.3, 9.3	73.0
5⁗′	5.03 dq	9.3, 6.2	70.0	5⁗′	3.57 m		77.1	5⁗′	5.04 dq	9.3, 6.2	70.0
6⁗′	1.63 d	6.2	18.4	6⁗′ a	4.16 br d	10.5	61.2	6⁗′	1.66 d	6.2	18.4
				b	4.03 br d	10.5					
Glc″ 1⁗″	5.34 d	7.8	106.7	Rha′ 1⁗′	5.88 br s		102.5	Glc″ 1⁗″	5.37 d	7.8	106.8
2⁗″	4.07 dd	8.8, 7.8	76.0	2⁗′	4.88 br d	3.0	72.0	2⁗″	4.09 dd	8.8, 7.8	76.0
3⁗″	4.19 dd	8.8, 8.8	78.4	3⁗′	4.67 dd	9.1, 3.0	84.0	3⁗″	4.21 dd	8.8, 8.8	78.4
4⁗″	4.26 dd	8.8, 8.5	71.3	4⁗′	4.50 dd	9.6, 9.1	73.0	4⁗″	4.26 dd	8.8, 8.8	71.3
5⁗″	3.83 m		78.4	5⁗′	5.06 dq	9.6, 6.1	70.0	5⁗″	3.85 m		78.4
6⁗″ a	4.40 br d	11.2	62.3	6⁗′	1.67 d	6.1	18.5	6⁗″ a	4.41 br d	11.8	62.3
b	4.34 br d	11.2						b	4.35 br d	11.8	
				Glc‴ 1⁗″	5.38 d	7.8	106.8				
				2⁗″	4.07 dd	8.8, 7.8	76.1				
				3⁗″	4.20 dd	8.8, 8.8	78.4				
				4⁗″	4.28 dd	8.8, 8.8	71.3				
				5⁗″	3.86 m		78.4				
				6⁗″ a	4.41 br d	11.6	62.4				
				b	4.34 br d	11.6					

a) Spectra were measured in C_5D_5N.

sugar chain attached at C-3 of the aglycone moiety was made up of four monosaccharides and differed from that of **1** by the presence of an additional glucosyl unit. In a comparison of the ^{13}C-NMR spectrum of **2** with that of **1**, a set of six additional signals corresponding to a terminal β-D-glucopyranosyl unit (Glc) [$\delta_{H\text{-}1}$ 5.14 (1H, d, J=7.8 Hz); δ_C 106.8, 75.5, 78.8, 71.3, 78.8, and 62.6] appeared, and the signal due to C-4 of the arabinosyl moiety shifted downfield by 11.0 ppm to δ 80.4, suggesting that the C-4 hydroxyl group of the arabinosyl moiety is the glycosylated position at which the additional D-glucosyl unit is linked. In the HMBC spectrum of **2**, long-range correlations were observed between H-1 of Rib at δ 5.98 and C-3 of Rha at δ 81.1, H-1 of Rha at δ 6.32 and C-2 of Ara at δ 75.8, H-1 of Glc at δ 5.14 and C-4 of Ara at δ 80.4, H-1 of Ara at δ 4.72 and C-3 of the aglycone at δ 88.7, H-1 of Glc‴ at δ 5.38 and C-3 of Rha′ at δ 84.0, H-1 of Rha′ at δ 5.88 and C-4 of Glc″ at δ 77.3, H-1 of Glc″ at δ 4.90 and C-6 of Glc′ at δ 69.3, and between H-1 of Glc′ at δ 6.26

Table 2. Cytotoxic Activities of Compounds **1**—**11** against Four Human Cancer Cell Lines[a,b]

