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TWO NEW TRITERPENE SAPONINS FROM THE TUBERS OF *STACHYS SIEBOLDII*

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Abstract – Chemical investigation of the MeOH extract of tubers of *Stachys sieboldii* MIQ. (Labiatae) led to two new triterpene saponins (**1** and **2**) named sieboldii saponin B and C, together with six known triterpenes (**3**-**8**). The structural elucidation of the new compounds was based on MS, ^{1}H- and ^{13}C-NMR, and 2D NMR analysis (^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY) as well as acid hydrolysis. All the isolated compounds **1-8** were reported from this source for the first time.

Stachys sieboldii MIQ. (Labiatae) is widely distributed in North America, Asia, and Europe,[1] and has been used for the treatment of various gastrointestinal problems, senile dementia, and ischemic stroke.[2] Antioxidant, antitumor, and antimicrobial activities on this plant have been reported.[3] However, only a few phytochemical studies on *S. sieboldii* have been reported. We have recently reported the isolation of triterpene saponins in the EtOAc and $CHCl_3$-soluble layers from this plant.[3] In continuing research on this source, two new triterpene saponins (**1** and **2**) and six known triterpene saponins (**3**-**8**) were isolated from the BuOH-soluble layer in the MeOH extract. The compounds **3**-**8** were reported from this source for the first time. The chemical structures of these new compounds were determined on the basis of 1D and 2D NMR spectroscopic data analysis (^{1}H- and ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY), as well as chemical means.

Compound **1** was obtained as a colorless gum, and its molecular formula $C_{42}H_{68}O_{15}$ was inferred from the positive ion HR-ESI-MS *m/z* 835.4457 $[M + Na]^+$ (calcd. for 835.4456). The ^{1}H-NMR spectrum of **1** (Table 1) showed the signals of olefinic proton at δ_H 5.47 (1H, br t, J = 5.9 Hz, H-12), oxygenated methine protons at δ_H 4.27 (1H, m, H-2), 3.73 (1H, m, H-3), and 3.53 (1H, d, J = 3.0 Hz, H-19), one methine proton at δ_H 2.06 (1H, d, J = 3.0 Hz, H-18), seven tertiary methyl protons at δ_H 1.49 (3H, s, H-27), 1.20 (3H, s, H-23), 1.15 (3H, s, H-29), 1.09 (3H, s, H-26), 0.97 (3H, s, H-25), 0.96 (3H, s, H-30),

and 0.83 (3H, s, H-24), and two sugar anomeric protons at δ_H 6.26 (1H, d, J = 8.4 Hz, H-1′) and 5.75 (1H, d, J = 7.7 Hz, H-1″). The ^{13}C-NMR spectrum of **1** displayed a total of 42 carbon signals, of which 30 carbons were to be assigned to the aglycone and the remaining 12 carbons to the sugar moieties. The ^{13}C-NMR and DEPT spectra included one carboxylic carbon at δ_C 177.2, seven methyl carbons at δ_C 29.3, 28.8, 24.6, 24.5, 22.0, 17.5, and 16.4, two olefinic carbons at δ_C 144.3 and 123.4, three oxygenated methine carbons at δ_C 80.9, 79.2, and 65.9, three methine carbons at δ_C 48.7, 48.0, and 44.4, eight methylene carbons at δ_C 42.5, 33.1, 32.5, 29.7, 28.5, 27.5, 24.0, and 18.4, six quaternary carbons at δ_C 46.3, 42.0, 40.2, 38.6, 38.6, and 35.3, as well as 12 remaining signals at δ_C 104.3, 78.9, 78.8, 75.7, 72.6, and 63.6, and at δ_C 93.6, 78.4, 78.1, 78.0, 70.4, and 61.7 assignable to two glucose moieties, respectively. From these data, compound **1** was presumed to be of ole-12-ene-28-oic acid glycosyl ester. The ^{1}H and ^{13}C NMR data of **1** were found to be very similar to those of rivaloside D[4] with major differences in the downfield shift of C-2′ (δ_C 78.4 for **1**; δ_C 74.0 for rivaloside D), indicating that **1** possessed a glucopyranosyl-(1→2)-glucopyranoside unit. The full NMR assignments and connectivities were determined by ^{1}H-^{1}H COSY, HMQC, and HMBC (Figure 2A). The sugar sequence was determined on the basis of 1D and 2D NMR spectrum. The positions of the glucoses were confirmed by the HMBC correlations of H-1′/C-28 and H-1″/C-2′ (Figure 2A).

