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Re-evaluation of physicochemical and NMR data of triol ginsenosides Re, Rf, Rg2, and 20-gluco-Rf from *Panax ginseng* roots

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ABSTRACT

Ginseng roots were extracted with aqueous methanol, and extracts were suspended in water and extracted successively with ethyl acetate and *n*-butanol. Column chromatography using the *n*-butanol fraction yielded four purified triol ginseng saponins: the ginsenosides Re, Rf, Rg2, and 20-gluco-Rf. The physicochemical, spectroscopic, and chromatographic characteristics of the ginsenosides were measured and compared with reports from the literature. For spectroscopic analysis, two-dimensional nuclear magnetic resonance (NMR) methods such as ^{1}H-^{1}H correlation spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum correlation, and heteronuclear multiple bond connectivity were employed to identify exact peak assignments. Some peak assignments for previously published ^{1}H- and ^{13}C-NMR spectra were found to be inaccurate. This study reports the complete NMR assignment of 20-gluco-Rf for the first time.

1. Introduction

Saponins are key constituents of *Panax ginseng* Meyer to exhibit various pharmacological activities [1]. To date, approximately 80 kinds of saponin have been isolated from *P. ginseng*. Most have two kinds of dammarane-type triterpenoid moieties as aglycones: protopanaxdiol (diol, PPD) and protopanaxtriol (triol, PPT). Only ginsenoside Ro analogs have oleanolic acid as an aglycone [2].

Nuclear magnetic resonance (NMR) is the most common method for identifying ginsenosides, but many variations and inaccuracies can be found in the published NMR data. We previously described the several physicochemical and spectroscopic characteristics of four major diol-ginsenosides, Rb1, Rb2, Rc, and Rd, and the ginsenoside Rg1, all of which were measured using standard methods. We also identified their signals using two-dimensional NMR spectroscopic methods [3,4].

In this study, we obtained physicochemical and spectroscopic data for four major triol- saponins: the ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4). The aglycone of the ginseng triol-saponins is a PPT, which is a dammarane triterpenoid hydroxylated to C-3, C-12, and C-20 via β-linkage and to C-6 via α-linkage with a double bond between C-24 and C-25. In triol-saponins, sugars are attached to the hydroxyl groups at C-6 and C-20. The ginsenosides Re (1) and 20-gluco-ginsenoside Rf (4) are bisdesmosidic and the ginsenosides Rf (2) and Rg2 (3) are monodesmosidic saponins. The ginsenoside Re (1) has an α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranose moiety at 6-OH and a β-D-glucopyranose moiety at 20-OH. The 20-gluco-ginsenoside Rf (4) has a sophorose moiety (β-D-glucopyranosyl-(1→2)-β-D-glucopyranose) at 6-OH and a β-D-glucopyranose moiety at 20-OH. The monodesmoside ginsenosides Rf (2) and Rg2 (3) have sophorose and α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranose moieties, respectively, at 6-OH (Fig. 1). The literature has varying

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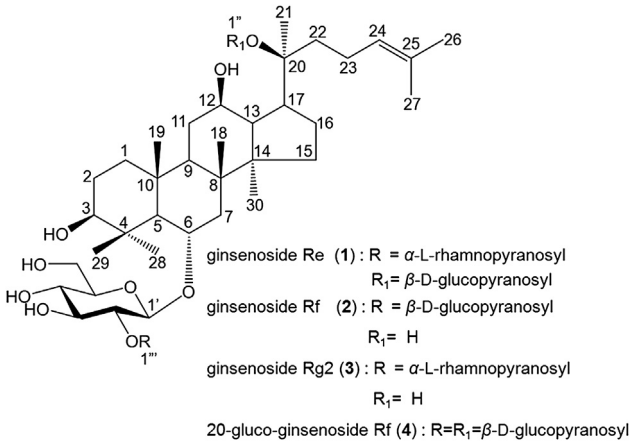

Fig. 1. Chemical structures of ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4) from the roots of *Panax ginseng*.

assignments for the NMR signals for the hydroxyl group-linked atoms, the methyl carbon atoms, the olefinic carbon atoms, and for protons linked to individual carbon atoms [5–15].

This study definitively identified individual proton and carbon signals using the two-dimensional NMR techniques of correlation spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond connectivity (HMBC). Melting points, specific rotation, IR absorbance, and fast atom bombardment (FAB)/MS data were obtained using standard methods and data were compared to findings in the literature [5,7,10,15–21]. The retention factor (R_f) of each saponin in both normal and reverse-phase TLC experiments and the retention time of each ginsenoside by carbohydrate-based HPLC were also determined.