Compound	IC_{50} (μM)			
	HL-60	A549	HSC-2	HSC-4
4	3.1±0.15	2.9±0.20	5.9±0.16	13.0±0.14
5	2.3±0.09	1.5±0.14	5.7±0.46	11.7±0.20
6	5.7±0.58	9.6±0.03	16.3±0.06	>20
Oleanolic acid	>10	—[c]	—	—
Hederagenin	>10	—	—	—
Cisplatin[d]	1.6±0.03	6.4±0.43	16.9±0.60	>20
Etoposide[d]	0.38±0.02	>20	>20	>20
Doxorubicin[d]	0.07±0.01	1.7±0.03	0.25±0.03	0.88±0.04

a) Data represent the mean±S.E.M. of three independent experiments. *b*) Compounds **1**—**3** and **7**—**11** were not cytotoxic to HL-60 cells (IC_{50}>10 μM). *c*) Not determined. *d*) Positive control substances.

and C-28 at the aglycone at δ 176.5. Thus, the structure of **2** was determined to be 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid *O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester.

Compound **3** was shown to have a molecular formula $C_{70}H_{114}O_{35}$ on the basis of HR-ESI-TOF-MS analysis (*m*/*z*: 1515.7216 $[M+H]^+$). Analysis of the ^{1}H- and ^{13}C-NMR spectra of **3** and comparison with that of **1** indicated that the triglycoside structure attached to C-3 of the aglycone and the tetraglycoside attached to C-28 are identical to those of **1**; however, **3** slightly differs from **1** in terms of the aglycone moiety. The molecular weight of **3** was higher than that of **1**, with the difference corresponding to one oxygen atom. In the ^{13}C-NMR spectrum, the signal due to the C-23 methyl group, which was observed at δ 28.2 in **1**, appeared as a signal due to a hydroxymethyl carbon at δ 64.0. Furthermore, ABq signals at δ 4.30 and 3.93 (J=10.3 Hz) were associated with the hydroxymethyl carbon signal by analysis of the HMQC spectrum. Acid hydrolysis of **3** yielded 3β,23-dihydroxyolean-12-en-28-oic acid (hederagenin, **3a**), L-rhamnose, D-ribose, L-arabinose, and D-glucose. On the basis of these data, the structure of **3** was found to be 3β-[(*O*-β-D-ribopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid *O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester.

The isolated compounds (**1**—**11**) were evaluated for their cytotoxic activity against HL-60 cells (Table 2). The triterpene monodesmosides (**4**—**6**) showed moderate cytotoxic activity against HL-60 cells with IC_{50} values of 3.1, 2.3, and 5.7 μM, respectively. Oleanolic acid (the aglycone of **4** and **5**), hederagenin (the aglycone of **6**), and bisdesmosides with the sugar units at both the C-3 hydroxy group and C-28 carboxy group (**1**—**3** and **7**—**11**) did not exhibit cytotoxicity even at a sample concentration of 10 μM. Compounds **4**—**6** also exhibited cytotoxic activities against A459 and HSC-2 solid tumor cells greater than or equal to those of cisplatin, which was used as a positive control. A459 and HSC-2 cells were not sensitive to etoposide. Compounds **4** and **5** were moderately cytotoxic to HSC-4 cells, which were resistant to both cisplatin and etoposide. Compound **6** is the C-23 hydroxy derivative of **4**, and it was presumed that the hydroxy group in **6** diminishes its cytotoxicity against HSC-4 cells.