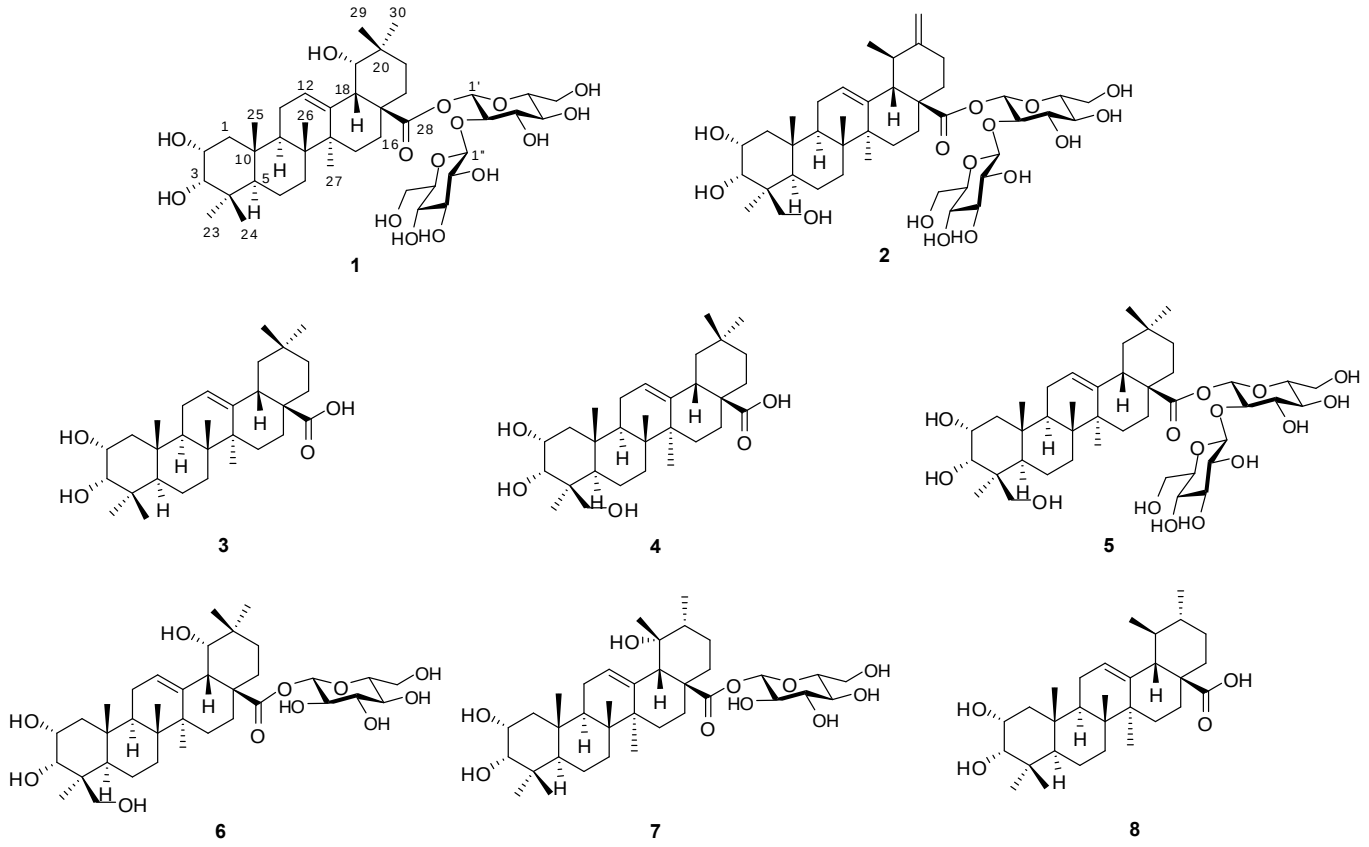

Figure 1. Structures of compounds **1**-**8**

The relative stereochemistry of the aglycone was assumed to be similar with that of 2α,3α,19α-trihydroxy-olean-12-en-28-oic acid by comparing ^{13}C-NMR[5] and corroborated by NOESY cross-peaks of H-2/H-3 and H-25, H-3/H-24, H-5/H-9, H-9/H-27, H-24/H-25, H-25/H-26, H-18/H-19 and H-29, and H-19/H-29 (Figure 2B). Acid hydrolysis of **1** with 1 N HCl yielded 2α,3α,19α-trihydroxy-olean-12-en-28-oic acid and D-glucose {$[\alpha]_D^{25}$ +49.4° (*c* 0.04 in H_2O)}. The anomeric configurations for two glucoses were defined as *β* for glucose from the coupling constant of 8.4 and 7.7 Hz.[6] These data indicated the structure of **1** to be 28-O-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl]-2α,3α,19α-trihydroxy-olean-12-en-28-oic acid, named sieboldii saponin B.

