Triterpenoid Saponins from the Roots of *Pulsatilla koreana*

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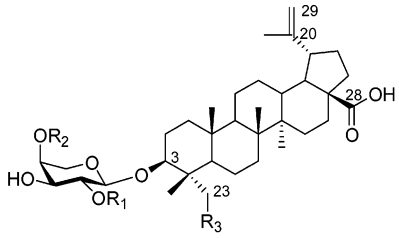
Six new saponins, five lupanes (**1**−**5**) and one oleanane (**6**), along with 11 known saponins, were isolated from the roots of *Pulsatilla koreana*. The structures of the new saponins were found to be 23-hydroxy-3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid (**1**), 23-hydroxy-3*β*-[(*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid (**2**), 3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid (**3**), 3*β*-[(*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid (**4**), 23-hydroxy-3*β*-[(*O*-*β*-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid (**5**), and hederagenin 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**6**). Their structures were determined on the basis of 1D and 2D NMR (^{13}C NMR, ^{1}H NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC) methods, FABMS, and hydrolysis. All isolated compounds were evaluated for their cytotoxic activity against A-549 human lung carcinoma cells.

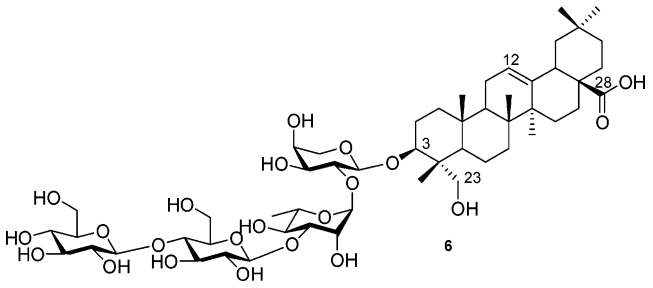
A preparation made from the roots of *Pulsatilla koreana* ("Pulsatillae Radix"), *Panax ginseng*, and *Glycyrrhiza glabra* has been used in traditional medicinal practice to treat various cancers.[1] Currently, this preparation, which is called SB31, is undergoing phase II clinical studies in Korea. It was previously reported that SB31 exhibited cytotoxic activity against some human cancer cell lines[2,3] and potent antitumor activity on a mouse tumor model.[4] An active antitumor constituent from the roots of *Pulsatilla koreana* Nakai (Ranunculaceae) as the main component of the recipe was isolated and found to be deoxypodophyllotoxin (DPT), which exhibited antiangiogenic activity and good antitumor activity against mice bearing Lewis lung carcinoma (LLC).[5] However, the DPT content of Pulsatillae Radix, which is an active constituent of SB31, is too low for discernible antitumor activity. This suggests that DPT is not a major constituent responsible for the antitumor activity of *P. koreana* roots.

The above highlights the need to isolate additional antitumor constituents from *P. koreana* roots. In the first step of this study, we isolated a total 17 triterpenoid saponins, comprising 11 already known saponins and six new saponins (**1**−**6**) from *P. koreana* roots. These new monodesmodic triterpene glycosides (**1**−**6**) were determined structurally by 1D and 2D NMR (^{13}C NMR, ^{1}H NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC) methods, FABMS, and hydrolysis. The cytotoxic activity of the new isolated compounds as well as of the known compounds was evaluated against A-549 cells.

The 50% MeOH extract of the roots of *P. koreana* was purified by centrifugation. The combined upper layer extracts were suspended in H_2O and defatted with *n*-hexane. The aqueous layer was then partitioned with *n*-BuOH. The *n*-BuOH layer was dried and chromatographed on a Sephadex LH-20 column to give four fractions, each of which was purified sequentially on a silica gel column and HPLC to give 17 saponins as detailed in the Experimental Section. Compounds **1**−**6**, which were obtained in fraction 3, were found to be new saponins,



