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Dammarane-type Saponins from the Black Ginseng

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Ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae) is one of the most commonly used traditional medicines in the Orient for the treatment of various diseases.[1,2] Biologically active constituents of ginseng have been pursued extensively and many dammarane-type triterpene oligoglycosides, generally known as ginsenosides, have been characterized as the principal ingredients.[1-4]

Traditionally, ginseng has been processed to make white ginseng (WG, roots air-dried after peeling) and red ginseng (RG, roots steamed at 98 - 100 °C without peeling) to enhance its preservation and efficacy, which is associated with the changes in the chemical constituents, especially newly formed ginsenosides as results of steaming process, considerably.

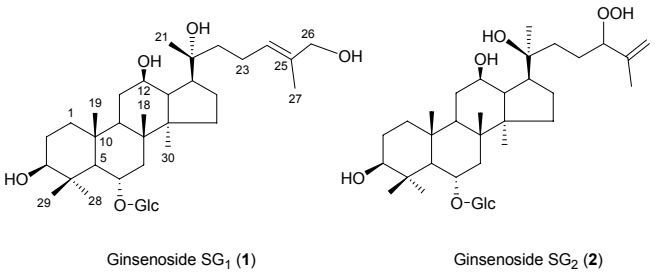

Figure 1. New Ginsenosides SG_1 (**1**) and SG_2 (**2**)

Recently, there have been reported that black ginseng, which is steamed at a higher temperature (120 °C) under higher pressure (0.15 MPa), increased significantly biological effects.[5-7] Meanwhile, its constituents have been analyzed, but not extensively in respect to that of other conventional ginsengs.[7] Subsequently, in our ongoing systematic ginseng-research, the current study on chemical components of the black ginseng led to the isolation of two new saponins, named ginsenosides SG_1 (**1**) and SG_2 (**2**) (Fig. 1), along with twenty compounds, including ginsenoside Rh_2 (**3**),[8] 20*R*-ginsenoside Rh_1 (**4**),[9] ginsenoside Rk_3 (**5**),[10] ginsenoside Rh_4 (**6**),[10] 6'-acetyl ginsenoside Rg_1 (**7**),[11] ginsenoside Rg_6 (**8**),[12] 20*E*-ginsenoside F_4 (**9**),[13] ginsenoside Rg_2 (**10**),[14] ginsenoside Rg_1 (**11**),[15] ginsenoside Rf (**12**),[16] ginsenoside Rg_3 (**13**),[17] ginsenoside Rk_1 (**14**),[10] 6'''-acetyl ginsenoside Re (**15**),[11] ginsenoside Re (**16**),[15] ginsenoside Rs_2 (**17**),[18] ginsenoside Rd (**18**),[15] ginsenoside F_3 (**19**),[15] ginsenoside Rb_1 (**20**),[15] ginsenoside Rc (**21**),[15] and ginsenoside Rb_2 (**22**)[15] (Fig. 2).

Ginsenoside SG_1 (**1**), an amorphous powder, has the molecular formula $C_{36}H_{62}O_{10}$ deduced by a high-resolusion electrospray-

