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Physicochemical Characterization and NMR Assignments of Ginsenosides Rb_1, Rb_2, Rc, and Rd Isolated from *Panax ginseng*

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The fresh ginseng roots were extracted with aqueous methanol, and the obtained extracts were partitioned using ethyl acetate, *n*-butanol, and water, successively. The repeated silica gel and octadecyl silica gel column chromatogaraphy for *n*-butanol fraction afforded four diol ginseng saponins, ginsenosides Rb_1, Rb_2, Rc, and Rd. The physicochemical, spectroscopic, and chromatographic characteristics of these ginsenosides were measured and compared with those reported in the literature. Some of the peak assignments in previously published ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were inaccurate. This study employed two-dimensional NMR experiments, including ^{1}H–^{1}H correlation spectroscopy, heteronuclear single quantum correlation, and heteronuclear multiple bond connectivity, to determine exact peak assignments.

Keywords: Diol ginsenoside, Fast atom bombardment/mass spectrometry, High-performance liquid chromatography, Nuclear magnetic resonance

INTRODUCTION

Saponin is one of the most important compounds in *Panax ginseng* C.A. Meyer which exhibit various pharmacological activities. To date, approximately 70 kinds of saponin have been isolated from *Panax ginseng*. Most of them are protopanaxdiol and protopanaxtriol, which are aglycones of dammarane-type triterpenoids. Only a few ginsenosides, such as ginsenoside Ro, are aglycones of oleanolic acid [1,2]. Identification of ginsenosides is usually performed by nuclear magnetic resonance (NMR) analyses, but several discrepancies and/or inaccuracies exist in the published NMR data. Among ginseng saponins, we previously described the various physicochemical properties of ginsenoside Rg_1, measured with standardized methods, and further identified signals in its two-dimensional (2-D) NMR spectrum [3].

In the present study, we acquired physicochemical and spectroscopic data from four major diol-saponins, i.e., ginsenosides Rb_1, Rb_2, Rc, and Rd. The aglycone of ginseng diol-saponins is protopanaxdiol, which is a dammarane moiety hydroxylated via β-linkages to carbon atoms C3, C12, and C20 with a double bond between C24 and C25. In diol-saponins, a sugar group is attached to the hydroxyl groups at C3 and C20. In ginsenosides Rb_1, Rb_2, Rc, and Rd, sophorose [β-D-glucopyranosyl-(1→2)-D-glucopyranose] is β-linked to the hydroxyl group at C3. In ginsenoside Rb_1, β-D-glucopyranosyl-(1→6)-D-glucopyranose is β-linked to the hydroxyl group at C20. α-L-Arabinopyranosyl-(1→6)-D-glucopyranose

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is β-linked to the hydroxyl group at C20 in ginsenoside Rb_2, and α-L-arabinofuranosyl-(1→6)-glucopyranose is β-linked in ginsenoside Rc, and β-D-glucopyranose is β-linked in ginsenoside Rd (Fig. 1). All four of these saponins are bisdesmoides, in which a sugar is attached at C3 and C20. NMR peak assignments for the hydroxyl group and the methyl-linked carbon atom, the olefine carbon atom, and protons linked to individual carbon atoms differ significantly in the literature [4-17].

Although ginsenosides were identified in the 1970s, the lack of 2-D NMR techniques limited the amount of structural information available. As a result, past reports may contain inaccurate peak assignments. In the present study, individual signals were identified using modern 2-D NMR techniques. These included heteronuclear single quantum correlation (HSQC), which provides information related to hydrogen atoms bound to a given carbon atom, and heteronuclear multiple bond connectivity (HMBC), which yields information on neighboring hydrogen and carbon atoms. Melting points, specific rotation, infrared (IR) absorbance, and fast atom bombardment/mass spectrometry (FAB/MS) data were also collected using standardized methods and data were discussed relative to literature values [17–23]. Retention factors (R_f) of each saponin in both normal and reversed-phase thin layer chromatography (TLC) experiments and the standardized retention times of each ginsenoside through a carbohydrate-based high-performance liquid chromatography (HPLC) column are also presented herein.

MATERIALS AND METHODS

Ginseng samples

Six-year-old fresh ginseng roots were purchased from the Geumsan ginseng market in Chungnam, Korea in October 2007.