	R_1	R_2	R_3
1	rha	glc	OH
2	H	glc-(1→3)-rha	OH
3	rha	glc	H
4	H	glc-(1→3)-rha	H
5	H	glc	OH



whereas the remaining 11 already known isolates were identified as 23-hydroxy-3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]-lup-20(29)-en-oic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester,[6] 3-*O*-α-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester,[7] 23-hydroxy-3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl)oxy]lup-20(29)-en-oic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester,[6] 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester,[7] hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside,[7] hederagenin 3-*O*-

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β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside,[8] oleanolic acid 3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside,[8] oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside,[8] hederagenin 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside,[9] 23-hydroxy-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl)oxy]lup-20(29)-en-oic acid,[6] and hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside,[8] respectively.

Compound **1** was obtained as an amorphous powder, and the FABMS showed a quasi-molecular ion at *m/z* 935 $[M + Na]^+$, which is consistent with a molecular formula of $C_{47}H_{76}O_{17}$. Acid hydrolysis of **1** afforded 23-hydroxybetulinic acid,[10] which was identified by TLC and HPLC comparison with an authentic sample, and the sugars L-arabinose, D-glucose, and L-rhamnose. The absolute configurations of the component monosaccharides were determined by direct HPLC analysis of the hydrolysate using a combination of RI and optical rotary detectors.[8] The IR spectrum showed a characteristic absorption attributable to a carboxylic acid at 1680 cm^{-1}, as well as broad absorption of a carboxyl group near 3400 cm^{-1}. The ^1H and ^{13}C NMR spectra of **1** clearly showed the presence of a triterpene bearing an olefinic group and a carbonyl group. The DEPT spectrum of **1** revealed signals for six methyls, 14 methylenes, 20 methines, and seven quaternary carbons. The NMR data also suggested the presence of three sugar residues, clearly indicated by three anomeric carbon signals at δ 107.0 (C-1‴ of glucose), 104.7 (C-1′ of arabinose), and 102.1 (C-1″ of rhamnose), and three anomeric proton signals at δ 6.26 (1H, br, s, H-1″ of rhamnose), 5.10 (1H, d, J = 8.0 Hz, H-1‴ of glucose), and 4.96 (1H, d, J = 7.0 Hz, H-1′ of arabinose), as well as six methyl proton signals at δ 1.74, 1.63 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.06, 1.02, 1.00, 0.85, and an exomethylene group at δ 4.90 and 4.71 (each br, s). These data indicated a β configuration at the anomeric position of the glucose residue. The C-28 carbonyl carbon was observed at δ 179.3 in the ^{13}C NMR spectrum of **1**, which suggested that no sugar linkage was formed at C-28 and that a triglycoside was attached at C-3 of 23-hydroxybetulinic acid in **1**. Glycosylation shifts were observed only at C-2 (−1.1 ppm), C-4 (−1.1 ppm), and C-3 (+8.1 ppm), by comparison with the ^{13}C NMR spectrum of authentic 23-hydroxybetulinic acid. This led to the conclusion that the arabinose unit is connected to the hydroxyl at C-3 of 23-hydroxybetulinic acid. On comparison of the ^{13}C NMR spectrum of **1** with that of 3β,23-dihydroxylup-20(29)-en28-oic acid 3-*O*-α-L-arabinopyranoside,[10] the signals due to C-2′ and C-4′ of the arabinosyl moiety were displaced downfield by 3.6 and 11.0 ppm, which were observed at δ 76.8 and 80.6, respectively, suggesting that the C-2′ and C-4′ hydroxyl groups of the arabinosyl moiety were the positions at which the additional L-rhamnose and D-glucose units were linked. Finally, the HMQC spectrum was used to correlate all the proton resonances with those of the corresponding one-bond coupled carbons, leading to the unambiguous assignments of the carbon chemical shifts. Accordingly, the structure of **1** was elucidated as 23-hydroxy-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid.