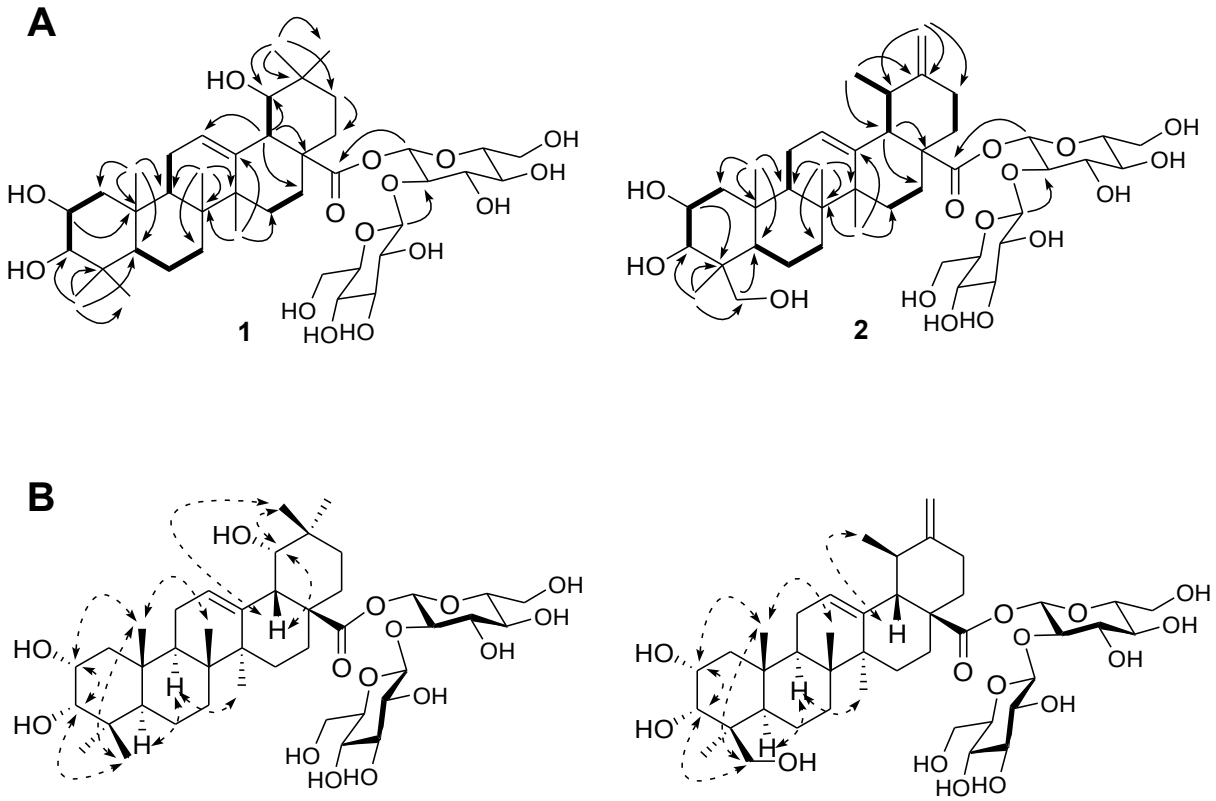

Figure 2. Key HMBC (plain arrows), ^{1}H-^{1}H COSY (bold) correlations (A) and NOESY (dashed arrows) correlations of **1** and **2** (B)

Compound **2** was obtained as a colorless gum, and its molecular formula $C_{42}H_{66}O_{15}$ was inferred from the positive ion HR-FAB-MS *m/z* 811.4480 $[M + H]^+$ (calcd. for 811.4480). The ^{1}H-NMR spectrum of **2** (Table 1) showed the signals of olefinic proton at δ_H 5.43 (1H, brt, *J* = 3.5 Hz, H-12), exomethylene protons at δ_H 4.76 (1H, br s, H-30a) and 4.71 (1H, br s, H-30b), oxygenated methine protons at δ_H 4.60 (1H, m, H-3) and 4.41 (1H, m, H-2), oxymethylene protons at δ_H 4.08 (1H, d, *J* = 10.9 Hz, H-24a) and 3.71 (1H, d, *J* = 10.9 Hz, H-24b), four tertiary methyl protons at δ_H 1.63 (3H, s, H-23), 1.06 (3H, s, H-27), 1.05 (3H, s, H-26), and 0.96 (3H, s, H-25), one secondary methyl proton at δ_H 1.02 (3H, d, *J* = 6.4 Hz,