2. Materials and methods

2.1. Ginseng samples

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

2.2. Reagents, instrumentation, and measurement methods

Kieselgel 60 and LiChroprep RP-18 were used for column chromatography (Merck, Darmstadt, Germany). Kieselgel 60 F_{254} and RP-18 F_{254S} were used as TLC solid phases (Merck). The former used a mobile phase of $CHCl_3$–methanol (MeOH)–H_2O (65:35:10) and the latter used MeOH–H_2O (2:1). Detection of substances on TLC plates was by observation under a UV lamp (Spectroline, model ENF-240 C/F; Spectronics Corp., New York, NY, USA) or by spraying developed plates with 10% aqueous H_2SO_4 followed by heating and observing color development. HPLC was at 50°C and 30 psi using an LC-20A (Shimadzu, Kyoto, Japan) with an evaporative light scattering detector (ELSD; Shimadzu). HPLC analytical columns were Carbohydrate ES (5 μm, 250 × 46 mm; Grace, Deerfield, IL, USA) eluted with step-wise gradients 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). B was 25% from 0 min to 12 min, 25 → 80% from 12 min to 32 min, 80 → 90% from 32 min to 33 min, 90% from 33 min to 38 min, 90–25% from 38 min to 40 min.

NMR spectra were recorded on a Varian Inova AS 400 spectrometer (400 MHz; Varian, Palo Alto, CA, USA) with 0.0625 mol of each ginsenoside (59.1 mg Re, 50.0 mg Rf, 49.0 mg Rg2, and 60.1 mg 20-gluco-Rf) 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 standard adjusted to 0 ppm. IR spectra were measured with an IR spectrometer (model 599B; PerkinElmer, Waltham, MA, USA). For each sample, 2 mg were dissolved in 100 uL of MeOH and a drop of the solution was added to a CaF_2 salt plate (Spectral Systems, Hopewell Junction, NY, USA) and evaporated. Measurements were at room temperature. FAB/MS was carried out with a JMS-700 mass spectrometer (JEOL, Tokyo, Japan) using glycerol as a 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 (JASCO). Melting points were obtained using an EZ-Melt MPA 120 automated melting point apparatus (Stanford Research Systems, Sunnyvale, CA, USA), and values obtained were uncorrected.

2.3. Isolation of ginsenosides

Six-year-old fresh ginseng roots (20 kg fresh weight) were cut into pieces and extracted with 90% MeOH (5.45 L) for 24 h at room temperature. Extracts were filtered through filter paper and residues were extracted twice more with 80% MeOH (4 L). Filtrates were evaporated under reduced pressure at 45°C to yield 2.2 kg of dried extract. Dried extract was partitioned between ethyl acetate (3 L × 3) and H_2O (3 L). The remaining H_2O layer was extracted with *n*-butanol (*n*-BuOH, 2.8 L × 3). Each layer was concentrated under reduced pressure to obtain ethyl acetate (25 g), *n*-BuOH (169 g), and H_2O fractions. The *n*-BuOH extract (160 g) was applied to a silica gel column (φ 10 cm × 24 cm) and eluted in three steps with $CHCl_3$–MeOH–H_2O (step 1 = 65 L of 10:3:1, step 2 = 55 L of 8:3:1, and step 3 = 30 L of 6:4:1) to yield 24 fractions (PGB1–PGB24). Fractions PGB9 and PGB10 were combined (18.08 g, Ve/Vt = 0.35–0.43, where Ve was volume of eluent for the fraction and Vt was total elution volume), and separated on a silica gel column (φ 6.5 cm × 15 cm) with $CHCl_3$–MeOH–H_2O (65:35:10, 111 L) as eluent to obtain 14

Table 1
Physicochemical Characteristics for Ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4)

	Ginsenoside Re	Ginsenoside Rf	Ginsenoside Rg2	20-glucoginsenoside Rf
Crystals	Colorless powder	White powder	Colorless powder	White powder
Melting point (°C)	186–187	180–181	191–192	204–205
$[\alpha]_D$	−1.80° (28°C, *c* = 0.50, MeOH)	+13.80° (28°C, *c* = 0.50, MeOH)	−3.84° (28°C, *c* = 0.50, MeOH)	+64.00° (28°C, *c* = 0.50, MeOH)
IR (cm^{-1})	3359, 2929, 1642, 1072, 1045	3360, 2924, 1637, 1071, 1031	3391, 2930, 1635, 1070, 1048	3360, 2930, 1635, 1074, 1032
FAB/MS (*m*/*z*)	945, 765, 475, 265	799, 475, 325	765, 281, 255	961, 799
TLC (R_f)	0.27[1], 0.57[2]	0.37[1], 0.29[2]	0.51[1], 0.13[2]	0.28[1], 0.65[2]
HPLC (R_t, min)	27.1[3]	20.6[3]	10.3[3]	30.2[3]

FAB, fast atom bombardment; R_f, retention factor; R_t, retention time.
[1] Kieselgel 60 F_{254}, $CHCl_3$:MeOH:H_2O (65:35:10).
[2] Kieselgel RP-18 F_{254S}, MeOH:H_2O (2:1).
[3] Carbohydrate ES (5 μm, 250 × 4.6 mm), solvent A (acetonitrile:H_2O:isopropanol = 80:5:15), solvent B (acetonitrile:H_2O:isopropanol = 60:25:15), gradient elution: B 25% at 0–12 min, 25–80% at 12–25 min, 80% at 25–32 min, 80–90% at 32–33 min, and 90% at 33–38 min. Flow rate = 0.8 mL/min.