The FABMS of compound **2** displayed a quasi-molecular ion peak at *m/z* 935 $[M + Na]^+$, suggesting the same molecular formula as **1**. Acid hydrolysis of **2** yielded 23-hydroxybetulinic acid, arabinose, glucose, and rhamnose. The ^{13}C and DEPT spectra of **2** displayed 47 signals, of which 30 were assigned to a triterpene moiety and 17 to the saccharide portion. The ^1H and ^{13}C NMR spectra showed the presence of three sugar residues, indicated by three anomeric carbon signals at δ 106.9 (C-1‴ of glucose), 105.0 (C-1′ of arabinose), and 101.7 (C-1″ of rhamnose), and three sugar residues, indicated by three anomeric proton signals at δ 6.24 (1H, br, s, H-1″ of rhamnose), 5.48 (1H, d, J = 8.0 Hz, H-1‴ of glucose), and 5.00 (1H, d, J = 6.5 Hz, H-1′ of arabinose), along with six methyl proton signals and an exomethylene group at δ 4.90 and 4.72 (each br, s). Analysis of the ^{13}C NMR data of **2** and comparison with those of **1** indicated these compounds are different in the saccharide portions. The chemical shift of the C-4′ position of arabinose, which was linked to the hydroxyl at C-3 of aglycon, showed a significant upfield shift (δ 69.9). Instead, the C-3″ position of rhamnose which was linked to the C-2′ of arabinopyranosyl unit was shifted downfield (δ 83.2). This indicated that the terminal glucosyl residue must be attached to the C-3″ position of rhamnose, not the C-4′ position of the arabinose residue. These findings led to the assignment of **2** as 23-hydroxy-3β-[(*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid.

The FABMS of **3** displayed a $[M + Na]^+$ peak at *m/z* 919, consistent with a molecular formula of $C_{47}H_{76}O_{16}$. Analysis of the ^{13}C NMR spectrum of **3** revealed that the triglycoside structure was identical to that of **1**, but differed slightly from **1** in terms of the aglycon structure. Acid hydrolysis of **3** liberated the known triterpenoid, betulinic acid,[6] as well as L-arabinose, D-glucose, and L-rhamnose. Thus, **3** was shown to be a new compound and its structure was assigned as 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-[*O*-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid.

The FABMS of **4** displayed a $[M + Na]^+$ peak at *m/z* 919, consistent with a molecular formula of $C_{47}H_{76}O_{16}$. Comparison of the ^{13}C NMR spectrum of **4** with those of **1** and **2** suggested that the triglycoside structure attached at C-3 of the aglycon was identical to that of **2** with respect to the sugar moieties, but the aglycon of **4** was betulinic acid as **3**. Upon acid hydrolysis of **4**, betulinic acid was afforded, along with the sugars L-arabinose, D-glucose, and L-rhamnose. Thus, the structure of **4** was elucidated as 3β-[(*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid.

The FABMS of **5** displayed a $[M + Na]^+$ peak at *m/z* 766, consistent with a molecular formula of $C_{41}H_{66}O_{13}$, and was lower by $C_6H_{10}O_4$ than that of **1**. Acid hydrolysis of **5** yielded 23-hydroxybetulinic acid, L-arabinose, and D-glucose. On comparison of the ^{13}C NMR spectrum of **5** with that of 3β,23-dihydroxylup-20(29)-en-28-oic acid 3-*O*-α-L-arabinopyranoside, the signal due to C-4′ of the arabinosyl moiety was displaced downfield 10.3 ppm to δ 79.9, suggesting that the C-4′ hydroxyl group of the arabinosyl moiety was the position to which the additional D-glucose was linked. Thus, the structure of **5** was elucidated as 23-hydroxy-3β-[(*O*-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid.