H-29), as well as two sugar anomeric protons at δ_H 6.15 (1H, d, J = 8.2 Hz, H-1′) and 5.63 (1H, d, J = 7.7 Hz, H-1″). The ^{13}C-NMR spectrum of **2** displayed a total of 42 carbon signals, 30 of which were assigned to the aglycone and the remaining 12 carbons to the sugar moieties. The ^{13}C-NMR and DEPT spectra included one carboxylic carbon at δ_C 176.9, five methyl carbons at δ_C 25.1, 24.9, 18.7, 18.7, and 17.7, four olefinic carbons at δ_C 154.7, 139.6, 127.5, and 106.3, two oxygenated methine carbons at δ_C 75.5 and 67.5, one oxygenated methylene carbon at δ_C 66.4, four methine carbons at δ_C 56.8, 50.8, 49.5, and 38.8, eight methylene carbons at δ_C 44.6, 39.9, 33.8, 35.3, 30.5, 25.7, 25.3, and 20.1, five quaternary carbons at δ_C 49.8, 46.5, 43.8, 41.7, and 39.8, together with 12 remaining signals at δ_C 106.3, 80.6, 80.4, 77.4, 73.9, and 64.9, and at δ_C 95.0, 80.1, 79.6, 79.5, 72.1, and 63.5 assignable to two glucose moieties. The NMR data of **2** was similar to those of urs-12-en-28-oic acid,[7,8] except for the presence of oxymethylene group and additional two glucose groups terminal double bond.