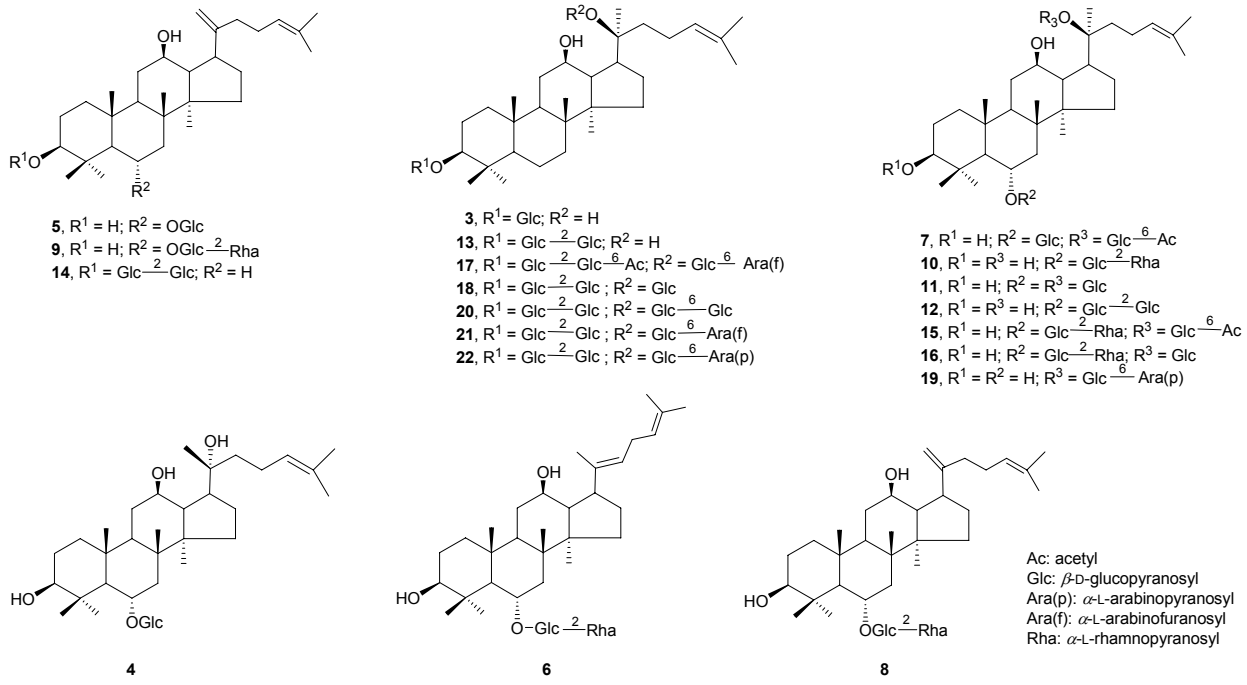

Figure 2. Known Saponins **3-22**

ionization time-of-flight mass spectrometry (HRESITOFMS) experiment (found at *m/z* $[M-H]^+$ 653.4254, calcd. for $C_{36}H_{61}O_{10}$ 653.4265). The IR spectrum of **1** showed absorption bands at ν_{max} 3454, 1060, 1633 cm^{-1} due to hydroxy groups, glycosidic linkage, and double bond. Acid hydrolysis of **1** liberated D-glucose confirmed by GC experiment. From the ^{1}H- and ^{13}C-NMR spectra (Table 1), **1** was proposed to be a β-D-glucopyranosyl and an aglycone with five oxygenated carbons and one double bond. The configuration of the anomeric position was determined to be β on the basis of the large coupling constant (J = 7.8 Hz) of the anomeric proton at δ 5.07 in the ^{1}H-NMR spectrum. Additionally, the ^{1}H-NMR spectrum of **1** showed signals assignable to the aglycone part [δ 0.84, 1.06, 1.26, 1.37, 1.64, 1.83, 2.10 (3H each, all s, H_3-30, 19, 18, 21, 29, 27, 28), 3.54 (1H, dd, J = 11.4, 4.8 Hz, H-3), 3.98 (1H, m, H-12), 4.26 (2H, overlapped, H-26), 4.41 (1H, m, H-6), 5.58 (1H, br t, J = 7.2 Hz, H-24)]. The ^{13}C-NMR spectrum of **1** disclosed thirty-six carbon signals including the set of six signals (δ 106.3, 75.7, 78.4, 72.0, 79.9, and 63.3) accounting for a β-D-glucopyranosyl unit and thirty remaining carbons of a sapogenol moiety. The signal of C-5 at δ 61.7 is a characteristic of a protopanaxatriol-type aglycone common among dammarane-type saponins in *P. ginseng* with variations in its side-chain.[4,8,15] Furthermore, ^{1}H- and ^{13}C-NMR data of **1** were similar to those of 20*R*-ginsenoside Rh_1[9] except for the signals belonging to the side-chain part (C-22 − C-27) of the aglycone. The structure of **1**, especially the side-chain, was assigned by ^{1}H-^{1}H COSY, HMBC, and ROESY spectra, respectively. As shown in Fig. 3, ^{1}H-^{1}H COSY experiment on **1** indicated the presence of partial structures written in bold lines; and in the HMBC experiment, the long-range correlations were observed between the following protons and carbons: H-6 and C-8; H-12 and C-9; H-18 and C-7,9,14; H-19 and C-1,5,9; H-21 and C-17; H-23 and C-20,25; H-24 and C-22, 26; H-26 and C-24; H-27 and C-26; H-1' and C-6. 20*R*-Configuration of **1** was concluded on the basis of the carbon signals C-17 and C-21 at δ 22.9 and 51.1, which were compatible with