The FABMS of **6** displayed a $[M + Na]^+$ peak at *m/z* 1097, consistent with a molecular formula of $C_{53}H_{86}O_{22}$, which was supported by the ^{13}C NMR and DEPT spectroscopic 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 and ^{13}C NMR spectra of **6** clearly showed the presence of a triterpene bearing an olefinic group and carbonyl group. The DEPT spectra of **6** revealed

signals for seven methyls, 14 methylenes, 24 methines, and eight quaternary carbons. The ^{1}H NMR spectra of **6** exhibited signals due to seven tertiary methyl groups at δ 1.53 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.23, 1.09, 1.01, 0.99, 0.95, and 0.91, and an olefinic proton at δ 5.44 (1H, br, s, H-12). Also, the ^{1}H NMR spectrum of **6** indicated the presence of four anomeric proton signals at δ 6.21 (1H, br, s, H-1″ of rhamnose), 5.37 (1H, d, J = 7.8 Hz, H-1‴ of inner glucose), 5.13 (1H, d, J = 7.8 Hz, H-1⁗ of terminal glucose), and 5.00 (1H, d, J = 6.6 Hz, H-1′ of arabinose), which were correlated with the ^{13}C NMR signals for anomeric carbons at δ 106.7 (C-1‴ of inner glucose), 105.1 (C-1′ of arabinose), 104.9 (C-1⁗ of terminal glucose), and 101.6 (C-1″ of rhamnose) in the ^{1}H−^{1}H COSY and HMQC spectra. The corresponding six tertiary methyl carbons at δ 24.0 (C-30), 18.6 (C-6″ of rhamnose), 16.3 (C-25), 17.7 (C-26), 26.4 (C-27), and 14.3 (C-24) and two olefinic carbons at δ 145.3 (C-13) and 122.6 (C-12) appeared in the ^{13}C NMR spectum. On comparison of the ^{13}C NMR spectrum of **6** with that of oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside,[8] the chemical shifts of the carbohydrate moieties were very similar, except that the chemical shift of C-23 of the aglycon was displaced downfield 36.1 ppm, due to a hydroxyl group, to δ 64.3, suggesting that the aglycon of **6** is hederagenin. By acid hydrolysis of **6** hederagenin could be confirmed as the aglycon by comparison with published spectral data.[11] In addition, L-arabinose, D-glucose, and L-rhamnose were observed as the sugar moieties by TLC and HPLC. Thus, the structure of **6** was elucidated as hederagenin 3-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside.

The cytotoxic activities of the isolated six new compounds (**1**−**6**) and the 11 known compounds were evaluated against A-549 human lung carcinoma cells. Although these new saponins did not show any apparent cytotoxic activity compared with doxorubicin used as a positive control (ED_{50} 0.02 μg/mL), three known compounds, oleanolic acid 3-*O*-α-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside, oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside, and hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside exhibited cytotoxic activity, with ED_{50} values of 2.6, 4.2, and 9.6 μg/mL, respectively. All other compounds exhibited ED_{50} values of >10 μg/mL. Even though the additional investigation of other compounds still has to be pursued, it may be suggested that the anticancer activity of SB31 is due in part to the activity of these known saponins. To validate this, a preclinical study on the saponins is currently in progress, and these results will be reported in due course.

Experimental Section

General Experimental Procedures. Melting points were measured on an Electrothermal melting point apparatus. Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were obtained on KBr disks using a JASCO Report 100 spectrophotometer. ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra were recorded on a Bruker MSL-300 or -500 instrument operating at 300 and 500 MHz for ^{1}H and 75.5 MHz for ^{12}C, respectively. FABMS were recorded on a JMS-HX110/110A spectrometer. HPLC was performed using a Shimadzu liquid chromatograph model Class-vp version 6.12, equipped with a SPD-10A UV−vis detector (Shimadzu), an RI-8010 detector (Tosoh, Japan), and a Shodex OR-2 detector (Showa-Denko, Japan). HPLC was performed using a Mightysil column (250 mm × 10 mm, RP-C_{18}, 5 μm, Kanto, Japan) and a Spherisorb S5 ODS2 column (250 mm × 10 mm, RP-C_{18}, 5 μm, Waters, Milford, MA), and all solvents for HPLC were filtered through a 0.45 μm membrane filter (Waters). Merck silica gel was used for column chromatography and thin-layer chromatography. The following materials and reagents were used for cell culture and the sulforhodamine B (SRB) assay: microplate reader, Tecan A-5082 (Salzburg, Austria); 96-well flat-bottom plate, Falcon (Bedford, MA); RPMI-1640 medium and FBS, Gibco BRL (Rockville, MD). All other chemicals used were of biochemical reagent grade.