Experimental

Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H-NMR, Karlsruhe, Germany) and a Bruker DRX-600 (600 MHz for ^{1}H-NMR) spectrometer using standard Bruker pulse programs. 1D-TOCSY and HSQC-TOCSY spectra were recorded at the mixing time of 120 ms. Chemical shifts are given as δ values in reference to tetramethylsilane (TMS) as an internal standard. ESI-TOF-MS spectra were recorded on a Waters-Micromass LCT mass spectrometer (Manchester, U.K.). Diaion HP-20 (Mitsubishi Chemical, 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 Silica gel 60 F_{254} (thickness: 0.25 mm, Merck, Darmstadt, Germany) and RP_{18} F_{254S} plates (thickness: 0.25 mm, Merck), and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed by using a system composed of a CCPM pump (Tosoh, Tokyo, Japan), a PX-8010 controller (Tosoh), an RI-8010 (Tosoh) and a Shodex OR-2 (Showa Denko, Tokyo, Japan) detector, and a Rheodyne injection port. The following reagents were obtained from the indicated companies: RPMI 1640, minimum essential medium (MEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, U.S.A.); fetal bovine serum (FBS) (BioWhittaker, Walkersville, MO, U.S.A.); Dulbecco's modified Eagle's medium (DMEM), penicillin G sodium salt, and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material The young plants of *Anemone hupehensis* var. *japonica* were purchased from Sakata Seed Co., Ltd. (Kanagawa, Japan) in June 2002. The plants were cultivated in the botanical garden of Tokyo University of Pharmacy and Life Sciences, and identified by Dr. Yutaka Sashida, emeritus professor of medicinal pharmacognosy at Tokyo University of Pharmacy and Life Sciences. A voucher specimen has been deposited in our laboratory (voucher No. AH-2003-001, Laboratory of Medicinal Pharmacognosy).

Extraction and Isolation The plant material (dry weight, 3.3 kg) was extracted with hot MeOH (26 l). The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (180 g) was passed through a Diaion HP-20 column (85 mm inner diameter (i.d.).×200 mm) that was successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc (each 7 l). Column chromatography of the MeOH eluate portion (50 g) on silica gel (70 mm i.d.×330 mm) and elution with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (9 : 1 : 0; 30 : 10 : 1; 20 : 10 : 1; 7 : 4 : 1), and finally with MeOH alone, yielded eight fractions (A—H). Fraction D was chromatographed on an ODS silica gel column (60 mm i.d.×240 mm) eluted with MeOH–H_2O (4 : 1) to give 14 subfractions (D-1—D-14). Fraction D-7 was suspended in MeOH and the insoluble solid was filtrated to give **6** (1.00 g). Fraction D-11 was chromatographed on silica gel (25 mm i.d.×200 mm) eluted with $CHCl_3$–MeOH–H_2O (50 : 10 : 1) to give **4** (15.1 mg). Fraction D-12 was chromatographed on silica gel (35 mm i.d.×200 mm) eluted with $CHCl_3$–MeOH–H_2O (70 : 10 : 1) to give **5** (137 mg). Fraction E was chromatographed on an ODS silica gel column (40 mm i.d.×220 mm) eluted with MeOH–H_2O (7 : 3) to give seven subfractions (E-1—E-7). Fraction E-3 was subjected to column chromatography on silica gel (35 mm i.d.×200 mm) eluted with $CHCl_3$–MeOH–H_2O (30 : 10 : 1; 20 : 10 : 1) and on ODS silica gel (25 mm i.d.×240 mm) eluted with MeOH–H_2O (2 : 1) to give **9** (6.2 mg). Fraction E-4 was subjected to column chromatography on silica gel (35 mm i.d.×200 mm) eluted with $CHCl_3$–MeOH–H_2O