Reagents, instrumentation, and measurement methods

Kieselgel 60 was purchased from Merck Co. (Darmstadt, Germany). Kieselgel 60 F_{254} and RP-18 F_{254S} were used as solid phases in TLC. The former utilized a mobile phase composed of $CHCl_3$-MeOH-H_2O (65:35:10); the latter mobile phase consisted of a MeOH-H_2O blend (3:1). Detection of substances on the TLC plate was performed by observation under a UV lamp (ENF-240C; Spectronics Corp., New York, NY, USA) or by spraying the developed plate with 10% aqueous H_2SO_4 followed by heating and observing color development. HPLC was performed at 50°C and 30 psi using an LC-20A (Shimadzu, Kyoto, Japan) equipped with an evaporative light scattering detector (ELSD; Shimadzu). The HPLC analytical column was a Carbohydrate ES (4.6×250 mm, 5 μm; Grace, Deerfield, IL, USA). The column was eluted in a step-wise gradient at a flow rate of 0.8 mL/min using solvent A (acetonitrile-H_2O-isopropanol=80:5:15) and solvent B (acetonitrile-H_2O-isopropanol=60:25:15). The elution schedule was as follows: 25% solvent A for 28 minutes, 75% A for 75 minutes, 90% A for 50 minutes, and 100% A for 60 minutes.

NMR spectra were recorded on a Varian Inova AS 400 spectrometer (400 MHz; Varian, Palo Alto, CA, USA). Then 0.0625 mol of each ginsenoside (69.3 mg Rb_1, 67.4 mg Rb_2, 67.4 mg Rc, and 59.1 mg Rd) was dissolved in 0.75 mL (0.083 M) pyridine-d_5 and placed in a 5-mm-diameter NMR tube (Norell, Landisville, NJ, USA) with a tetramethylsilane internal-standard adjusted to 0 ppm. IR spectra were measured with an IR spectrometer (599B; Perkin Elmer, Waltham, MA, USA). Two milligrams of each sample was dissolved in 100 uL of MeOH and one drop of the solution was poured onto

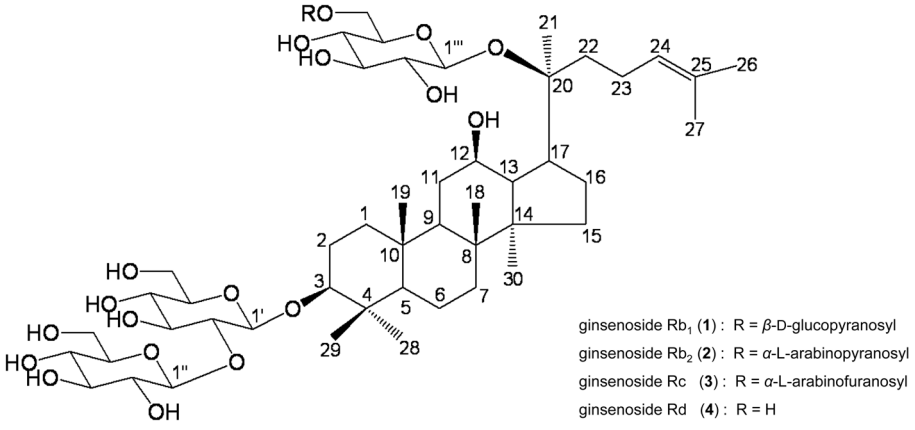

Fig. 1. Chemical structures of ginsenosides Rb_1, Rb_2, Rc, and Rd isolated from the roots of *Panax ginseng*.

a CaF_2 salt plate (Spectral Systems, Hopewell Junction, NY, USA) and allowed to evaporate. Measurements were performed at room temperature. FAB/MS was carried out with a JMS-700 mass spectrometer (JEOL, Tokyo, Japan) using glycerol as the matrix. Optical rotation was measured with a P-1020 polarimeter (JASCO, Tokyo, Japan) on 10 mg of each ginsenoside, dissolved in MeOH in a 1 mL sample cell at a depth of 1 dm.