Plant Material. The roots of *Pulsatilla koreana* were collected at Geumsan-gun, Chungnam, Korea, in May 2003 and authenticated by Professor Bae, Chungnam National University. A voucher specimen was deposited in the herbarium of Chungnam College of Pharmacy.

Extraction and Isolation. Dried roots (500 g) were extracted with 1 L of 50% MeOH at room temperature for 12 h and then filtered. The extraction was repeated twice. The combined filtrates were concentrated in a vacuum evaporator to afford a syrupy brown residue (63 g). This residue was suspended in water (250 mL) and extracted successively with *n*-hexane (250 mL × 3) and *n*-BuOH (250 mL × 3). A light brown residue remained (12 g) after evaporating the *n*-BuOH.

The residue (2 g) was dissolved in 80% MeOH (15 mL) and loaded into a Sephadex LH-20 column (400 g, 60 × 5 cm) and eluted with 80% MeOH (1 mL/min). The fractionation was performed on silica gel plates (Kieselgel, eluting solvent; *n*-BuOH−AcOH−H_2O, 4:1:1, sprayed with 10% H_2SO_4 and heated). The fractions were divided as follows: fraction 1, tube No. 1−45; fraction 2, 46−96; fraction 3, 97−170; fraction 4, 171−240. This fractionation was repeated in order to collect a sufficient amount of each fraction, to give final amounts as follows: fraction 1 (3.1 g), fraction 2 (2.4 g), fraction 3 (3.7 g), and fraction 4 (2.0 g). Since fractions 1, 2, and 4 contained only already known saponins, the isolation procedure is described in the Supporting Information. Fraction 3 (3.7 g) was dissolved in MeOH (20 mL), loaded into a silica gel column (300 g, 80 × 4.8 cm), and eluted using a mixture of $CHCl_3$−MeOH−EtOAc−H_2O (2:2:4:1, lower phase) to give subfractions 3-1 (0.85 g, R_f = 0.30 with the same solvent), 3-2 (1.23 g, R_f = 0.16), and 3-3 (0.55 g, R_f = 0.09). Fraction 3-2 (1.23 g) was dissolved in MeOH (15 mL), loaded in the same column, and eluted with 40% MeCN (flow rate: 1.5 mL/min) to give compound **1** (149.4 mg, t_R = 33.4 min). Fraction 3-1 (0.85 g) was dissolved in 10 mL of MeOH and filtered on a 0.45 μm membrane filter. An aliquot (100 μL) of the filtrate was loaded on a Spherisorb S5 ODS2 column and subjected to preparative HPLC elution with 43% MeCN (flow rate: 3 mL/min) to give compounds **2** (26.0 mg, t_R = 15.1 min), **3** (31.0 mg, t_R = 23.7 min), **4** (18.6 mg, t_R = 30.5 min), and **5** (16.7 mg, t_R = 39.3 min). Fraction 3-3 (0.55 mg) was dissolved in MeOH (15 mL), loaded in the same column, and eluted with 36% MeCN (flow rate: 3.5 mL/min) to give compound **6** (60.2 mg, t_R = 26.3 min).