Table 2
^{1}H-NMR Data for Ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4) (400 MHz, pyridine-d_5, δ_H)

H-No.	Ginsenoside Re		Ginsenoside Rf	Ginsenoside Rg2		20-gluco-ginsenoside Rf
	PGB16+17-9-15	Refs[3)]	PGB16+17-7-16	PGB9+10-10+11-5-13	Refs[3)]	PGB16+17-9-12
1	0.92 1.63[1)]	–	0.98, 1.68	0.84, 1.57	0.95, 1.62 [5] 0.96, 1.65 [8]	0.76, 1.67
2	1.78, 1.85	–	1.62, 1.79	1.79, d, 12.4 1.88, d, 7.6		1.81, 1.86
3	3.41, dd, 11.2, 4.8[2)]		3.44, dd, 4.4, 10.8	3.40, dd, 10.8, 4.8		3.43, dd, 10.8, 4.4
5	1.35, d, 10.4		1.35	1.33, d, 10.0		1.34
6	4.66		4.39	4.59	4.68 [5], 4.69 [8]	4.29
7	1.97, 2.23		1.92, 2.39	0.88, 2.19	1.97, 2.26 [5] 1.98, 2.26 [8]	1.89, 2.34
9	1.48		1.50	1.48		1.45
11	1.48, 2.04		1.49, 2.03	1.51, 1.96		1.44, 2.02
12	4.13		3.87, m	3.83		4.10, m
13	1.91		2.01	1.93		1.96
15	0.82, 1.48	1.04, 1.60 [8]	1.13, 1.63	0.83, br.dd, 5.6 1.41	0.92, 1.53 [8]	1.09, 1.44
16	1.19, 1.72		1.32, 1.38	1.38, 1.73	1.26, 1.78 [5] 1.29, 1.77 [8]	1.26, 1.72
17	2.48		2.28	2.21		2.49
18	1.14, s		1.14, s	1.13, s		1.09, s
19	0.93, s		0.94, s	0.91, s		0.92, s
21	1.56, s		1.37, s	1.35, s		1.57, s
22	1.75, 2.34	1.71 [5]	1.69, 1.99	1.57, 1.91		1.78, 2.36
23	2.20, 2.45, br.dd, 19.6, 9.2S		2.26, 2.58	2.18, 2.52	2.28, 2.59 [5] 2.27, 2.59 [8]	2.19, 2.46
24	5.22, dd, 6.4, 6.4		5.31, dd, 6.8, 6.8	5.27, dd, 6.8, 6.8		5.22, dd, 6.4, 6.8
26	1.58, s		1.65, s	1.64, s		1.57, s
27	1.58, s		1.62, s	1.59, s		1.57, s
28	2.04, s		2.03, s	1.99, s	2.11 [5], 2.10 [8]	2.04, s
29	1.33, s		1.42, s	1.29, s		1.44, s
30	0.92, s		0.81, s	0.89, s		0.76, s
6-O-glc-1′	5.22, d, 6.4		4.90, d, 7.6	5.17, d, 6.0		4.86, d, 7.2
2′	4.32		4.38	4.24	4.34 [5, 8]	4.43
3′	4.37		4.31	4.29		4.33
4′	4.15		4.09	4.10	4.21 [5], 4.20 [8]	4.13 *
5′	3.89		3.90	3.86	3.97 [5], 3.96 [8]	4.21
6′	4.28, 4.49		4.26, 4.42	4.30, 4.47		4.31, 4.43 *
2′-O-rha-1″	6.47, br. s			6.40, br.s		
2″	4.77, br.d, 3.2			4.73		
3″	4.64			4.59		
4″	4.28			4.25		
5″	4.90, dq, 9.2, 6.0			4.85, dq, 10.4, 6.4	4.98 [5], 4.92 [8]	
6″	1.74, d, 6.0			1.72, d, 6.4		
2′-O-glc-1″	–		5.84, d, 7.2	–		5.89 d, 7.2
2″	–		4.13	–		4.17
3″	–		4.18	–		4.20
4″	–		4.12	–		4.12 *
5″	–		3.81	–		3.81 *
6″	–		4.29, 4.42	–		4.27, 4.44 *
20-O-glc-1‴	5.13, d, 8.4		–	–		5.13 d, 7.6
2‴	3.95		–	–		3.97
3‴	4.19		–	–		4.19
4‴	4.12		–	–		4.11 *
5‴	3.88		–	–		3.90 *
6‴	4.23, 4.44		–	–		4.27, 4.44 *

br, broad; d, doublet; dd, doublet of doublet; dq, doublet of quartet; s, singlet; NMR, nuclear magnetic resonance.
1) Signals, the coupling pattern of which was not described, overlapped with other signals.
2) Chemical shift, coupling pattern, J in Hz.
3) The chemical shifts showing the difference bigger than 0.1 ppm from those of this study were exhibited. * Assignments with the same superscript in a single column might be reversed.