Isolation of diol ginsenosides

Twenty kilograms (fresh weight) of 6-year-old fresh ginseng roots were cut into pieces and extracted with 90% MeOH (50 L) for 24 hours at room temperature. The extracts were passed through filter paper and the residues were extracted twice more with 80% MeOH (6 L). The filtrate was evaporated under reduced pressure at 45°C to yield 2.2 kg of dried extract. The dried extract was partitioned between ethyl acetate (EtOAc, 3 L×3) and H_2O (3 L). The remaining H_2O layer was extracted again with normal butanol (*n*-BuOH, 2.8 L×3). Each layer was concentrated under reduced pressure to obtain EtOAc (25 g), *n*-BuOH (169 g), and H_2O fractions. The BuOH extract (*n*-BuOH fraction of *Panax ginseng*, PGB) (160 g) was applied to a silica gel column (φ 10×24 cm) and eluted in three steps with a mixture of $CHCl_3$-MeOH-H_2O (step 1, 65 L of 10:3:1; step 2, 55 L of 8:3:1; step 3, 30 L of 6:4:1) to yield 24 fractions (PGB1–PGB24). Fractions PGB16 and PGB17 were combined (12 g, Ve/Vt=0.65–0.73, where Ve refers to the volume of eluent for the corresponding fraction and Vt represents the total elution volume). The combined fractions were separated on a silica gel column (φ 7×17 cm) with a $CHCl_3$-MeOH-H_2O (65:35:10) eluent (101 L) to obtain 20 fractions (PGB16-1–PGB16-20). PGB16-12 (3.85 g, Ve/Vt=0.62–0.72) was further fractionated over an octadecyl silica gel (ODS) column (φ 4.5×14 cm, MeOH-H_2O=3:2, 25 L) to afford 18 additional fractions (PGB16-12-1–PGB16-12-18) including ginsenoside Rb_2 [PGB16-12-5, 70 mg, Ve/Vt=0.22–0.33, TLC R_f=0.32 (RP-18 F_{254S}, MeOH-H_2O=3:1), R_f=0.46 (Kieselgel 60 F_{254}, $CHCl_3$-MeOH-H_2O=65:35:10)] and ginsenoside Rb_1 [PGB16-12-10, 70 mg, Ve/Vt=0.5–0.61, TLC R_f=0.40 (RP-18 F_{254S}, MeOH-H_2O=3:1), R_f=0.42 (Kieselgel 60 F_{254}, $CHCl_3$-MeOH-H_2O=65:35:10)]. PGB16-7 (370 mg, Ve/Vt=0.16–0.21) was also separated over an ODS column (φ 4×6 cm, 3:1 MeOH-H_2O, 2.4 L) to yield 20 additional fractions (PGB16-7-1–PGB16-7-20) including ginsenoside Rd [PGB16-7-16, 104 mg, Ve/Vt=0.31–0.46, TLC R_f=0.25 (RP-18 F_{254S}, MeOH-H_2O=3:1), R_f=0.50 (Kieselgel 60 F_{254}, $CHCl_3$-MeOH-H_2O=65:35:10)]. Fraction PGB16-9 (1.7 g, Ve/Vt=0.25–0.29) was also fractionated on an ODS colum (φ 4×6 cm) with 10 L of 3:1 MeOH-H_2O to yield 36 fractions (PGB16-9-1–PGB16-9-36) including ginsenoside Rc [PGB16-9-26, 19 mg, Ve/Vt=0.49–0.53, TLC R_f=0.40 (RP-18 F_{254S}, MeOH-H_2O=3:1), R_f=0.48 (Kieselgel 60 F_{254}, $CHCl_3$-MeOH-H_2O=65:35:10)]. Physicochemical and spectroscopic data from each ginsenoside are shown in Tables 1-3.

RESULTS AND DISCUSSION

The purity of the isolated compounds was over 99% as determined by HPLC and ^{1}H-NMR. Most of the saponins were obtained as white powders. This agrees

Table 1. Physicochemical characteristics for ginsenosides Rb_1, Rb_2, Rc, and Rd

	Ginsenoside Rb_1	Ginsenoside Rb_2	Ginsenoside Rc	Ginsenoside Rd
Crystals	White powder(H_2O-MeOH)	White powder (H_2O-MeOH)	White powder (H_2O-MeOH)	White powder (H_2O-MeOH)
Mp (°C)	170-171	181-183	168-169	182-183
$[\alpha]_D$	+6.49° (20°C, *c*=0.50, MeOH)	+14.08° (20°C, *c*=0.50, MeOH)	+1.91° (20°C, *c*=0.50, MeOH)	+18.1° (20°C, *c*=0.50, MeOH)
IR (cm^{-1})	3365, 2919, 1590, 1079, 1032	3389, 2942, 1640, 1077, 1029	3367, 2943, 1649, 1078, 1031	3366, 2943, 1647,1077, 1034
FAB/MS (*m/z*)	1131, 789, 425, 407, 365, 325	1101, 789, 425, 407	1101, 789, 425, 407	969, 789, 425
TLC (R_f)	0.42[1)], 0.40[2)]	0.46[1)], 0.32[2)]	0.48[1)], 0.40[2)]	0.50[1)], 0.25[2)]
HPLC (r.t. min)	54.3[3)]	47.7[3)]	48.1[3)]	36.5[3)]