Compound 1: amorphous white solid; mp 250−252 °C; $[\alpha]^{25}_D$ −11.3° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930, 1680 (C=O), 1640 (C=C), 1050 (C−O−C) cm^{-1}; ^{1}H NMR (500 MHz, C_5D_5N) δ 6.26 (1H, br, s, H-1″ of rhamnose), 5.10 (1H, d, J = 8.0 Hz, H-1‴ of glucose), 4.96 (1H, d, J = 7.0 Hz, H-1′ of arabinose), 4.90 and 4.72 (each 1H, br, s, H_2-29), 1.74 (3H, s, Me-30), 1.63 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.06 (3H, s, Me-24), 1.04 (3H, s, Me-26), 1.00 (3H, s, Me-27), 0.85 (3H, s, Me-25); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables 1 and 2; FABMS *m/z* 935 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 936.1103 (calcd for $C_{47}H_{76}O_{17}Na$, 936.1071).

Compound 2: amorphous white solid; mp 252−254 °C; $[\alpha]^{25}_D$ −11.0° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930, 1700 (C=O), 1645 (C=C), 1060 (C−O−C) cm^{-1}; ^{1}H NMR (500 MHz, C_5D_5N) δ 6.24 (1H, br, s, H-1″ of rhamnose), 5.48 (1H, d, J = 8.0 Hz, H-1‴ of glucose), 5.00 (1H, d, J = 6.5 Hz, H-1′ of arabinose), 4.90 and 4.75 (each 1H, br, s, H_2-29), 1.74 (3H, s, Me-30), 1.52 (3H, d, J = 6.5 Hz, H-6″ of rhamnose), 1.09 (3H, s, Me-24), 1.04 (3H, s, Me-26), 1.01 (3H, s, Me-27), 0.85 (3H, s, Me-25); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables

Table 1. ^{13}C NMR Data of the Aglycon Moieties of Compounds **1**−**6** (75.5 MHz, in C_5D_5N)

carbon	1	2	3	4	5	6
C-1	39.6	39.5	39.3	39.6	39.2	39.2
C-2	26.8	26.7	27.0	27.2	26.4	26.5
C-3	81.6	81.5	89.0	89.3	82.2	81.4
C-4	44.0	43.8	39.8	40.1	43.7	43.8
C-5	48.1	48.0	56.3	48.2	47.9	47.8
C-6	18.5	18.3	18.7	18.9	18.3	18.3
C-7	34.8	34.6	34.9	35.2	34.6	33.0
C-8	41.5	41.3	41.3	41.5	41.3	39.9
C-9	51.4	51.2	51.0	51.3	51.1	48.4
C-10	37.4	37.2	37.3	37.6	37.3	37.1
C-11	21.6	21.5	21.4	21.7	21.4	24.0
C-12	26.5	26.3	26.3	26.5	26.3	122.6
C-13	38.4	38.8	38.7	38.9	38.8	145.3
C-14	43.2	43.0	43.0	43.3	43.0	42.4
C-15	30.7	30.5	30.5	30.4	30.5	28.6
C-16	33.3	33.2	33.2	33.3	33.1	24.0
C-17	57.0	56.9	56.9	56.5	56.9	46.9
C-18	50.2	50.0	50.0	50.2	50.0	42.3
C-19	48.4	48.0	48.0	48.2	48.0	46.8
C-20	151.6	151.6	151.6	151.0	151.6	31.1
C-21	31.6	31.5	31.5	31.5	31.4	34.4
C-22	38.0	37.9	37.9	38.2	37.8	30.1
C-23	64.3	64.2	28.1	28.5	64.5	64.3
C-24	14.1	14.0	17.0	17.3	13.6	14.3
C-25	17.3	17.1	16.6	16.8	17.1	16.3
C-26	16.8	16.6	16.6	16.8	16.6	17.7
C-27	15.3	15.1	15.0	15.3	15.0	26.4
C-28	179.3	180.0	180.0	180.0	180.0	181.1
C-29	110.3	110.0	110.0	110.1	110.1	33.5
C-30	19.8	19.6	19.7	19.9	19.6	24.0