fractions (PGB9+10-1–PGB-9+10-14). Fractions PGB9+10-10 and PGB9+10-11 were combined (13.4 g, Ve/Vt = 0.675–0.781), and separated on a silica gel column (φ 7 cm × 16 cm) with $CHCl_3$:*n*-BuOH:MeOH:H_2O (10:1:3:1, 104 L) as eluent to obtain eight fractions (PGB-9+10-10+11-1–PGB-9+10-10+11-8). Fraction PGB9+10-10+11-5 (434 mg, Ve/Vt = 0.41–0.49) was fractionated over an octadecyl silica gel (ODS) column (φ 4 cm × 6 cm, MeOH–H_2O = 6:5, 2.6 L) into 16 fractions (PGB9+10-10+11-5-1–PGB9+10-10+11-5-16) including ginsenoside Rg2 [3, PGB9+10-10+11-5-13, 36.1 mg, Ve/Vt = 0.77–0.84, TLC R_f = 0.31 (RP-18 F_{254S}, MeOH–H_2O = 3:1), and R_f = 0.45 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–-H_2O = 65:35:10)]. Fractions PGB16 and PGB17 were combined (12 g, Ve/Vt = 0.65–0.72), and separated on a silica gel column (φ 4 cm × 6 cm) with a $CHCl_3$–MeOH–H_2O (65:35:10, 98 L) as eluent for 20 fractions (PGB16+17-1–PGB16+17-20). PGB16+17-7 (370 mg, Ve/Vt = 0.18–0.20) was fractionated over the ODS column (φ 4 cm × 5 cm, MeOH–H_2O = 3:1, 2 L) for 20 fractions (PGB16+17-7-1–PGB-16+17-7-20) including ginsenoside Rf [2, PGB16+17-7-16, 3.4 mg, Ve/Vt = 0.712–0.798, TLC R_f = 0.42 (RP-18 F_{254S}, MeOH-H_2O = 3:2), and R_f = 0.44 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 65:35:10)]. Fraction

Table 3
^{13}C-NMR Data for Ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4) (100 MHz, pyridine-d_5, δ_C)

Carbon No.	Ginsenoside Re		Ginsenoside Rf		Ginsenoside Rg2		20-gluco-ginsenoside Rf
	PGB16+17-9-15	Refs[1)]	PGB16+17-7-16	Refs[1)]	PGB9+10-10+11-5-13	Refs[1)]	PGB16+17-9-12
1	39.438	–	39.542		39.625		39.499
2	27.842	–	27.888		27.736		27.836
3	78.395	–	78.706		78.304		78.694
4	40.065	–	40.308		39.997		40.249
5	60.849	–	61.501		60.788		61.420
6	74.550	78.7 [8]	79.911		74.308		79.945
7	46.025		45.176		46.048		44.973
8	41.248		41.278		41.157	39.3 [9]	41.212
9	49.589		50.241		49.703		49.986
10	39.458		39.769		39.390	41.0 [9]	39.703
11	31.012		32.195	31.1 [14]	32.050		30.983
12	70.228		71.085		71.002		70.201
13	49.103		48.368		48.156		49.204
14	51.454		51.773		51.682		51.426
15	30.822		31.391		31.330	29.9 [13]	30.756
16	26.720		26.963		26.864		26.707
17	51.727		54.851		54.654		51.578
18	17.386	17.4 [7], 17.7 [8], 17.4 [11]	17.568	17.6 [14]	17.196	17.7 [8], 17.6 [9]. 17.46 [13]	17.660
19	17.628	17.2 [4], 17.8 [8]	17.757	17.5 [14]	17.667	17.7 [8], 17.5 [9], 17.29 [13]	17.600
20	83.270		73.072		72.996		83.274
21	22.421		27.160		27.023		22.407
22	36.092		35.940		35.811		36.155
23	23.338		23.149		23.020		23.302
24	125.891		126.316		126.202		125.896
25	130.850		130.767		130.691		130.855
26	25.886		25.969		25.901		25.865
27	17.886		17.848		17.757	16.8 [9], 17.34 [13]	17.865
28	32.285		32.195		32.187		32.128
29	17.780	17.3 [8], 17.1 [11]	16.916	17.6 [14]	17.667	17.0 [8], 16.87 [13]	16.857
30	17.325	17.6 [8]	16.969	16.7 [14]	16.969	17.2 [8]	17.228
6-O-glc-1′	101.832		103.872		101.703		103.793
2′	79.433		79.911		79.312	78.18 [13]	79.505
3′	78.569		79.722	78.7 [14]	78.516	79.61 [13]	79.884
4′	72.556		71.790		72.576		72.332 [a]
5′	78.288		77.917	79.8 [14]	78.304		78.507 [b]
6′	63.086		63.010		63.040		63.369 [c]
2′-O-rha-1″	101.908				101.817		
2″	72.458				72.336		
3″	72.291				72.177		
4″	74.164				74.057		
5″	69.508				69.417		
6″	18.872				18.766		
2′-O-glc-1″	–		103.872		–		103.762
2″	–		76.074		–		76.009
3″	–		78.478		–		78.254
4″	–		72.404		–		71.627 *
5″	–		78.122	79.8 [14]	–		77.875 *
6″	–		63.434		–		62.899 *
20-O-glc-1‴	98.253		–		–		103.762
2‴	75.172		–		–		76.009
3‴	79.221		–		–		78.398
4‴	71.570		–		–		72.332
5‴	78.395		–		–		78.254
6‴	62.843		–		–		62.899