[1)]Kieselgel 60 F_{254}, $CHCl_3$-MeOH-H_2O (65:35:10).
[2)]Kieselgel RP-18 F_{254S}, MeOH-H_2O (3:1).
[3)]Carbohydrate ES (4.6×250 mm, 5 μm), solvent A (acetonitrile-H_2O-isopropanol=80:5:15), solvent B (acetonitrile-H_2O-isopropanol=60:25:15), gradient elution: The concentration of A was 25 to 50% at 0 to 28 minutes, 50 to 75% at 28 to 47 minutes, 75 to 90% at 47 to 50 minutes, 90 to 100% at 50 to 52 minutes, and 100 to 0% at 52 to 60 minutes. The flow rate was 0.8 mL/min.
Mp, melting point; IR, infrared; FAB/MS, fast atom bombardment/mass spectrometry; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography.

Table 2. ^{1}H NMR data for ginsenosides Rb_1, Rb_2, Rc, and Rd (400 MHz, pyridine-d_5, δ_H)

H-No.	Ginsenoside Rb_1	Ginsenoside Rb_2	Ginsenoside Rc	Ginsenoside Rd
1	1.51[1)]	0.74, 1.56	0.70	0.82, 1.47
2	1.81	1.81, 2.17	1.81, 2.18	1.79, 2.17
3	3.25 dd, 11.6, 4.4[2)]	3.25, dd, 11.2, 3.6[2)]	3.25, dd, 11.6, 4.4[2)]	3.26, dd, 11.6, 3.6[2)]
5	0.67	0.67	0.63	0.67
6	1.47	1.47, 1.36	1.46	1.46. 1.59
7	1.20	1.21, 1.48	1.16, 1.43	1.20, 1.46
9	1.33	1.35	1.33	1.95
11	1.30	1.97, 1.56	1.48, 1.95	1.00, 1.95
12	4.28	4.09	4.1	4.06
13	1.97	1.97	1.98	1.35
15	0.90, 1.97	1.00, 1.56	0.95, 1.48	1.00, 1.54
16	1.82, 2.18	1.36, 1.82	1.33, 1.81	1.35, 1.76
17	2.55	2.53	2.51	2.53
18	0.94	0.95	0.92	0.95
19	0.79	0.81	0.77	0.82
21	1.62	1.61	1.61	1.60
22	1.84, 2.36	1.83, 2.34	1.81, 2.33	1.83, 2.32
23	2.18, 2.55	2.34, 2.53	2.33, 2.51	2.46, 2.23
24	5.30	5.30	5.29	5.24
26	1.59	1.65	1.59	1.60
27	1.64	1.62	1.64	1.60
28	1.25	1.26	1.25	1.27
29	1.08	1.07	1.07	1.09
30	0.94	0.94	0.92	0.95
3-O-glc-1’	4.89 d, 7.6[2)]	4.86 d, 7.6[2)]	4.88 d, 7.2[2)]	4.88 d, 7.2[2)]
2’	4.18	4.13	4.84	4.10
3’	4.12	4.22	4.22	4.25
4’	4.01	3.97	3.95	4.08
5’	3.88	3.85	4.28	3.85
6’	4.42, 4.48	4.18, 4.37	4.53	4.25, 4.40
2’-O-glc-1”	5.33 d, 7.6[2)]	55.29 d, 7.6[2)]	5.35 d, 7.6[2)]	5.31 d, 7.2[2)]
2”	4.01	4.03	4.10	4.02
3”	4.12	4.11	4.15	4.14
4”	4.16	4.23	4.30	4.22
5”	4.12	4.17	3.91	4.16
6”	4.41, 4.49	4.31, 4.50	4.22, 4.46	4.40, 4.42
20-O-glc-1’”	5.08 d, 8.0[2)]	5.07 d, 7.6[2)]	5.11 d, 8.0[2)]	5.14 d, 8.0[2)]
2’”	3.88	3.87	3.94	3.92
3’”	4.28	4.24	3.91	4.26
4’”	4.09	4.09	4.10	4.08
5’”	4.06	3.98	4.10	3.85
6’”	4.42, 4.70	4.19, 4.62	4.08, 4.64	4.42, 4.47
6’”-O-sugar-1””	5.04 d, 8.0[2)]	4.94 d, 6.0[2)]	5.63 d, 7.2[2)]	
2””	3.99	4.37	4.21	
3””	4.18	4.18	4.77	
4””	4.29	4.29	4.73	
5””	4.19	3.76, 4.28	4.18, 4.22, 4.45	
6””	4.42, 4.49			

[1)]The signals, the coupling pattern of which was not described, were overlapped each other.
[2)]Chemical shift, coupling pattern, *J* in Hz.