Table 2. ^{13}C NMR Data of the Sugar Moieties of Compounds **1**−**6** (75.5 MHz, in C_5D_5N)

carbon	1	2	3	4	5	6
ara-1′	104.7	105.0	105.1	105.7	106.5	105.1
2′	76.8	75.8	76.7	76.2	73.8	75.6
3′	75.2	75.1	74.0	75.0	74.8	74.9
4′	80.6	69.9	79.6	70.2	79.9	69.8
5′	65.6	66.4	64.5	66.2	66.4	66.3
rha-1″	102.1	101.7	102.0	102.1		101.6
2″	72.8	71.8	72.6	71.8		71.9
3″	72.6	83.2	72.5	83.8		83.7
4″	74.5	73.1	74.0	73.4		73.1
5″	70.0	69.8	70.0	69.8		69.8
6″	19.0	18.6	18.8	18.9		18.6
glc-1‴	107.0	106.9	106.5	107.2	106.9	106.7
2‴	75.8	76.0	75.6	76.3	75.9	75.6
3‴	78.9	78.6	78.7	78.9	78.8	76.8
4‴	71.7	71.9	71.5	72.1	71.6	81.1
5‴	79.1	78.7	78.9	79.0	78.5	76.9
6‴	62.9	62.7	62.7	62.9	62.8	61.9
glc-1⁗						104.9
2⁗						75.1
3⁗						78.4
4⁗						71.6
5⁗						78.5
6⁗						62.5

1 and 2; FABMS *m/z* 935 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 936.1137 (calcd for $C_{47}H_{76}O_{17}Na$, 936.1071).

Compound 3: amorphous white solid; mp 247−250 °C; $[\alpha]^{25}_D$ −5.9° (*c* 0.05, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930, 1700 (C=O), 1640 (C=C), 1060 (C−O−C) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 6.17 (1H, br, s, H-1″ of rhamnose), 5.13 (1H, d, J = 8.0 Hz, H-1‴ of glucose), 4.76 (1H, d, J = 6.0 Hz, H-1′ of arabinose), 4.92 and 4.72 (each 1H, br, s, H_2-29), 1.76 (3H, s, Me-30), 1.62 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.17 (3H, s, Me-23), 1.08 (3H, s, Me-27), 1.07 (3H, s, Me-24), 1.01 (3H, s, Me-26), 0.77 (3H, s, Me-25); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables 1 and 2; FABMS *m/z* 919 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 920.1061 (calcd for $C_{47}H_{76}O_{16}$-Na, 920.1077).

Compound 4: amorphous white solid; mp 245−250 °C; $[\alpha]^{25}_D$ −6.0° (*c* 0.05, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930, 1685 (C=O), 1640 (C=C), 1055 (C−O−C) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 6.17 (1H, br, s, H-1″ of rhamnose), 5.46 (1H, d, J = 7.8 Hz, H-1‴ of glucose), 4.81 (1H, d, J = 6.0 Hz, H-1′ of arabinose), 4.91 and 4.73 (each 1H, br, s, H_2-29), 1.76 (3H, s, Me-30), 1.53 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.31 (3H, s, Me-23), 1.10 (3H, s, Me-27), 1.09 (3H, s, Me-24), 1.01 (3H, s, Me-26), 0.76 (3H, s, Me-25); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables 1 and 2; FABMS *m/z* 919 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 920.1094 (calcd for $C_{47}H_{76}O_{16}$-Na, 920.1077).

Compound 5: amorphous white solid; mp 267−269 °C; $[\alpha]^{25}_D$ −27.7° (*c* 0.09, MeOH); IR (KBr) ν_{max} 3400 (OH), 2950, 1690 (C=O), 1640 (C=C), 1050 (C−O−C) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 5.16 (1H, d, J = 7.5 Hz, H-1″ of glucose), 4.88 (1H, overlap, H-1′ of arabinose), 4.92 and 4.74 (each 1H, br, s, H_2-29), 1.75 (3H, s, Me-30), 1.04 (3H, s, Me-24), 0.99 (3H, s, Me-26), 0.88 (3H, s, Me-27), 0.84 (3H, s, Me-25); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables 1 and 2; FABMS *m/z* 789 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 789.9627 (calcd for $C_{41}H_{66}O_{13}Na$, 789.9632).