NMR, nuclear magnetic resonance.
[1)] The chemical shifts showing the difference > 1.0 ppm from those of this study were exhibited and methyl signals that are listed in wrong order were exhibited. * Assignments with the same alphabetical superscript in a single column might be reversed.

PGB16+17-9 (1.7 g, Ve/Vt = 0.25–0.29) was separated over the ODS column (φ 4 × 6 cm, MeOH–H_2O = 3:1, 7 L) into 36 fractions (PGB16+17-9-1–PGB16+17-9-36) including the 20-gluco-ginsenoside Rf [4, PGB16+17-9-12, 223 mg, Ve/Vt = 0.22–0.27, TLC R_f = 0.54 (RP-18 F_{254S}, MeOH–H_2O = 2:1), R_f = 0.31 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 65:35:10)] and the ginsenoside Re [1, PGB16+17-9-15. 68.3 mg, Ve/Vt = 0.38–0.40, TLC R_f = 0.50 (RP-18 F_{254S}, MeOH–H_2O = 2:1), and R_f = 0.36 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 65:35:10)]. Physicochemical and spectroscopy data from each ginsenoside are in Tables 1–3.

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, in agreement with most of the literature in which ginsenosides were obtained as white or colorless powders [7,10,15,19]. Preliminary experiments showed that more precise and accurate melting points were obtained with the Stanford Research Systems melting point apparatus we used than with the Fisher-John instrument used previously. As a result, melting points