Table 1. ^{1}H- and ^{13}C-NMR data of **1** and **2** in C_5D_5N

1						**2**					
No.	Aglycone		No.	Sugar		No.	Aglycone		No.	Sugar	
	δ_H	δ_C		δ_H	δ_C		δ_H	δ_C		δ_H	δ_C
1a	1.88 (m)	42.5	1′	6.26 (d, J = 8.4 Hz)	93.6	1a	1.93 (m)	44.6	1′	6.15 (d, J = 8.2 Hz)	95.0
1b	1.75 (m)					1b	1.86 (m)				
2	4.27 (m)	65.9	2′	4.51 (m)	78.4	2	4.41 (m)	67.5	2′	4.40 (m)	80.1
3	3.73 (m)	79.2	3′	4.12 (m)	78.1	3	4.60 (m)	75.5	3′	4.00 (m)	79.6
4	-	38.6	4′	4.30 (m)	70.4	4	-	46.5	4′	4.20 (m)	72.1
5	1.60 (m)	48.7	5′	4.12 (m)	78.0	5	1.77 (m)	50.8	5′	4.00 (m)	79.5
6a	1.51 (m)	18.4	6′a	4.46 (dd, J = 11.2, 2.1 Hz)	61.7	6a	1.65 (m)	20.1	6′a	4.50 (dd, J = 11.2, 2.1 Hz)	63.5
6b	1.34 (m)		6′b	4.31 (m)		6b	1.41 (m)		6′b	4.33 (m)	
7	1.65 (m)	33.1	1″	5.75 (d, J = 7.7 Hz)	104.3	7	1.59 (m)	35.3	1″	5.63 (d, J = 7.7 Hz)	106.3
8	-	40.2	2″	4.13 (m)	75.7	8	-	39.8	2″	4.08 (m)	77.4
9	2.10 (br s)	48.0	3″	3.92 (m)	78.9	9	1.80 (m)	49.5	3″	3.78 (m)	80.6
10	-	38.6	4″	4.21 (m)	72.6	10	-	41.7	4″	4.11 (m)	73.9
11		24.0	5″	4.30 (m)	78.8	11	1.90 (m)	25.3	5″	4.30 (m)	80.4
12	5.47 (br t, J = 5.9)	123.4	6″a	4.71 (dd, J = 11.2, 2.1 Hz)	63.6	12	5.43 (br t, J = 3.5 Hz)	127.5	6″a	4.68 (dd, J = 11.2, 2.1 Hz)	64.9
			6″b	4.35 (m)					6″b	4.33 (m)	
13	-	144.3				13	-	139.6			
14	-	42.0				14	-	43.8			
15a	2.25 (m)	29.7				15a	2.38 (m)	30.5			
15b	1.25 (m)					15b	1.35 (m)				
16a	2.82 (m)	27.5				16a	2.31 (m)	25.7			
16b	2.45 (m)					16b	2.25 (m)				
17	-	46.3				17	-	49.8			
18	2.06 (d, J = 3.0)	44.4				18	2.61 (d, J = 11.8 Hz)	56.8			
19	3.53 (d, J = 3.0)	80.9				19	2.38 (m)	38.8			

20	-	35.3	20	-	154.7
21a 21b	2.10 (m) 0.98 (m)	28.5	21a 21b	2.25 (m) 2.12 (m)	33.8
22a 22b	2.00 (m) 1.98 (m)	32.5	22a 22b	2.01 (m) 1.73 (m)	39.9
23	1.20 (s)	29.3	23	1.63 (s)	24.9
24	0.83 (s)	22.0	24a 24b	4.08 (d, *J* = 10.9 Hz) 3.71 (d, *J* = 10.9 Hz)	66.4
25	0.97 (s)	16.4	25	0.96 (s)	17.7
26	1.09 (s)	17.5	26	1.05 (s)	18.7
27	1.49 (s)	24.6	27	1.06 (s)	25.1
28	-	177.2	28	-	176.9
29	1.15 (s)	28.8	29	1.02 (d, *J* = 6.4 Hz	18.7
30	0.96 (s)	24.5	30a 30b	4.76 (br s) 4.71 (br s)	106.3

