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Research Article

Six new dammarane-type triterpene saponins from *Panax ginseng* flower buds and their cytotoxicity

Ke-Ke Li [1,*], Sha-Sha Li [2], Fei Xu [2], Xiao-Jie Gong [1,**]

[1] *Department of Biological Engineering, College of Life Science, Dalian Minzu University, Dalian, China*
[2] *Department of Traditional Chinese Medicine, College of Medical, Dalian University, Dalian, China*

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ABSTRACT

Background: *Panax ginseng* has been used for a variety of medical purposes in eastern countries for more than two thousand years. From the extensive experiences accumulated in its long medication use history and the substantial strong evidence in modern research studies, we know that ginseng has various pharmacological activities, such as antitumor, antidiabetic, antioxidant, and cardiovascular system–protective effects. The active chemical constituents of ginseng, ginsenosides, are rich in structural diversity and exhibit a wide range of biological activities.
Methods: Ginsenoside constituents from *P. ginseng* flower buds were isolated and purified by various chromatographic methods, and their structures were identified by spectroscopic analysis and comparison with the reported data. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide method was used to test their cytotoxic effects on three human cancer cell lines.
Results: Six ginsenosides, namely 6'–malonyl formyl ginsenoside F_1 (**1**), 3β–acetoxyl ginsenoside F_1 (**2**), ginsenoside Rh_{24} (**6**), ginsenoside Rh_{25} (**7**), 7β–hydroxyl ginsenoside Rd (**8**) and ginsenoside Rh_{26} (**10**) were isolated and elucidated as new compounds, together with four known compounds (**3**–**5** and **9**). In addition, the cytotoxicity of these isolated compounds was shown as half inhibitory concentration values, a tentative structure–activity relationship was also discussed based on the results of our bioassay.
Conclusion: The study of chemical constituents was useful for the quality control of *P. ginseng* flower buds. The study on antitumor activities showed that new Compound **1** exhibited moderate cytotoxic activities against HL-60, MGC80-3 and Hep-G2 with half inhibitory concentration values of 16.74, 29.51 and 20.48 μM, respectively.

1. Introduction

Panax ginseng Meyer (Araliaceae) is a precious botanical drug that has been used as a general tonic in eastern countries for more than two thousand years. It is famous for the efficacy in reinforcing vital energy, invigorating spleen for benefiting the lung, and recuperating from diseases. Ginseng saponins (ginsenosides), the major biologically active components of *P. ginseng*, were contributed to its various pharmacological effects and have been administrated to treat cancer [1,2], diabetes [3], cardiovascular disorders [4], aging [5], and so on. Based on the diverse structure differentiation of the sapogenins, the known ginsenosides can be further classified into six different subtypes: the protopanaxadiol (PPD) type, protopanaxatriol type, octillol type, oleanolic acid type, C17 side chain–varied type, and miscellaneous triterpenes. The PPD, protopanaxatriol, oleanolic acid, and octillol types are the four most common subtypes among the saponins from ginseng plant [6,7].

Recent studies have been focusing on the synthesis of more active PPD derivatives from dammarane-type triterpene saponins, conversion products using artificial gastric juice, acidic and alkaline hydrolysis from total ginseng saponins, and extraction of malonylginsenosides under room temperature. The new products obtained have showed promising antidiabetic and antitumor properties [8–13]. Researchers never stop to report new compounds related to ginsenosides [14,15]. In view of the wide range of

* Corresponding author. College of Life Science, Dalian Minzu University, No. 18 Liaohe West Road, Dalian Economic and Technological Development Zone, Dalian 116600, China.
** Corresponding author. College of Life Science, Dalian Minzu University, Dalian 116600, China.
E-mail addresses: like905219@163.com (K.-K. Li), gxjclr@163.com (X.-J. Gong).

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potent activities, ginseng (ginsenosides) can be regarded as the source of active lead compounds research.

Owing to our continued interests in the chemical diversity and structure–bioactivity relationship of ginsenosides [16–21], we investigated the flower buds of *P. ginseng* and obtained 6 new ginsenosides, **1**, **2**, **6**–**8**, and **10**, and 4 known ginsenosides. Herein, we report the isolation and structure elucidation of these new ginsenosides, along with their antiproliferative activities against three human cancer cell lines (HL-60, MGC80-3, and Hep-G2).

2. Materials and methods

2.1. Plant materials

The flower buds of *P. ginseng* were collected in Tonghua city, Jilin province of China in July 2014 and identified by one of the authors (Xiao-Jie Gong). A voucher specimen (No.2014003) of this plant was deposited at our laboratory.