Table 1. ^{1}H- and ^{13}C-NMR Data for **1** and **2**

Position	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	39.7	1.03 m 1.73 m	39.2	1.03 m 1.73 m
2	28.9	1.87 m 1.95 m	27.8	1.87 m 1.95 m
3	78.8	3.54 dd (11.4, 4.8)	78.3	3.54 dd (11.4, 4.2)
4	40.7		40.2	
5	61.7	1.42 d (10.2)	61.3	1.45 d (10.8)
6	80.4	4.41 m	80.0	4.43 m
7	45.6	1.97 m 2.54 m	45.1	1.97 m 2.54 m
8	41.6		41.0	
9	50.8	1.61 m	50.0	1.61 m
10	39.9		39.6	
11	30.2	1.46 m 2.13 m	31.5	1.46 m 2.13 m
12	72.0	3.98 m	70.8	3.98 m
13	50.8	2.02 m	48.0	2.02 m
14	51.1		51.5	
15	32.6	1.10 m 1.61 m	31.0	1.10 m 1.61 m
16	28.1	1.29 m 1.85 m	26.7	1.29 m 1.85 m
17	51.1	2.38 m	54.5	2.38 m
18	17.9	1.26 s	16.6	1.20 s
19	17.7	1.06 s	17.4	1.06 s
20	72.8		72.8	
21	22.9	1.37 s	27.0	1.39 s
22	45.5	1.86, m 2.48, m	40.2	2.16 m 2.54 m
23	23.9	2.66, m	26.4	1.83 m 2.23 m
24	125.8	5.58, br t (7.2)	90.0	4.76 m
25	139.8		146.5	
26	69.7	4.26, overlapped	112.8	5.07 br s 5.23 br s
27	13.3	1.83, s	17.5	1.95 s
28	32.0	2.10 s	31.7	2.12 s
29	16.6	1.64 s	16.2	1.63 s
30	17.0	0.84 s	17.2	0.82 s
Glc-1'	106.3	5.07 d (7.8)	105.9	5.08 d (7.2)
Glc-2'	75.7	4.14 t (8.4)	75.3	4.13 t (7.8)
Glc-3'	78.4	4.28 m	79.5	4.28 m
Glc-4'	72.0	4.23 m	71.7	4.22 m
Glc-5'	79.9	3.98 m	78.1	3.97 m
Glc-6'	63.3	4.41 m 4.56 dd (11.4, 2.4)	63.0	4.43 m 4.57 dd (12.0, 2.4)

Assignments were confirmed by COSY, HMQC, HMBC, and ROESY spectra

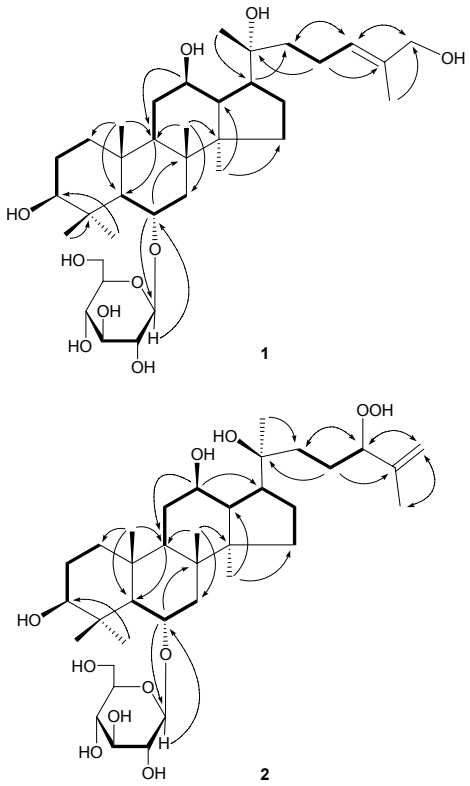

Figure 3. H-H COSY (bold lines) and Selected HMBC (arrows) Correlations of **1** and **2**

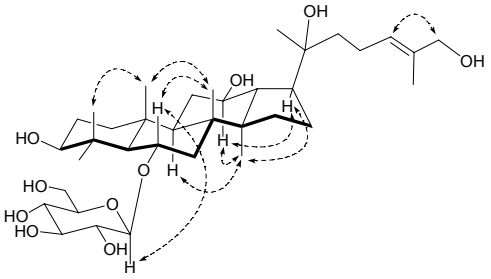

Figure 4. Selected ROESY Correlations of **1**

those of related structures.[8,9] Consequently, geometry of $\Delta^{24,25}$ was proposed as *E* form based on the ^{13}C-NMR agreements of C-24, 26, and 27 with those in the literature[15] and, furthermore, the NOE correlation H-26/H-24 in the ROESY spectrum of **1** (Fig. 4). On the basis of the above evidence, the structure of ginsenoside SG_1 (**1**) was characterized as (20*R*,24*E*)-3β,6α,12β, 20α,26-pentahydroxydammar-24-ene 6-*O*-β-D-glucopyranoside.