Table 3. ^{13}C-NMR data for ginsenosides Rb_1, Rb_2, Rc, and Rd (100 MHz, pyridine-d_5, δ_C)

Carbon no.	Ginsenoside Rb_1	Ginsenoside Rb_2	Ginsenoside Rc	Ginsenoside Rd
1	39.241	39.635	39.256	39.589
2	26.707	27.062	26.722	27.060
3	89.021	89.332	89.006	89.301
4	40.075	40.059	39.771	40.082
5	56.400	56.802	56.438	56.764
6	18.510	18.866	18.540	18.866
7	35.176	35.555	35.199	35.540
8	39.756	40.438	40.090	40.431
9	50.251	50.592	50.243	50.700
10	36.951	37.314	36.973	37.307
11	30.824	31.134	30.862	31.309
12	70.209	70.565	70.299	70.557
13	49.508	49.826	49.477	49.840
14	51.654	51.790	51.472	51.805
15	30.824	31.134	30.862	31.187
16	26.707	27.062	26.722	27.055
17	51.441	52.078	51.699	52.048
18	16.091	16.439	16.371	16.394
19	16.356	16.667	16.091	16.697
20	83.281	83.842	83.372	83.652
21	22.468	22.748	22.460	22.816
22	36.268	36.571	36.230	36.518
23	23.287	23.605	23.264	23.643
24	125.889	126.161	125.964	126.191
25	131.007	131.340	130.969	131.166
26	25.895	26.160	25.880	26.153
27	18.062	18.282	17.979	18.183
28	28.162	28.503	28.193	28.511
29	16.697	17.000	16.712	17.023
30	17.494	17.819	17.478	17.789
3-O-glc-1’	105.082	105.286	105.081	105.362
2’	83.486	83.721	83.440	83.758
3’	77.973	78.397	77.988	78.564
4’	71.505	72.119	72.127	72.013
5’	78.140	78.246	78.307	78.382
6’	62.815	63.088	62.914	63.224
2’-O-glc-1”	105.923	106.203	105.969	106.294
2”	77.124	77.298	77.131	77.412
3”	78.337	79.368	79.255	79.542
4”	71.665	72.043	71.710	72.013
5”	78.337	78.200	78.132	78.261
6”	62.724	63.232	62.709	63.224
20-O-glc-1”’	98.068	98.363	98.083	98.568
2”’	74.880	75.198	75.069	75.471
3”’	78.337	78.595	78.132	78.648
4”’	71.665	71.998	71.710	72.013
5”’	77.124	76.919	76.578	78.564
6”’	69.746	69.480	68.540	63.080
6”’-O-sugar-1””	105.324	104.717	110.101	
2””	75.251	72.392	83.372	
3””	78.337	74.333	78.868	
4””	71.665	68.752	86.003	
5””	79.262	65.749	62.709	
6””	62.815			

with most literature sources in which ginsenosides were obtained as white or colorless powders [4,12,19]. Preliminary experiments showed that more precise and accurate melting points were obtained with the melting point apparatus supplied by Standard Research System as opposed to the Fisher-John unit that had been used previously. As a result, melting points determined in the current study often differed significantly from values found in the literature. The melting point of ginsenoside Rb_1 has been reported in the literature anywhere from 168°C to 198°C [4,16,19]. The current study indicated a melting point at the lower end of this range at 170°C to 171°C. Literature values for ginsenoside Rb are 197°C to 199°C [4]; the current study yielded 181°C to 183°C. Liu *et al*. [12] and Wang *et al*. [23] state melting points of ginsenoside Rc of 193°C and 201°C, respectively, while the current study found 168°C to 169°C. Dong *et al*. [4] and Wang *et al*. [14] reported melting points of 204°C and 209°C, respectively, for ginsenoside Rd, while the current study gave 182°C to 183°C.

Significant differences with literature sources were also found with optical rotation. Ginsenoside Rb_1 has an optical rotation of +11.5° or +12.42° according to Dong *et al*. [4] or Sanada *et al*. [19], respectively, while the current study measured +6.49°. Likewise, the optical rotation of ginsenoside Rb_2 has been stated as +15.1° or +28.8° according to Dong *et al*. [4] or Yahara *et al*. [20], respectively, while a value of +14.08° was obtained herein. The specific rotation of ginsenoside Rc was measured as +1.91°, similar to the literature value of +2.1 [23]. In the case of ginsenoside Rd, the literature value is +16.8° [4], while the result obtained herein was +18.1°.