(30 : 10 : 1) and on ODS silica gel (25 mm i.d.×250 mm) eluted with MeOH–H_2O (7 : 3) to give **7** (17.6 mg). Fraction F was separated by an ODS silica gel column (40 mm i.d.×240 mm) eluted with MeOH–H_2O (7 : 3) to give **8** (371 mg). Fraction G was subjected to silica gel column chromatography (60 mm i.d.×250 mm) eluted with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) and ODS silica gel column chromatography (40 mm i.d.×240 mm) eluted with MeOH–H_2O (7 : 3) to give **10** (527 mg). Fraction H was chromatographed on an ODS silica gel column (60 mm i.d.×300 mm) eluted with MeOH–H_2O (7 : 3) to give 12 subfractions (H-1—H-12). Fraction H-3 was subjected to column chromatography on ODS silica gel (40 mm i.d.×220 mm) eluted with MeOH–H_2O (3 : 2) and on silica gel (25 mm i.d.×270 mm) eluted with $CHCl_3$–MeOH–H_2O (7 : 4 : 1) to give **11** (37.8 mg). Fraction H-4 was chromatographed on silica gel (40 mm i.d.×250 mm) eluted with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) and on ODS silica gel (25 mm i.d.×240 mm) eluted with MeOH–H_2O (3 : 2) to give **3** (35.3 mg). Fraction H-6 was subjected to column chromatography on silica gel (35 mm i.d.×230 mm) eluted with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) and on ODS silica gel (25 mm i.d.×230 mm) eluted with MeOH–H_2O (2 : 1) to give **2** (6.7 mg). Fraction H-9 was chromatographed on silica gel (20 mm i.d.×220 mm) eluted with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) to give **1** (45.3 mg).

Compound **1**: Amorphous solid. $[\alpha]_D^{25}$ −33.6° (c=0.10, MeOH). HR-ESI-TOF-MS m/z: 1521.7109 $[M+Na]^+$ (Calcd for $C_{70}H_{114}O_{34}Na$: 1521.7089). IR ν_{max} (film) cm^{-1}: 3376 (OH), 2925 (CH), 1740 (C=O), 1260 and 1064 (C–O). ^{1}H-NMR (500 MHz, C_5D_5N) δ: 5.39 (1H, br s, H-12), 3.26 (1H, dd, J=11.5, 3.8 Hz, H-3), 3.17 (1H, dd, J=10.1, 3.3 Hz, H-18), 1.28 (3H, s, Me-23), 1.23 (3H, s, Me-27), 1.13 (3H, s, Me-24), 1.06 (3H, s, Me-26), 0.89 (3H, s, Me-29), 0.87 (3H, s, Me-30), 0.85 (3H, s, Me-25); for signals of sugar moieties, see Table 1. ^{13}C-NMR (125 MHz, 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), 33.1 (C-7), 39.9 (C-8), 48.0 (C-9), 37.0 (C-10), 23.8 (C-11), 122.8 (C-12), 144.1 (C-13), 42.1 (C-14), 28.3 (C-15), 23.3 (C-16), 47.0 (C-17), 41.7 (C-18), 46.2 (C-19), 30.7 (C-20), 34.0 (C-21), 32.5 (C-22), 28.2 (C-23), 17.1 (C-24), 15.6 (C-25), 17.4 (C-26), 26.1 (C-27), 176.5 (C-28), 33.1 (C-29), 23.7 (C-30); for signals for the sugar moieties, see Table 1.

Acid Hydrolysis of 1 A solution of **1** (12.0 mg) in 1 M HCl (dioxane–H_2O, 1 : 1, 3 ml) was heated at 95 °C for 1.5 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-96SB (Organo, Tokyo, Japan) column and chromatographed on Diaion HP-20 eluted with MeOH–H_2O (2 : 3) followed by EtOH–Me_2CO (1 : 1), to yield an aglycone fraction (3.7 mg) and a sugar fraction (8.6 mg). The aglycone fraction was chromatographed on silica gel eluted with hexane–Me_2CO (4 : 1) to give **1a** (oleanolic acid, 2.3 mg). The sugar fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d.×250 mm, 5 μm, Shiseido, Tokyo, Japan); solvent, MeCN–H_2O (17 : 3); flow rate, 0.9 ml/min; detection, refractive index (RI) and optical rotation (OR). Identification of L-rhamnose, D-ribose, D-arabinose, and D-glucose present in the sugar fraction was carried out by comparison of their retention times (t_R) and optical rotations with those of authentic samples. t_R (min): 7.6 (L-rhamnose, negative optical rotation), 8.2 (D-ribose, negative optical rotation), 9.0 (D-arabinose, positive optical rotation), 15.6 (D-glucose, positive optical rotation).