Ginsenoside SG_2 (**2**), also an amorphous powder, has the molecular formula $C_{36}H_{62}O_{11}$ on the basis of HR-ESI-TOF-MS experiment. Compound **2** was proposed to have a hydroperoxyl group due to positive response to *N*,*N*-dimethyl-*p*-phenylenediammonium dichloride reagent.[4] On the acid hydrolysis, it yielded D-glucose as identified by the GC procedure. The ^{1}H- and ^{13}C-NMR (Table 1) spectra of **2** due to the dammarane-type triterpene part and 6-*O*-β-D-glucopyranosyl moiety were superimposable on those of 20*S*-ginsenoside Rh_1[10] except for the signals of the side-chain part (C-24 − C-27), which was identical to that of floralginsenosides A and C.[4] 20*S*-Configuration was suggested based on the ^{13}C-NMR evidence of C-17 at δ 54.5 and C-21 at δ 27.0, which were downfield-shifted as compared with those of ginsenoside SG_1 (**1**) and other ginsenosides with 20*R*-configuration.[8,9] Moreover, comprehensive analyses of the ^{1}H-^{1}H COSY, HMQC, and HMBC (Fig. 3) permitted complete assignments of its NMR data as well as partial structures. As shown in Fig. 3, interpretation of the ^{1}H-^{1}H COSY spectra indicated the connectivity of partial structures written in bold lines, with key HMBC correlations observed between the following protons and carbons: H-6 and C-8; H-12 and C-9, 17; H-18 and C-7, 9, 14; H-19 and C-1, 5, 9; H-21 and C-22; H-22 and C-24; H-24 and C-22, 26; H-26 and C-24, 27; H-1' and C-6. Hence, the structure of ginsenoside SG_2 (**2**) was identified as (20*S*)-24ξ-hydroperoxyl-3β,6α,12β,20β-tetrahydroxydammar-25-ene 6-*O*-β-D-glucopyranoside.

In conclusion, the present study demonstrates that steaming of ginseng at higher temperatures produces significant changes in its chemical constituents, especially with formation of unique minor components. Biological evaluation of ginsenosides from black ginseng is now in progress.

Experimental

General procedures. Optical rotations were obtained using a DIP-360 digital polarimeter (Jasco, Easton, MD). IR spectra were measured using a Perkin-Elmer 577 spectrometer (Perkin Elmer, Waltham, MA). NMR spectra were recorded on Bruker DRX 400 and 500 NMR spectrometers (Bruker, Billerica, MA). ESI-MS spectra were recorded on a Model 1100 LC-MSD Trap spectrometer (Agilent, Santa Clara, CA). HRESITOFMS measurements utilized a JEOL AccuTOF^{TM} LC mass spectrometer (Jeol, Tokyo, Japan). GC (Shimadzu-2010, Tokyo, Japan) using a DB-05 capillary column (0.5 mm i.d. × 30 m) [column temperature: 210 °C; detector temperature: 300 °C; injector temperature: 270 °C; He gas flow rate: 30 mL/min (splitting ratio: 1/20)] was used for sugar determination. Column chromatography was performed on silica gel (70 - 230 and 230 - 400 mesh, Merck), YMC RP-18 resins (30 - 50 μm, Fuji Silysia Chemical Ltd., Aichi, Japan), and Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). TLC was performed on Kieselgel 60 F_{254} (1.05715; Merck, Darmstadt, Germany) or RP-18 F_{254s} (Merck) plates. Spots were visualized by spraying with 10% aqueous H_2SO_4 solution, followed by heating.

Plant material. The ginseng roots were collected in Geumsan province, which is well-known for ginseng cultivation in Korea, in August 2008, and were taxonomically identified by one of us (Young Ho Kim). Voucher specimens (CNU 08202) have been deposited at the College of Pharmacy, Chungnam National University. The air-dried sample (2.0 kg) was then steamed at 120 °C for 4 h under 0.15 MPa pressure, without mixing with water, to give the steamed sample, which was used for extraction and isolation in this study.