The hydroxyl groups on these ginsenosides render them marginally soluble in nonpolar solvents like CCl_4 or $CHCl_3$. Therefore, each compound was mixed with KBr and compressed under reduced pressure to form a pellet for IR absorbance measurements. However, due to water absorption by the pellet and the accompanying spectral interference, a new method was employed in which the saponin was dissolved in MeOH, cast onto KBr or CaF_2 plates, and allowed to evaporate. Preliminary results showed less interference in the latter method. Ginsenosides Rb_1, Rb_2, Rc, and Rd all exhibited absorption peaks corresponding to the O–H stretch of each hydroxyl group (3365, 3389, 3367, 3366, respectively), C–H stretching (2919, 2942, 2943, 2943), C=C stretching (1590, 1640, 1649, 1647), C–H bending (1079, 1077, 1078, 1077), and C–O bending (1032, 1029, 1031, 1034).

Glycosides like saponin contain multiple hydroxyl groups and exhibit very low volatilities. Thus, mass spectra are usually obtained with FAB/MS. Relative to EI/MS, FAB/MS is a soft ionization method that tends to yield high abundances of molecular ions and relatively smaller proportions of fragment ions. This study employed a positive ionization method. Ginsenoside Rb_1 exhibited a molecular ion at *m*/*z* 1131 $[M+Na]^+$ and fragment peaks at *m*/*z* 789, 425, 407, 365, and 325. The molecular ion of ginsenoside Rb_2 was observed at *m*/*z* 1101 $[M+Na]^+$ with fragment peaks at *m*/*z* 789, 425, and 407. Ginsenoside Rc revealed *m*/*z* 1101 $[M+Na]^+$ as a molecular ion peak and *m*/*z* 789, 425, and 407 were observed as fragment ion peaks. Ginsenoside Rd exhibited a molecular ion peak at *m/z* 969 $[M+Na]^+$ with fragment peaks at *m/z* 789 and 425.

NMR spectra were obtained at 40°C from 0.08 M solutions of each compound dissolved in pyridine-d_5. Each spectrum represents the accumulation of 8 scans for ^{1}H-NMR and over 1024 scans for ^{13}C-NMR.

The chemical name of ginsenoside Rb_1 is 3-*O*-[*β*-D-glucopyranosyl (1→2)-*β*-D-glucopyranosyl]-20-*O*-[*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranosyl]-3*β*,12*β*,20*β*-trihydroxydammar-24-ene. Since ginsenoside Rb_1 contains four attached sugar moieties, it dissolved easily in methanol, pyridine, and dimethyl sulfoxide (DMSO). The few double bonds and many oxygen-linked carbon atoms made pyridine-d_5 the better suited solvent for NMR measurements due to less overlap of ginsenosides and solvent-derived signals, although both methanol and DMSO have been used. ^{13}C-NMR measured in the former solvent exhibited peaks that were generally shifted to lower magnetic fields relative to those encountered when acquiring spectra in pyridine-d_5 [8,16]. The extent of this shift was approximately 1.2 to 1.5 ppm and could be as high as 2.0 ppm. In particular, oxygen-linked carbon atoms C3 and C20 were shifted to lower magnetic fields with chemical shifts of 2.6 and 2.1 ppm, respectively. Among the eight methyl groups, C21 and C28 showed the largest shifts of 5.1 and 3.7 ppm, respectively, as confirmed by HSQC measurements. C21 and C28 were identified as the peaks at 22.468 ppm and at 28.162 ppm, respectively. In contrast, NMR spectra measured in DMSO-d_6 were generally shifted to higher magnetic fields by 0.5 to 1.0 ppm [16]. Although C20 was observed at lower magnetic fields than those indicated in the literature when measured in pyridine-d_5 [5,9,17], C2' and C20 gave rise to peaks at δ_C 83.486 and δ_C 83.281 ppm, respectively, with C2' at the lower magnetic field. In this