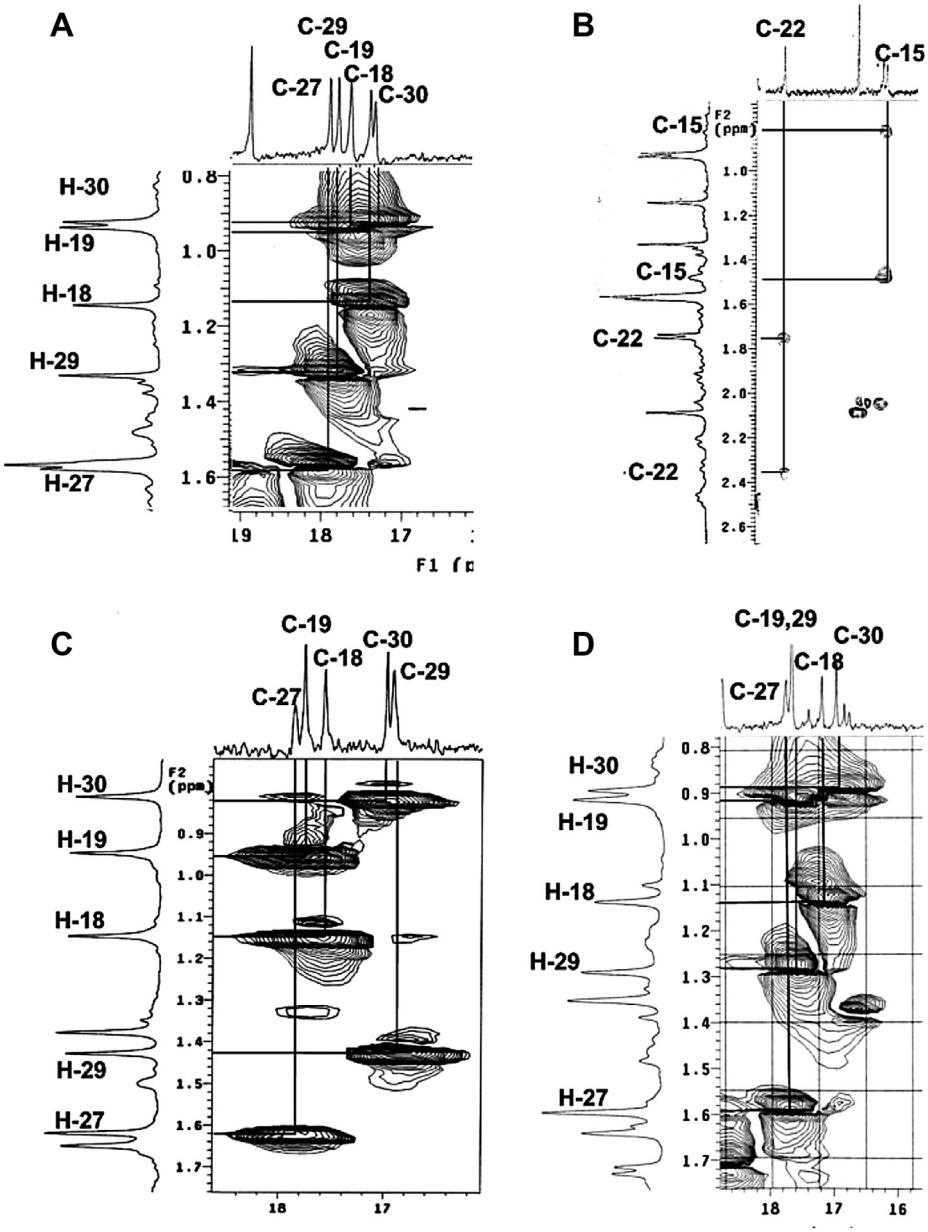

Fig. 2. Heteronuclear single quantum correlation spectra of ginsenosides. (A,B) Re (1). (C) Rf (2). (D) Rg2 (3).

determined in this study often differed significantly from values found in the literature. The melting points of ginsenoside Re (1) in the literature are from 168°C to 198°C [7,15], whereas the results of this study indicated a melting point of 186–187°C. The literature value for ginsenoside Rf (2) is 197–198°C [15], whereas this study found that it was 180–181°C. The reference-state [15] melting point of ginsenoside Rg2 (3) is 187–189°C in the literature, whereas it was found to be 191–192°C in this study. The reported melting point for 20-gluco-ginsenoside Rf (4) is 204°C [19], whereas this study found that it was 204–205°C.

Significant differences from the values in the literature were also found for optical rotation. Ginsenoside Re (1) has an optical rotation of −1.0° according to previous studies [11], whereas it measured −1.80° in this study. Likewise, the optical rotation of ginsenoside Rf (2) is +6.99° in other studies [15], whereas a value of +13.80° was obtained here. The specific rotation of ginsenoside Rg2 (3) measured −3.84°, whereas the literature value is +6.0° [15]. For 20-gluco-ginsenoside Rf (4), the literature value is +21.0° [19], whereas the result obtained here was +64.00°.

The many hydroxyl groups of the ginsenosides studied here make them insoluble in nonpolar solvents such as $CHCl_3$ or CCl_4. Therefore, each compound was mixed with KBr and compressed under reduced pressure to form a pellet for IR absorbance measurement. However, spectroscopic interference derived from water absorption by the pellet required the use of an alternative method, in which the saponin was dissolved in MeOH, cast onto CaF_2 or LiF plates, and allowed to evaporate. Ginsenosides Re (1), Rf (2), Rg2 (3), and 20-gluco-Rf (4) exhibited absorption peaks corresponding to the O–H stretching of each hydroxyl group (3359, 3360, 3391, and 3360, respectively), C–H stretching (2929, 2924, 2930, and 2930), C=C stretching (1642, 1637, 1635, and 1635), C–H bending (1072, 1071, 1070, and 1074), and C–O bending (1045, 1031, 1048, and 1032).

The multiple hydroxyl groups of ginsenosides also result in very low volatility. Thus, mass spectra are usually obtained with FAB/MS

