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New Dammarane Saponins from the Steamed Ginseng Leaves

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Extracts from ginseng root and leaf-stem have similar multi-faceted pharmacological activities (e.g. CNS and cardiovascular system). Moreover, in terms of costs and source availability, ginseng leaf has advantages over its root.[1] Especially, biologically active constituents of ginseng leaves have been studied and dammarane-type triterpene oligoglycosides have been characterized as the principal ingredients as ginseng roots.[2,3] Consequently, ginseng leaf is a valuable production of ginseng saponins.

Traditionally, the ginseng root 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.[4,5]

In this regard, there has been no study concerning processed leaves in respect to that of ginseng roots in traditional use. Subsequently, in continuation of our research on *P. ginseng*,[6,7] our current work to study on chemical components of the steamed leaves resulted in the isolation of two new damarane-type saponins, named ginsenosides ST_1 (**1**) and ST_2 (**2**), and notoginsenoside R_{10} (**3**),[8] which was isolated for the first time from *P. ginseng* (Fig. 1).

Ginsenoside ST_1 (**1**), an amorphous powder, has the molecular formula $C_{36}H_{60}O_{10}$ on the basis of a HR-ESI-TOF-MS experiment (found at *m/z* $[M-H]^-$ 651.4116, calcd for $C_{36}H_{59}O_{10}$ 651.4108). The molecule of **1** was proposed to have hydroperoxyl group due to positive response to *N*,*N*-dimethyl-*p*-phenylenediammonium dichloride.[9] On acid hydrolysis, it yielded D-glucose as identified by GC experiment. The ^{1}H-NMR spectrum of **1** (Table 1) showed signals due to the aglycone part [δ 0.82, 1.07, 1.24, 1.65, 1.81, 1.88, 2.12 (3H each, s, H_3-30, 19, 18, 21, 29, 27, 28), 3.54 (1H, dd, J = 11.6, 4.8 Hz, H-3), 3.98 (1H, m, H-12), 4.42 (1H, m, H-6), 5.08 and 5.26 (1H each, br s, H-26)], 5.80 (1H, m, H-22), and an anomeric proton at δ 5.08 (d, J = 7.6 Hz, H-1'), which was assignable to a β-glucopranosyl unit. The ^{13}C-NMR spectrum of **1** exhibited 36 signals including a set of six signals (δ 105.9, 75.3, 79.5, 71.7, 78.0, and 62.9) revealing a β-D-glucopyranosyl unit and 30 remaining ones of a sapogenol moiety. The signal of C-5 at δ 61.3 is a feature of a protopanaxatriol-type aglycone common among dammarane-type saponins from *P. ginseng* with variations in its side-chain. Furthermore, the ^{1}H- and ^{13}C-NMR data of **1** were similar to those of ginsenoside Rh_4[5] except for the signals belonging to the side-chain part (C-24 − C-27) of the aglycone, which was identical to that of floralginsenosides A and C.[10] *E*-geometry of the double bond at C-20(22) of **1** was concluded on the basis of the methyl carbon signal C-21 at δ 13.0; whereas in case of *Z*-form, the chemical shift of C-21 is expected at *ca*. δ 30.0,[11] respectively. The proposed structure of **1** was further confirmed by the ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) (Fig. 2), and rotating frame Overhauser effect spectroscopy (ROESY) (Fig. 3) spectra, respectively. As shown in Fig. 2, the ^{1}H-^{1}H COSY experiment on **1** indicated the presence of partial structures written in bold lines; and in the HMBC spectrum, the long-range correlations were 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-17,24; H-24 and C-22,26; H-26 and C-24; H-27 and C-24; H-1' and C-6, respectively. Consequently, the structure of ginsenoside ST_1 (**1**) was characterized as (20*E*)-24-hydroperoxyl-3β,6α,12β-trihydroxy-dammar-20(22),25-diene 6-*O*-β-D-glucopyranoside.

Ginsenoside ST_2 (**2**), also an amorphous powder, has the molecular formula $C_{36}H_{62}O_{10}$ on the basis of HR-ESI-TOF-MS experiment. Like compound **1**, on the acid hydrolysis, it yielded D-glucose as confirmed by the GC procedure. The ^{1}H- and ^{13}C-NMR (Table 1) spectra of **2** were superimposable on those of **1** except for the signals of the side-chain part (C-22 − C-27), which were similar to those of notoginseng ST_5.[12] 20*S*-Configuration was suggested on the basis of the ^{13}C-NMR evidence of C-21 at δ 27.1; whereas in case of 20*R*-form, the chemical shift of C-21 is expected at *ca*. δ 22.0.[13,14] Moreover, compre-