case, since these two carbon atoms were representative of a methine group and a quarternary carbon, respectively, identification was easily made via distortionless enhancement by polarization transfer measurements. Also, the methyl carbon atoms C18 and C19 corresponded to peaks at δ_C 16.091 and δ_C 16.356 ppm, respectively. However, the order of the chemical shift differed from that of literature sources [5,7,9,17]. The significant difference in chemical shift between H18 (0.94 ppm) and H19 (0.79 ppm) indicated that the signals at 16.091 and 16.356 ppm, which were correlated with each proton signal in the HSQC spectrum, corresponded to C18 and C19, respectively. In the ^{1}H-NMR spectrum in reference [4], signals corresponding to H26 and H27; H28 and H29 yielded identical chemical shifts. In the current study, the allylic methyl protons H26 and H27 yielded peaks at 1.59 and 1.64 ppm, and H28 and H29 yielded peaks at 1.25 and 1.08 ppm, respectively. This relationship was confirmed by HMBC and HSQC spectra. Among the anomer hydrogen atoms H1''' and H1'''' of the sugar moieties, the latter was reported to appear at lower magnetic fields [4]. However, in the current study H1''' and H1'''' yielded peaks at 5.08 and 5.04 ppm, respectively, which differed significantly from the chemical shifts cited in the literature. The results of the current study were verified by a J_3 correlation between H1''' and C20 (δ_C 83.281), and between H1'''' and C6''' (δ_C 69.746) in the HMBC spectrum (Fig. 2). The chemical name of ginsenoside Rb_2 is 3-*O*-[*β*-D-glucopyranosyl (1→2)-*β*-D-glucopyranosyl]-20-*O*-[*β*-D-glucopyranosyl(1→6)-*α*-L-arabinopyranosyl]-3*β*,12*β*,20*β*-trihydroxydammar-24-ene. The observed chemical shifts of C18 and C19 in the ^{13}C-NMR spectrum differed from those in reference [17]. The significant difference in the chemical shifts of H18 (0.95 ppm) and H19 (0.81 ppm) indicated that the ^{13}C peaks at 16.439 ppm and 16.667 ppm, which were correlated with both protons in the HSQC spectrum, corresponded to C18 and C19. Although Oura *et al*. [17] gives 124.80 ppm as the chemical shift of the olefine methine carbon, C24, the current study yielded δ_C 126.16, a difference of 1.36 ppm. This finding was verified with correlations of C24 between H23 (δ_H 2.34, 2.53) via J_2 as well as among H26 (δ_H 1.65) and H27 (δ_H 1.62) via J_3 in the HMBC spectrum (Fig. 3). In the ^{1}H-NMR spectrum, the chemical shifts of the methyl protons H21, H26, and H27 were 1.61, 1.65, and 1.62 ppm, respectively, which represents a different order than that cited in reference [6]. However, since the chemical shifts of C21, C26, and C27 were absorbed at very different chemical

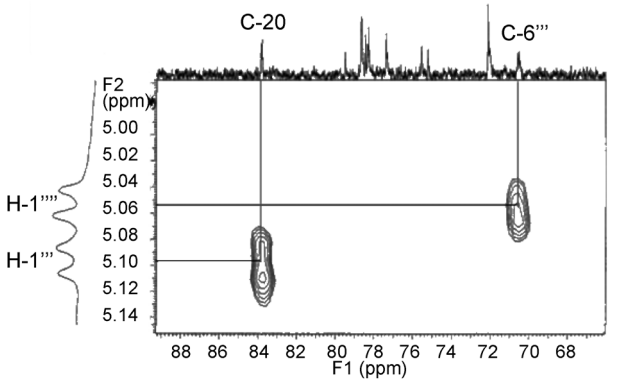

Fig. 2. Heteronuclear multiple bond connectivity spectrum of ginsenoside Rb_1.

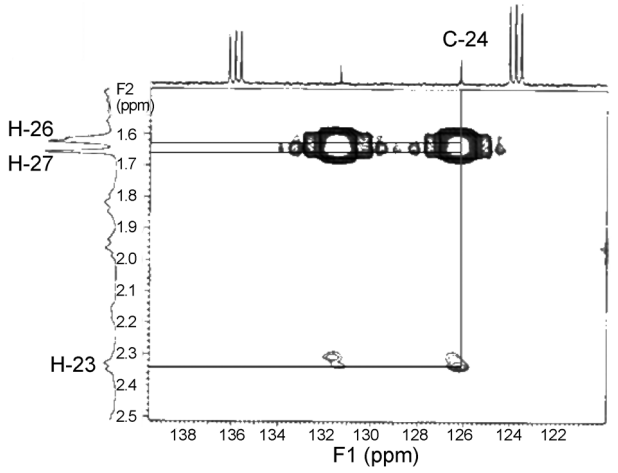

Fig. 3. Heteronuclear multiple bond connectivity spectrum of ginsenoside Rb_2.

shifts such as 22.748, 26.160, and 18.282 ppm, respectively, three methyl groups were easily identified using the HSQC experiment. Dong *et al*. [4] reports the same chemical shifts for the methyl protons H28 and H29. But there was a marked difference between the chemical shifts of C28 (28.503 ppm) and C29 (17.000 ppm), HSQC spectrum indicated the signals observed at 1.26 and 1.07 ppm as H28 and H29, respectively.