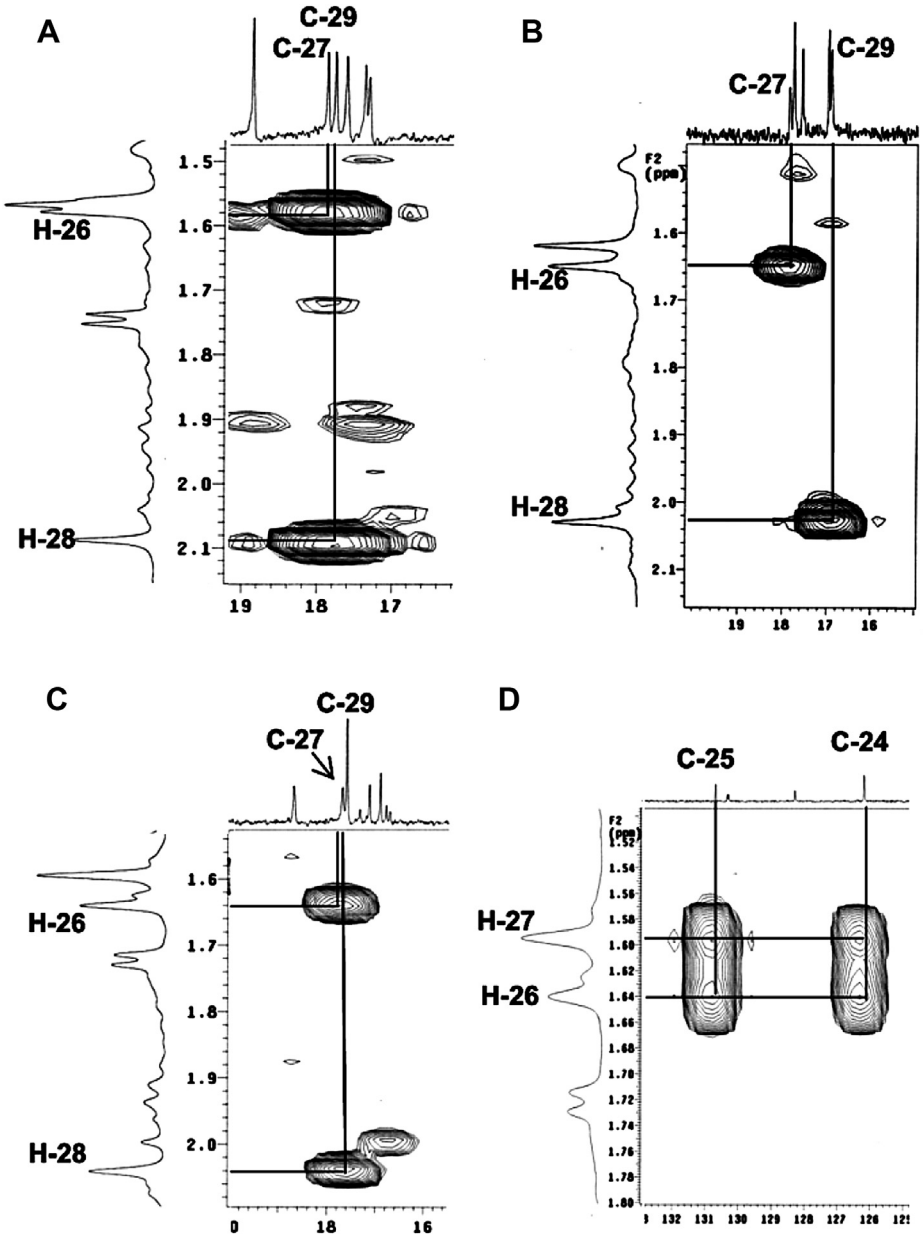

Fig. 3. Hetero nuclear multiple bond connectivity spectra of ginsenosides. (A) Re (1). (B) Rf (2). (C,D) Rg2 (3).

instead of EI/MS. The soft ionization method FAB/MS distinguishes between molecular ions and fragment ions of relatively smaller proportions. The negative ionization method showed better spectrums for the ginsenosides than a positive ionization method. Ginsenoside Re (1) showed a molecular ion at *m/z* 945 ($[M\text{-}H]^-$) and fragment ions peaks at *m/z* 765, 475, and 265. The molecular ion of ginsenoside Rf (2) was observed at *m/z* 799 ($[M\text{-}H]^-$) with fragment peaks at *m/z* 475 and 325. Ginsenoside Rg2 (3) showed *m/z* 765 ($[M\text{-}H_2O\text{-}H]^-$) as a pseudomolecular ion peak and *m/z* 281 and 255 as fragment ion peaks. 20-Gluco-ginsenoside Rf (4) revealed a molecular ion peak at *m/z* 961 ($[M\text{-}H]^-$) with a fragment ion peak at *m/z* 799.

NMR spectra were obtained at 40°C from 0.08 M solutions of compounds dissolved in pyridine-d_5. Each spectrum was the accumulation of eight scans for ^{1}H-NMR and > 1024 scans for ^{13}C-NMR. TMS was used as an internal standard adjusted to 0 ppm.

Because ginsenoside Re (1) contains two attached sugar moieties, it dissolved easily in methanol, pyridine, and DMSO. Pyridine-d_5 has few double bonds and many oxygen-linked carbon atoms so it was a better solvent for NMR measurements because it resulted in less overlap between the ginsenoside- and solvent-derived signals than deuterated methanol or DMSO-d_6. The methyl carbon atoms C-18, C-19, C-29, and C-30 of ginsenoside Re (1) in pyridine-d_5 corresponded to peaks at δ_C 17.386, 17.628, 17.780, and 17.325, respectively. However, the order of the chemical shifts differed from those in the literature [7,8,11]. The carbon signals were confirmed based on cross peaks with corresponding proton signals for C-18, C-19, C-29, and C-30 at δ_H 1.14, 0.93, 1.33, and 0.92, respectively, in the HSQC spectrum (Fig. 2A). Cross peaks were also seen in the HMBC spectrum, with H-26 at δ_H 1.58 showing cross peaks with the carbon signal at δ_C 17.886 (C-27), and H-28 at δ_H 2.04 with the carbon signal at δ_C 17.780 (C-29; Fig. 3A). Methylene proton signals H-15 (δ_H 0.82, 1.48) and H-22 (δ_H 1.75, 2.34) differed from the chemical shifts in the literature [5,8]. These signals were confirmed using the cross peaks with corresponding carbon signals for C-15 at δ_C 30.822 and C-22 at 36.092 in the HSQC spectrum