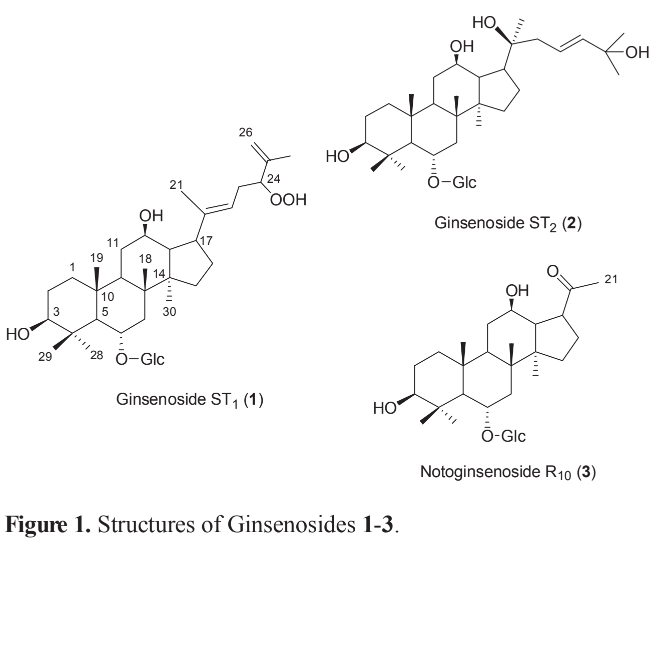

Figure 1. Structures of Ginsenosides **1**-**3**.

Table 1. ^{1}H- and ^{13}C-NMR Data for **1-3** in Pyridine-d_5

Position	**1**		**2**		**3**
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	39.2	1.03 m 1.73 m	39.3	1.03 m 1.73 m	39.6
2	27.8	1.87 m 1.95 m	27.8	1.87 m 1.95 m	28.1
3	78.3	3.54 dd (11.6, 4.8)	78.4	3.52 m	78.7
4	40.2		40.3		40.6
5	61.3	1.42 d (10.4)	61.4	1.42 d (8.0)	61.6
6	79.9	4.42 m	80.0	4.42 m	80.1
7	45.2	1.97 m 2.54 m	45.1	1.97 m 2.54 m	45.3
8	41.2		41.1		41.4
9	50.5	1.61 m	50.1	1.61 m	51.0
10	39.6		39.6		39.9
11	31.5	1.46 m 2.13 m	31.2	1.46 m 2.13 m	31.9
12	72.3	3.98 m	70.9	3.98 m	71.5
13	49.5	2.02 m	48.4	2.01 m	52.9
14	51.5		51.6		51.6
15	31.0	1.10 m 1.61 m	31.7	1.10 m 1.61 m	31.1
16	26.9	1.29 m 1.85 m	26.8	1.29 m 1.85 m	27.8
17	50.6	2.81 m	54.6	2.38 m	54.7
18	17.6	1.24 s	17.6	1.24 s	17.5
19	16.6	1.07 s	16.7	1.04 s	17.8
20	141.9		73.2		213.5
21	13.0	1.81 s	27.1	1.39 s	30.6
22	120.4	5.80 m	45.7	2.16 m 2.54 m	
23	30.6	2.25 m 2.80 m	123.7	6.50 m	
24	88.8	4.73 m	142.0	6.03 d (15.6)	
25	145.4		71.6		
26	113.3	5.08 br s 5.26 br s	19.8	1.54 s	
27	18.0	1.88 s	30.1	1.54 s	
28	31.6	2.12 s	31.7	2.08 s	31.9
29	16.2	1.65 s	16.4	1.60 s	16.5
30	17.2	0.82 s	17.4	0.81 s	16.9
Glc-1′	105.9	5.08 d (7.6)	105.9	5.02 d (7.2)	106.1
2′	75.3	4.13 t (8.0)	75.3	4.10 t (8.0)	75.6
3′	79.5	4.28 t (8.4)	79.5	4.28 t (8.4)	79.9
4′	71.7	4.22 m	71.6	4.22 m	72.0
5′	78.0	3.97 m	78.1	3.96 m	78.7
6′	62.9	4.40 m 4.57 br d (11.2)	62.9	4.38 m 4.52 br d (11.2)	63.2

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

hensive analyses of the ^{1}H-^{1}H COSY, HMQC, HMBC (Fig. 2) and ROESY spectra of **2** permitted complete assignments of its NMR data as well as partial structures. Hence, the structure of ginsenoside ST$_2$ (**2**) was identified as (20*S*)-3*β*,6*α*,12*β*-20*β*,25-pentahydroxy-dammar-23-ene 6-*O*-*β*-D-glucopyranoside.

Cytotoxic activity of **1-3** was tested against the HL-60 cell line, a type of human leukemia, using the 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[15] As result, ginsenosides ST$_1$ (**1**) exhibited potent activity with IC$_{50}$ values of 0.87 μM. Besides, the activity of ginsenosides ST$_2$ (**2**) and notoginsenoside R$_{10}$ (**3**) was relatively weak with IC$_{50}$

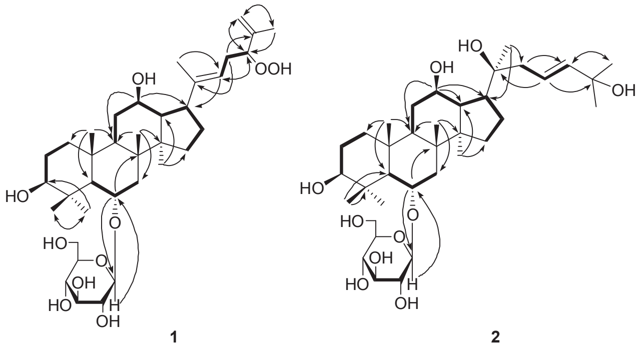

Figure 2. COSY (bold lines) and Selected HMBC (arrows) Correlations of New Ginsenosides **1** and **2**.