Compound 6: amorphous white solid; mp 260−262 °C; $[\alpha]^{25}_D$ −6.0° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930, 1680 (C=O), 1640 (C=C), 1050 (C−O−C) cm^{-1}; ^1H NMR (300 MHz, C_5D_5N) δ 6.27 (1H, br, s, H-1″ of rhamnose), 5.37 (1H, d, J = 7.8 Hz, H-1⁗ of terminal glucose), 5.13 (1H, d, J = 7.8 Hz, H-1‴ of inner glucose), 5.00 (1H, d, J = 6.6 Hz, H-1′ of arabinose), 5.45 (1H, br, s, H-12), 4.25 (1H, overlap, C-23b), 3.61 (1H, d, J = 10.8 Hz, C-23a), 1.53 (3H, d, J = 6.0 Hz, H-6″ of rhamnose), 1.23 (3H, s, Me-27), 1.12 (3H, s, Me-24), 1.01 (3H, s, Me-26), 0.99 (3H, s, Me-30), 0.94 (3H, s, Me-25), 0.91 (3H, s, Me-29); ^{13}C NMR (75.5 MHz, pyridine-d_5), see Tables 1 and 2; FABMS *m/z* 1097 $[M + Na]^+$; HRFABMS (positive mode) *m/z* 1098.2516 (calcd for $C_{53}H_{86}O_{22}Na$, 1098.2504).

General Acid Hydrolysis of Compounds 1−6. A solution of each compound (15 mg) in 0.4 M HCl (dioxane−H_2O, 1:1, 10 mL) was heated to 90 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ extract was evaporated and the residue was purified on a Si gel using a discontinuous gradient of $CHCl_3$−MeOH (99:1 to 1:1) to give an aglycon (compound **1**: 23-hydroxybetulinic acid (3.7 mg), compound **2**: 23-hydroxybetulinic acid (3.5 mg), compound **3**: betulinic acid (3.7 mg), compound **4**: betulinic acid (3.0 mg), compound **5**: 23-hydroxybetulinic acid (3.1 mg), and compound **6**: hederagenin (3.6 mg), which were identified by comparison of the spectroscopic data with literature values). Each of the H_2O layers was neutralized with Ag_2CO_3 and analyzed by TLC (Kieselgel, eluting solvent *n*-BuOH−AcOH−H_2O, 4:1:1, sprayed with 10% H_2SO_4 and heated) to reveal the presence of glucose (R_f = 0.10), rhamnose (R_f = 0.12), and arabinose (R_f = 0.19) for compounds **1**, **2**, **3**, **4**, and **6**, and glucose and arabinose for compound **5**. All the R_f values were coincident with those of authentic samples. 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 0.5 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) 11.65 (L-rhamnose, negative polarity); 14.38 (L-arabinose, positive polarity); 17.48 (D-glucose, positive polarity).

Cell Culture Assay. A SRB assay was carried out, as described previously.[12] A human cancer cell line, A-549 human lung carcinoma, was examined, and doxorubicin was used as the positive control. The cells were maintained routinely as an adherent cell culture in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified air incubator with 5% CO_2. Growth inhibition of 50% (ED_{50}) of the saponins was calculated using a published method.[13] Briefly, the cells were divided into 96-well plates and preincubated on the plates for 24 h. The compounds were added to the wells and incubated for 48 h. After incubation,

the culture medium in each well was removed, and the cells were fixed with cold 10% trichloroacetic acid. Subsequently, a 0.4% SRB solution in 1% acetic acid was added to each well. The optical density was measured in a microtiter plate reader at 540 nm.

Supporting Information Available: Isolation procedure for the known saponins isolated in the study. This information is available free of charge via the Internet at http://pubs.acs.org.

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