(Fig. 2B). ^{13}C-NMR measured in DMSO-d_6 showed peaks that were generally shifted upfield compared to those in spectra acquired in pyridine-d_5 [6]. The extent of this shift was 0.29–2.37 ppm. Also, ^{1}H-NMR measured in DMSO-d_6 exhibited peaks shifted upfield compared to those measured in pyridine-d_5 [6]. In particular, oxygen-linked proton atoms H-3, H-6, and H-12 of the aglycone moiety, as well as the hemiacetal proton atoms H-1′, H-1″, and H-1‴ of the sugar moieties, showed chemical shifts of 0.51 ppm for H-3, 0.67 for H-6, 0.60 for H-12, 0.75 for H-1′, 1.36 for H-1″, and 0.72 for H-1‴. Among the eight methyl groups, H-18, H-21, H-28, and H-29 showed the largest shifts upfield of 0.20 ppm, 0.33 ppm, 0.83 ppm, and 0.59 ppm, respectively. The chemical name of ginsenoside Re (1) is 6-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-3β,6α,12β,20β-tetrahydroxydammar-24-ene, and we could completely assign the ^{1}H and ^{13}C-NMR chemical shifts of the compound as in Tables 2 and 3.

The observed chemical shifts of C-18 (δ_C 17.568), C-19 (δ_C 17.757), C-27 (δ_C 17.848), C-29 (δ_C 16.916), and C-30 (δ_C 16.969) in the ^{13}C-NMR spectrum of ginsenoside Rf (2) differed from those in the literature [14]. These shifts were confirmed from cross peaks with corresponding proton signals at δ_H 1.14 for C-18, 0.94 for C-19, 1.62 for C-27, 1.42 for C-29, and 0.81 for C-30 in the HSQC spectrum (Fig. 2C). In addition, in the HMBC spectrum, H-26 at δ_H 1.65 showed a cross peak with the carbon signal at δ_C 17.848 (C-27), and H-28 at δ_H 2.03 with the carbon signal at δ_C 16.916 (C-29; Fig. 3B). The chemical name of ginsenoside Rf (2) is 6-O-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-3β,6α,12β,20β-tetrahydroxydammar-24-ene, and we could completely assign the ^{1}H and ^{13}C-NMR chemical shifts of the compound (Tables 2 and 3).

The methyl carbon atoms C-18, C-19, C-27, C-29, and C-30 of ginsenoside Rg2 (3) in pyridine-d_5 corresponded to peaks at δ_C 17.196, 17.667, 17.757, 17.667, and 16.969, respectively. However, the order of the chemical shifts differed from those in the literature [8,9,13]. The carbon signals were confirmed based on cross peaks with corresponding proton signals δ_H 1.13 for C-18, 0.91 for C-19, 1.59 for C-27, 1.29 for C-29, and 0.89 for C-30, in the HSQC spectrum (Fig. 2D). Carbon signals were also confirmed with the HMBC spectrum with methyl proton signals at δ_H 1.64 (H-26) and δ_H 1.99 (H-28) showing cross peaks with carbon signals at δ_C 17.757 (C-27) and δ_C 17.667 (C-29; Fig. 3C). Also, both methyl proton signals at δ_H 1.59 (H-27) and H-26 correlated with carbon signals at δ_C 126.202 (C-24) and δ_C 130.691 (C-25; Fig. 3D). The chemical name of ginsenoside Rg2 (3) is 6-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-3β,6α,12β,20β-tetrahydroxydammar-24-ene, and we could completely assign the ^{1}H and ^{13}C-NMR chemical shifts of the compound (Tables 2 and 3).

For tetracyclic triterpene glycosides, many of the methine and methylene proton signals overlapped upfield, and many of the oxygenated-methine and oxygenated-methylene proton signals of sugars overlapped in ^{1}H-NMR spectra. Thus, one-dimensional NMR techniques were not useful for identification of those protons. To date, peak assignments in NMR data for tetracyclic triterpene glycosides have been based on previously reported data. However, many of the earlier data might be erroneous because of instrument-resolution limitations. Little NMR data are available for 20-gluco-ginsenoside Rf (4), the chemical name of which is 6-O-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-3β,6α,12β,20β-tetrahydroxydammar-24-ene. In this study, the definite assignment of NMR data of the compound was established for the first time by extensive NMR experiments including correlation spectroscopy, nuclear Overhauser effect spectroscopy, HSQC, and HMBC (Tables 2 and 3).

By normal-phase silica gel TLC ($CHCl_3$–MeOH–H_2O = 65:35:10), R_f values were 0.27 for Re (1), 0.37 for Rf (2), 0.51 for Rg2 (3), and 0.28 for 20-gluco Rf (4). Reverse-phase ODS TLC (MeOH–H_2O = 2:1) yielded R_f values of 0.57, 0.29, 0.13, and 0.65, respectively. In 10% H_2SO_4 with heating, each compound was light purple on TLC. HPLC retention times were 27.1 min for Re (1), 20.6 min for Rf (2), 10.3 min for Rg2 (3), and 30.2 min for 20-gluco Rf (4).

Conflicts of interest

All contributing authors declare no conflicts of interest.

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