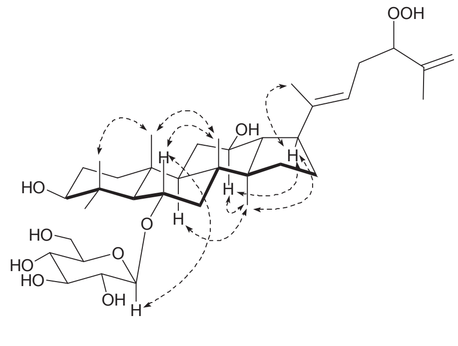

Figure 3. Selected ROESY Correlations of **1**.

values of 78.63 and 89.11 μM as compared with mitoxantrone (MX) used as the positive control with the IC$_{50}$ value of 7.90 μM. It is noteworthy that these components are unique in steamed leaves and not found in non-processed samples as reported previously.[6] These results warrant further studies concerning potential of saponin extracts of steamed ginseng-leaves for leukemia treatments.

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 NMR spectrometer (Bruker, Billerica, MA). HR-ESI-TOF-MS measurements utilized a JEOL AccuTOF™ 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 HP-20 Diaion (Mitsubishi Chemical, Tokyo, Japan). TLC was performed on Kieselgel 60 F$_{254}$ (Merck, Darmstadt, Germany) or RP-18 F$_{254s}$ (Merck, Darmstadt, Germany) plates. Spots were visualized by spraying with 10% aqueous H_2SO_4 solution, followed by heating.

Plant material. The leaves of *P. ginseng* were collected in

Geumsan province 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 (1.2 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 steamed-leaf sample of *P. ginseng* was extracted in MeOH (4.0 L × 3, 50 °C) and the combined extracts were concentrated in vacuo to dryness. The MeOH residue (160 g) was suspended in H_2O (2.0 L), then partitioned with CH_2Cl_2 (2.0 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 eight fractions (fr. 1.1 − fr. 1.8). Next, fr. 1.5 (4.5 g) was chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (5:1:0.1, v/v/v) to afford seven subfractions (fr. 2.1 − fr. 2.7). Fr. 2.2 (500 mg) was further chromatographed on a reversed-phase column with MeOH-H_2O (5:2) to obtain ginsenoside ST_2 (**2**, 40 mg). Fr. 1.7 (12.0 g) was subjected to a silica gel column with $CHCl_3$-MeOH-H_2O (4:1:0.1) to furnish ten subfractions (fr. 3.1 − fr. 3.10). Then, fr. 3.3 (370 mg) was repeatedly chromatographed on a reversed-phase column with MeOH-H_2O (5:3) to give ginsenoside ST_1 (**1**, 11 mg) and notoginsenoside R_{10} (**3**, 20 mg).

Ginsenoside ST_1 (1): White amorphous powder; $[\alpha]_D^{20}$ +12 (*c* 0.22, MeOH); IR (KBr) ν_{max}: 3448, 2922, 1637, 1262, 1054 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz): see Table 1; HR-ESI-TOF-MS *m/z*: $[M-H]^-$ 651.4116, calcd for $C_{36}H_{59}O_{10}$ 651.4108).

Ginsenoside ST_2 (2): White amorphous powder; $[\alpha]_D^{20}$ −9 (*c* 0.25, MeOH); IR (KBr) ν_{max}: 3436, 2931, 1634, 1260, 1068 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz): see Table 1; HR-ESI-TOF-MS *m/z*: 655.4410 $[M+H]^+$ (Calcd for $C_{36}H_{63}O_{10}$: 655.4421).

Notoginsenoside R_{10} (3): White amorphous powder; $[\alpha]_D^{20}$ −8 (*c* 0.20, MeOH); ^{1}H-NMR (pyridine-d_5, 400 MHz) δ 0.80, 1.01, 1.19, 1.61, 2.09 (3H each, all s, H-30,19,18,29,28), 2.36 (1H, m, H-17), 2.39 (3H, s, H-21), 3.54 (1H, dd, *J* = 11.6, 4.8 Hz, H-3), 3.98 (1H, m, H-12), 4.41 (1H, m, H-6), 5.03 (1H, d, *J* = 7.6 Hz, H-1'); ^{13}C-NMR (pyridine-d_5, 100 MHz): see Table 1; ESI-MS *m/z*: 555 $[M+H]^+$.

Acid hydrolysis and sugar determination of new ginsenosides 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 ginsenosides was detected at t_R 14.12 min for D-glucose. The retention times for the authentic samples (Sigma, St. Louis, MO, USA), 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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