The relative stereochemistry of **2** was presumed to be similar with that of 2α,3α,24-trihydroxy-ursa-12,20(30)-dien-28-oic acid by comparing ^{13}C-NMR,[5] and was reconfirmed by NOESY cross-peaks of H-2/H-3 and H-25, H-3/H-24, H-5/H-9, H-9/H-27, H-24/H-25, H-25/H-26 and H-18/H-29 (Figure 2B). The anomeric configurations for two glucoses of **2** were confirmed by the same method as **1**. And the positions of the glucoses were confirmed by the HMBC correlations of H-1′/C-28 and H-1″/C-2′ (Figure 2B). Thus, the structure of **2** was established as 28-O-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl]-2α,3α,24-trihydroxy-ursa-12,20(30)-dien-28-oic acid,[5] and named sieboldii saponin C.

The known compounds were identified as methyl 3-epimaslinate (**3**),[9] 2α,3α,24-trihydroxyolean-12-ene-28-oic acid (**4**),[9] 28-O-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl]-2α,3α,24-trihydroxy-olean-12-ene-28-oic acid (**5**),[5,10] 28-O-*β*-D-glucopyranosyl-2α,3α,19α,24-tetrahydroxy-olean-12-en-28-oic acid (**6**),[11] kaji-ichigoside F1 (**7**),[12,13] and 2α,3α-dihydroxyursan-12-en-28-oic acid (**8**)[9,14] by comparing their spectroscopic data with those in the literatures. The compounds **3**-**8** were reported from this source for the first time.

EXPERIMENTAL

General. HR-ESI-MS and HR-FAB-MS data were obtained on a JEOL JMS700 mass spectrometer. Preparative HPLC was conducted using a Gilson 306 pump with a Shodex refractive index detector and Econosil RP-C_{18} 10 μ column (250×10 mm). RP-C_{18} silica gel (YMG GEL ODS-A, 12 nm, S-75 *μ*m) and silica gel 60 (Merck, 70-230 mesh and 230-400 mesh) were used for column chromatography. TLC was performed using Merck precoated Silica gel F_{254} plates and RP-18 F_{254s} plates. NMR spectra were recorded on a Varian UNITY INOVA 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz

(^{13}C) with chemical shifts given in ppm (δ). A Hewlett-Packard (HP) GC system 6890 Series equipped with a 5973 Mass Selective Detector (MSD) system. The system was controlled by the Enhanced ChemStation Version B.01.00 program. The capillary column used for GC was an Agilent J&W HP-5MS UI (30.0 m×0.25 mm i.d., 0.25 μm film thickness coated 5% diphenyl 95% dimethylpolysiloxane).