The chemical name of ginsenoside Rc is 3-*O*-[*β*-D-glucopyranosyl (1→2)-*β*-D-glucopyranosyl]-20-*O*-[*β*-D-glucopyranosyl(1→6)-*α*-L-arabinofuranosyl]-3*β*,12*β*,20*β*-trihydroxy dammar-24-ene. The methyl protons H21 and H28 were assigned to the signals at 1.61 and 1.25 ppm, respectively, Wang *et al*. [23], however, reports the opposite assignment. The assignment for H21 reported herein was made using correlations observed among C20 (J_2, δ_C 83.372), C17 (J_3, δ_C 51.699), and C22 (J_3, δ_C 36.230) in the HMBC spectrum. Likewise, the assignment of H28 was based on

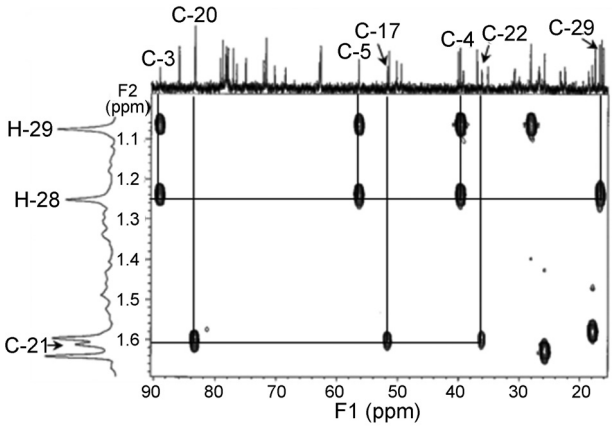

Fig. 4. Heteronuclear multiple bond connectivity spectrum of ginsenoside Rc.

observed correlations among C4 (J_2, δ_C 39.771), C29 (J_3, δ_C 16.712), C3 (J_3, δ_C 89.006), and C5 (J_3, δ_C 56.438) (Fig. 4).

The chemical name of ginsenoside Rd is 3-*O*-[*β*-D-glucopyranosyl (1→2)-*β*-D-glucopyranosyl]-20-*O*-*β*-D-glucopyranosyl-3*β*, 12*β*,20*β*-trihydroxydammar-24-ene. NMR peaks corresponding to C18 and C19 were observed at δ_C 16.394 and δ_C 16.679, respectively, which differed from the assignments reported in the literature [10,11,14,17]. The significant difference in chemical shift between H18 (0.95 ppm) and H19 (0.82 ppm) indicated that the ^{13}C peaks at 16.394 ppm and 16.697 ppm, which were correlated with both protons in the HSQC spectrum, corresponded to C18 and C19, respectively. The chemical shifts of the methyl carbon atoms C27, C29, and C30 differed from those in the literature [13-15]. The distinct difference in the chemical shifts of H27 (1.60 ppm), H29 (1.09 ppm), and H30 (0.95 ppm) indicated that the ^{13}C peaks at 18.183 ppm, 17.023 ppm, and 17.789 ppm, which were correlated with these protons in the HSQC spectrum, were C27, C29, and C30, respectively.

For the tetracyclic triterpene compounds characterized herein, many of the methine and methylene proton signals overlapped at higher magnetic fields. In addition, many of the oxygenated-methine proton signals of the four-sugar glycoside overlapped in the ^1H-NMR spectrum. Thus, identification of each signal would be very difficult based solely on one-dimensional NMR techniques. To date, peak assignments in NMR data for these types of materials has been based on previously reported data. However, much of the earlier data may be erroneous due to instrument-resolution limitations. The current study employed 2-D NMR techniques, including of HMBC and HSQC, to accurately assign the chemical shifts of each signal.

On a normal phase silica gel TLC plate ($CHCl_3$-MeOH-H_2O=65:35:10), R_f of 0.42, 0.46, 0.48, and 0.50 were observed for ginsenosides Rb_1, Rb_2, Rc, and Rd, respectively. Reverse-phase ODS TLC (MeOH-H_2O=3:1) yielded R_f values of 0.40, 0.32, 0.40, and 0.25, respectively. Although the ginsenosides did not exhibit fluorescence when excited at 254 or 365 nm, the location of each compound was revealed by a light purple color when sprayed with 10% H_2SO_4 and heating. HPLC retention times were 54.3, 47.7, 48.1, and 36.5 minutes for ginsenosides Rb_1, Rb_2, Rc, and Rd, respectively, by analysis methods described in Materials and Methods.

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