Compound **2**: Amorphous solid. $[\alpha]_D^{25}$ −20.2° (c=0.10, MeOH). HR-ESI-TOF-MS m/z: 1661.7778 $[M+H]^+$ (Calcd for $C_{76}H_{125}O_{39}$: 1661.7798). IR ν_{max} (film) cm^{-1}: 3376 (OH), 2925 (CH), 1739 (C=O), 1260, 1092 and 1027 (C–O). ^{1}H-NMR (500 MHz, C_5D_5N) δ: 5.42 (1H, br s, H-12), 3.25 (1H, dd, J=11.5, 3.8 Hz, H-3), 3.19 (1H, dd, J=13.4, 3.4 Hz, H-18), 1.31 (3H, s, Me-23), 1.26 (3H, s, Me-27), 1.18 (3H, s, Me-24), 1.09 (3H, s, Me-26), 0.92 (3H, s, Me-29), 0.90 (3H, s, Me-30), 0.88 (3H, s, Me-25); for signals of sugar moieties, see Table 1. ^{13}C-NMR (125 MHz, C_5D_5N) δ: 39.0 (C-1), 26.7 (C-2), 88.7 (C-3), 39.6 (C-4), 56.1 (C-5), 18.5 (C-6), 33.2 (C-7), 39.9 (C-8), 48.1 (C-9), 37.0 (C-10), 23.7 (C-11), 123.1 (C-12), 144.1 (C-13), 42.2 (C-14), 28.2 (C-15), 23.4 (C-16), 47.0 (C-17), 41.7 (C-18), 46.3 (C-19), 30.8 (C-20), 34.0 (C-21), 32.5 (C-22), 28.2 (C-23), 17.2 (C-24), 15.7 (C-25), 17.5 (C-26), 26.1 (C-27), 176.5 (C-28), 33.2 (C-29), 23.7 (C-30); for signals of sugar moieties, see Table 1.

Acid Hydrolysis of 2 A solution of **2** (6.0 mg) was subjected to acid hydrolysis using the above-mentioned procedure for the hydrolysis of **1** to afford an aglycone fraction (1.5 mg) and a sugar fraction (4.5 mg). The aglycone fraction was chromatographed on silica gel eluted with hexane–Me_2CO (4 : 1) to give **1a** (0.8 mg). HPLC analysis of the sugar fraction under the same conditions as in the analysis of **1** showed the presence of L-rhamnose, D-ribose, D-arabinose, and D-glucose. t_R (min): 7.7 (L-rhamnose, negative optical rotation), 8.3 (D-ribose, negative optical rotation), 9.7 (D-arabinose, positive optical rotation), 17.2 (D-glucose, positive optical rotation).

Compound **3**: Amorphous solid. $[\alpha]_D^{25}$ −29.3° (c=0.10, MeOH). HR-ESI-TOF-MS m/z: 1515.7216 $[M+H]^+$ (Calcd for $C_{70}H_{115}O_{35}$: 1515.7219). IR ν_{max} (film) cm^{-1}: 3376 (OH), 2936 (CH), 1732 (C=O), 1260 and 1061 (C–O). ^{1}H-NMR (500 MHz, C_5D_5N) δ: 5.41 (1H, br s, H-12), 4.29 (1H, m, H-3), 4.30 (1H, d, J=10.3 Hz, H-23a), 3.93 (1H, d, J=10.3 Hz, H-23b), 3.17 (1H, dd, J=13.4, 3.3 Hz, H-18), 1.19 (3H, s, Me-27), 1.14 (3H, s, Me-24), 1.11 (3H, s, Me-26), 0.97 (3H, s, Me-25), 0.88 (3H×2, s, Me-29 and Me-30); for signals of sugar moieties, see Table 1. ^{13}C-NMR (125 MHz, C_5D_5N) δ: 39.1 (C-1), 26.4 (C-2), 81.1 (C-3), 43.6 (C-4), 47.7 (C-5), 18.1 (C-6), 32.8 (C-7), 39.9 (C-8), 48.2 (C-9), 36.9 (C-10), 23.8 (C-11), 123.0 (C-12), 144.1 (C-13), 42.1 (C-14), 28.3 (C-15), 23.3 (C-16), 47.0 (C-17), 41.6 (C-18), 46.2 (C-19), 30.7 (C-20), 34.0 (C-21), 32.5 (C-22), 64.0 (C-23), 14.1 (C-24), 16.2 (C-25), 17.5 (C-26), 26.0 (C-27), 176.5 (C-28), 33.1 (C-29), 23.7 (C-30); for signals of sugar moieties, see Table 1.