Extraction and isolation. The black ginseng sample was extracted in MeOH (5.0 L × 3, 50 °C) and the combined extracts were concentrated in vacuo to dryness. The MeOH residue (650 g) was suspended in H_2O (2.5 L), then partitioned with CH_2Cl_2 (2.5 L × 3), and the water layer was subjected to a Diaion HP-20 column eluted with a gradient of MeOH in H_2O (25, 50, 75, and 100% MeOH; v/v) to give six fractions (fr. 1.1 − fr. 1.6). Next, fr. 1.3 (40 g) was chromatographed on a silica gel column using CH_2Cl_2-MeOH (20:1-1:1) to afford eight subfractions (fr. 2.1 − fr. 2.8). Fr. 2.3 (1.3 g) was further chromatographed on a silica gel column with $CHCl_3$-MeOH-H_2O (7:1:0.1), followed by a reversed-phase (RP) column with MeOH-H_2O (2:1) to obtain ginsenoside SG_1 (**1**, 18 mg) 20*R*-ginsenoside Rh_1 (**4**, 120 mg), ginsenoside Rk_3 (**5**, 35 mg), and ginsenoside Rh_4 (**6**, 25 mg). Fr. 2.5 (6.5 g) was rechromatographed on a silica gel column with $CHCl_3$-MeOH-H_2O (4:1:0.1) to afford nine subfractions (fr. 3.1 − fr. 3.9). Next, fr. 3.3 (420 mg) was subjected to a silica gel column with $CHCl_3$-MeOH-H_2O (4:1:0.1), followed by a RP column with MeOH-H_2O (5:2) to furnish ginsenoside SG_2 (**2**, 6 mg), ginsenoside Rg_1 (**7**, 100 mg), and ginsenoside Rg_3 (**13**, 7 mg). Similarly, fr. 3.5 (680 mg) was repeatedly chromatographed on a silica gel column with $CHCl_3$-MeOH-H_2O (4:1:0.1), followed by a RP column with MeOH-H_2O (3:1) to give ginsenoside Rh_2 (**3**, 11 mg), ginsenoside Rg_1 (**11**, 4 mg), and ginsenoside Rf (**12**, 44 mg). Again, fr. 3.6 (800 mg) was chromatographed on a a silica gel column with $CHCl_3$-MeOH-H_2O (4:1:0.1), followed by a RP column with MeOH-H_2O (2:1) to afford 6'''-acetyl ginsenoside Re (**15**, 8 mg), ginsenoside Re (**16**, 34 mg), ginsenoside Rs_2 (**17**, 30 mg), and ginsenoside Rd (**18**, 80 mg), respectively.

Fr. 2.7 (2.4 g) was subjected to a silica gel column with $CHCl_3$-MeOH-H_2O (7:3:0.4) to furnish five subfractions (fr. 4.1 − fr. 4.5). Then, fr. 4.3 (160 mg) was repeatedly chromatographed

on a silica gel column with $CHCl_3$-MeOH-H_2O (7:3:0.4), followed by a RP column with MeOH-H_2O (3:1) to afford ginsenoside F_3 (**19**, 8 mg) and ginsenoside Rb_1 (**20**, 12 mg). Finally, fr. 4.5 was rechromatographed on a silica gel column with $CHCl_3$-MeOH-H_2O (7:3:0.4), followed by a RP column with MeOH-H_2O (2:1) to afford ginsenoside Rc (**21**, 4 mg) and ginsenoside Rb_2 (**22**, 7 mg).

Ginsenoside SG_1 (1): white amorphous powder; $[\alpha]_D^{20}$ +8.0 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3454, 2922, 1633, 1262, 1060 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz): see Table 1; HR-ESI-TOF-MS *m*/*z* $[M-H]^-$ 653.4254, calcd for $C_{36}H_{61}O_{10}$ 653.4265).

Ginsenoside SG_2 (2): white amorphous powder; $[\alpha]_D^{20}$ −2.2 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3436, 2931, 1634, 1260, 1068 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz): see Table 1; HR-ESI-TOF-MS *m*/*z* 671.4357 $[M+H]^+$ (Calcd for $C_{36}H_{63}O_{11}$: 671.4370).

Acid hydrolysis and sugar determination of 1 & 2. A solution of each compound (2.0 mg) in 1.0 M HCl (4.0 mL) was heated under reflux for 4 h. Then, the reaction mixture was concentrated in vacuo to dryness. The residue was extracted with EtOAc and H_2O (5 mL each, 3 times). Next, the sugar residue, obtained by concentration of the water layer, was dissolved in dry pyridine (0.1 mL). Then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. After heating the reaction mixture at 60 °C for 2 h, 0.1 mL of trimethylsilylimidazole was added. Heating at 60 °C was continued for a further 2 h, and the mixture was evaporated in vacuo to give a dried product, which was partitioned between hexane and H_2O.[6] The hexane layer was analyzed by the GC procedure (General Procedures). The peak of the hydrolysate of the compound was detected at t_R 14.12 min for D-glucose. The retention times for the authentic samples (Sigma), after being treated in the similar manner, were 14.12 min (D-glucose) and 14.25 min (L-glucose), respectively. Co-injection of the hydrolysates of the ginsenoside with standard D-glucose gave single peaks.

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