Plant material. The tubers of *S. sieboldii* were collected at Yecheon, Gyeongsangbuk-Do, Korea, in June 2012, and identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL 1211) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation. The tubers of *S. sieboldii* (5 kg) were extracted with 80% MeOH three times at room temperature to yield 1 kg of material. The resultant MeOH extracts were suspended in distilled water (800 mL x 3) and then successively partitioned with hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding residues weighing 7 g, 20 g, 12 g, and 24 g, respectively. The *n*-BuOH-soluble layer (24 g) was chromatographed on a Diaion HP-20 column eluting with a gradient solvent system consisting of 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, and 100% MeOH to yield five subfractions (B1-B5). Fraction B3 (2.3 g) was purified using a silica gel (230-400 mesh, 50 g) column eluted with $CHCl_3$-MeOH (5:1) to yield four fractions (B31-B34). Fraction B31 (47 mg) and B34 (60 mg) was purified by reversed-phase preparative HPLC with MeCN-H_2O (3:7) and MeCN-H_2O (33:67), respectively to obtain compounds **1** (7 mg, t_R = 16.0 min) and **2** (3 mg, t_R = 10.9 min). The $CHCl_3$-soluble layer (20 g) was separated on RP-C_{18} silica gel open column (230-400 mesh, 550 g) eluting with a gradient solvent system of MeOH-H_2O (1:1 and 1:0), yielding nine fractions (C1-C9). Fraction C4 (8 g) was separated on a RP-C_{18} silica gel with 80% MeOH and further separated by silica gel column using *n*-hexane-EtOAc (1:1) to give six fractions (C41-C46). Fraction C45 (280 mg) was purified by reversed-phase preparative HPLC using 75% MeCN to yield compound **3** (9 mg, t_R = 20.0 min). Fraction C7 (273 mg) was chromatographed on RP-C_{18} silica gel open column with 80% MeOH and further separated by reversed-phase preparative HPLC using 60% MeCN to yield compound **4** (7 mg, t_R = 15.3 min). The EtOAc-soluble layer (12 g) was loaded on RP-C_{18} silica gel open column and eluted with a gradient of MeOH-H_2O (1:1 and 1:0) to yield eight fractions (E1-E8). Fraction E3 (1 g) was subjected to Lobar-A column using $CHCl_3$-MeOH (20:1) as the eluant and then purified by reversed-phase preparative HPLC with MeOH-H_2O (3:2) to give compound **6** (3 mg, t_R = 15.8 min). Fraction E6 (75 mg) was separated on silica gel column with $CHCl_3$-MeOH (20:1) and then purified by reversed-phase preparative HPLC using 40% MeCN to yield compound **5** (3 mg, t_R = 18.0 min) and **7** (3 mg, t_R = 18.8 min). Compound **8** (3 mg, t_R = 12.4 min) was obtained from fraction E7 (70 mg) by using reversed-phase preparative HPLC (85% MeOH).

Sieboldii saponin B (1): colorless gum; $[\alpha]_D^{25}$ +11.2 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3420, 2938, 1738, 1378, 1209, 1056 cm^{-1}; ^1H (C_5D_5N, 700 MHz) and ^{13}C-NMR (C_5D_5N, 175 MHz) see Table 1; HR-ESI-MS *m/z* 835.4457 $[M + Na]^+$ (calcd. for 835.4456).

Sieboldii saponin C (2): colorless gum; $[\alpha]_D^{25}$ +8.7 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3420, 2936, 1740, 1368, 1216, 1055 cm^{-1}; ^1H (C_5D_5N, 700 MHz) and ^{13}C-NMR (C_5D_5N, 175 MHz) see Table 1; HR-FAB-MS *m/z* 811.4480 $[M + H]^+$ (calcd. for 811.4480).

Acid Hydrolysis of Compounds 1 and 2. Compound **1** (1 mg) was shaken with 2 mL of 1 N HCl for 1.5 h at 80 °C**.** After cooling, the hydrolysate was extracted with $CHCl_3$ and the $CHCl_3$ extract was evaporated *in vacuo* to yield 2α,3α,19α-trihydroxy-olean-12-en-28-oic acid[5] as a colorless gum. The sugar in water layer was identified as D-glucose by co-TLC (EtOAc-MeOH-H_2O=9:3:1, *Rf* value: 0.2) with D-glucose standard (Aldrich Co., U.S.A.). Compound **2** (1 mg) was treated in the same method to give 2α,3α,24-trihydroxy-ursa-12,20(30)-dien-28-oic acid[5] and sugar.

Determination of the Sugar. The sugar obtained from the hydrolysis of compounds **1** and **2** was dissolved in anhydrous pyridine (0.1 mL) and L-cysteine methyl ester hydrochloride (2 mg) was added. The mixture was stirred at 60 °C for 1.5 h. After the reaction mixture was dried *in vacuo*, the residue was trimethylsilylated with 1-trimethylsilylimidazole (0.1 mL) for 2 h. The mixture was partitioned between *n*-hexane and H_2O (0.3 mL each), and the organic layer (1 μL) was analyzed by GC-MS. The identification of D-glucose was detected by co-injection of the hydrolysate with standard silylated samples, giving single peaks at 16.414 min. Retention time of authentic sample treated in the same way with 1-trimethylsilylimidazole in pyridine was 16.328 min.

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