Acid Hydrolysis of 3 A solution of **3** (12.0 mg) was subjected to acid hydrolysis using the above-mentioned procedure for the hydrolysis of **1** to afford an aglycone fraction (3.3 mg) and a sugar fraction (7.4 mg). The aglycone fraction was chromatographed on silica gel eluted with hexane–Me_2CO (4 : 1) to give **3a** (hederagenin, 1.7 mg). HPLC analysis of the sugar fraction under the same conditions as in the analysis of **1** showed the presence of L-rhamnose, D-ribose, D-arabinose, and D-glucose. t_R (min): 7.6 (L-rhamnose, negative optical rotation), 8.2 (D-ribose, negative optical rotation), 9.0 (D-arabinose, positive optical rotation), 15.6 (D-glucose, positive optical rotation).

Cell Culture and Assay for Cytotoxic Activity against HL-60 Cells HL-60 cells, which were obtained from the Human Science Research Resources Bank (JCRB 0085, Osaka, Japan) were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and antibiotics (100 units/ml penicillin sodium salt and 100 μg/ml streptomycin sulfate) in a 5% CO_2 humidified incubator at 37 °C. The cells were washed and resuspended in the medium at 4×10^4 cells/ml, and 196 μl of this cell suspension was seeded into 96-well flat bottom plates (Iwaki Glass, Chiba, Japan). 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 obtain final concentrations of 0.01—20 μM, and 4 μl of EtOH–H_2O (1 : 1) was added to the control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using a modified MTT reduction assay.[14] At the end of the incubation period, 10 μl of 5 mg/ml MTT in phosphate buffered saline (PBS) was added to each well, and the plate was further incubated in 5% CO_2/air for 4 h at 37 °C. Then, the plate was centrifuged at 1500 g for 5 min to precipitate MTT formazan. An aliquot of 150 μl of the supernatant was removed from each well, and 175 μl of dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The plate was mixed on a microplate mixer for 10 min and then read on a microplate reader (Spectra Classic, Tecan, Salzburg, Austria) at 550 nm. Each assay was performed in triplicate and cytotoxicity was expressed as the IC_{50} value, which reduces the number of viable cells by 50%.

Cell Culture and Assay for Cytotoxic Activity against A549, HSC-2, and HSC-4 Cells A549 (JCRB 0076) cells were incubated at 37 °C in MEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. HSC-2 and HSC-4 cells were maintained as monolayer cultures at 37 °C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Cells were trypsinized and resuspended in the medium at 1×10^5 cells/ml, and 100 μl of this cell suspension was seeded into 96-well flat bottom plates and incubated for 24 h. After washing once with PBS, the cells were treated for 24 h in the presence of the test compounds. The cells were washed once with PBS and incubated for 4 h with 0.2 mg/ml MTT in MEM (A549 cells) or DMEM (HSC-2 and HSC-4 cells), supplemented with 10% heat-inactivated FBS. After the medium was removed, the MTT formazan crystals were dissolved with 100 μl of DMSO. The plate was mixed on a microplate mixer for 10 min and then read on a microplate reader at 550 nm. Each assay was performed in triplicate and cytotoxicity was expressed as the IC_{50} value, which reduces the number of viable cells by 50%.

References and Notes

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