2.2. Apparatus and chemicals

Optical rotation values were measured using a P–2000 digital polarimeter (Jasco, Tokyo, Japan). IR spectra were recorded on a Nicolet 550 FT–IR spectrophotometer (Nicolet Instrument. Inc., Madison, USA) using KBr pellets. NMR spectra were measured uisng a DRX 500 MHz NMR spectrometer (Bruker Corporation, Bremen, Germany) using trimethylsilane as the internal standard. The high-resolution LC–MS spectra were recorded using a QTOF 6540 mass spectrometer (Agilent Technologies, Waldbronn, Germany) and LC–MS–2010EV mass spectrometer (Shimadzu Corporation, Kyoto, Japan). Column chromatography (CC) was performed on silica gel (200–400 mesh, Qingdao Marine Chemical co., Ltd, Qingdao, China), Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan) and MCI gel (75–150 μm, Chengdu Kepubio Co., Ltd, Chengdu, China). TLC was carried out on a precoated silica gel plate High-efficiency Silica Gel (HSG) F_{254} (0.25 mm, Qingdao Marine Chemical Co., Ltd, Qingdao, China); spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. The semi-preparative HPLC was performed on a Beijing CXTH LC–3000 system (Beijing Chuang Xin Tong Heng Science Technology Co. Ltd., Beijing, China) coupled with Daisogel C_{18} column (5 μm, 250 × 30 mm; Cherish Technology Co., Ltd, Beijing, China), and all UV detection was set at 203 nm with a flow rate of 5 mL/min. The sugar determinations were conducted using a 1200 HPLC system (Agilent Technologies) using a Kaseisorb LC NH_2–60–5 column (5 μm, 250 × 4.6 mm; Kasei Kogyo Co. Ltd, Tokyo, Japan). Analytical-grade reagents of *n*-hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$), ethanol (EtOH), and methanol (MeOH) were purchased from Kemiou Pure Chemical Co. Ltd. (Tianjin, China). HPLC-grade acetonitrile (MeCN) and MeOH were obtained from Sigma–Aldrich Chemical Co. (St. Louis, USA). Deionized water was purified using a Milli–Q system (Billerica, USA) in our laboratory.

The human cancer cell lines, HL-60, MCF-7 and Hep-G2, were obtained from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences/Peking Union Medical College (Beijing, China). The cell lines were cultured in an MCO-15 AC carbon dioxide (CO_2) incubator (Sanyo, Osaka, Japan). Dimethyl sulfoxide and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Ninety-six–well plates (Corning Costar) were purchased from Corning Inc. (Cambridge, USA). Nonessential amino acids, fetal bovine serum, Roswell Park Memorial Institute (RPMI) 1640 medium, and Dulbecco's modified Eagle's medium were purchased from Gibcol Laboratories (Grand Island, USA). Penicillin–streptomycin solution was purchased from Corning CellGro (Manassas, USA). Vinorelbine was purchased from Jianglaibio Co. Ltd. (Shanghai, China).

2.3. Extraction and isolation

The extraction of *P. ginseng* flower buds (4.1 kg) was performed with 70% EtOH (3 × 16 L) under reflux. The extract was concentrated on a rotary evaporator to give a dark brown residue (892 g), and then the residue was dissolved in H_2O (4 L) and subjected to liquid–liquid partitioning using *n*–hexane (3 × 12 L) and EtOAc (3 × 12 L) to provide the *n*-hexane (200 g), EtOAc (31 g), and water-soluble (645 g) fractions. The EtOAc fraction was separated on silica gel and eluted with $CHCl_3$/MeOH/H_2O (gradient, 100:10:1 to 10:10:1) to yield fractions E1–E9. Fraction E5 (2.8 g) was chromatographed on an MCI gel column using 75% EtOH to yield 6 subfractions E5A–E5F, and the subfraction E5E (62 mg) was purified by semipreparative HPLC with 80% MeOH to afford **1** (11.4 mg). Similarly, Fraction E6 (900 mg) was separated by 80% EtOH to yield 5 subfractions E6A–E6E, and the subfraction E6D (41 mg) was purified with 75% MeOH to afford **2** (10.2 mg). Fraction E7 (4.8 g) was separated by a gradient of EtOH-H_2O (60% to 100%), and **3** (524 mg) was afforded with 80% EtOH. The water fraction (200 g) was further fractionated on a Diaion HP-20 resin column, eluting with EtOH/H_2O (gradient, 0:100 to 100:0) to yield 5 fractions W1-W5. Fraction W3 (26 g) was separated on an MCI gel column eluting with EtOH/H_2O (gradient, 40:60 to 100:0), producing 11 subfractions W3A–W3K. And, the subfraction W3B (6 g) was separated by MCI gel column eluting with 70% EtOH, giving two subfractions W3B-1 and W3B-2. Semipreparative HPLC was used to purify subfraction W3B-2 (911 mg) to obtain **4** (25.4 mg) and **5** (661 mg). Subfraction W3D (3.2 g) was separated by combining silica gel ($CHCl_3$/MeOH/H_2O 6:1:0.1 to 2:1:0.1) and MCI gel (75% EtOH) column. After the MCI gel fractionation, four (subfraction W3D-1 to subfraction W3D-4) subfractions were obtained. Subfraction W3D-2 (77 mg) and W3D-4 (43 mg) were all further purified by semipreparative HPLC with 35% MeCN to afford **6** (8.6 mg), **7** (9.2 mg), and **8** (7.5 mg). Subfraction W3H (1.4 g) was separated by silica gel ($CHCl_3$/MeOH/H_2O 5:1:0.1) repeatedly to afford **9** (282 mg). Fraction W4 (21 g) was separated by MCI gel column eluting with 80% EtOH, giving 6 subfractions W4A–W4F. Further purification of subfraction W4D (92 mg) by semipreparative HPLC with 35% MeCN afforded **10** (12.5 mg).

2.4. Acid hydrolysis of new compounds

The acid hydrolysis of new compounds and sugar determination were based on our previously reported method by HPLC analysis [16]. All the glucose residues from those 6 new compounds showed the retention time of 12.6 min, which was the same as D-glucose [16]. In addition, the rhamnosyl moiety in **6** and the arabinosyl moiety in **6** and **7** were L-configured (t_R 9.6 and 10.7 min, respectively) [16,22], and the xylosyl moiety in **7** was D-configured (t_R 9.2 min) [22], showing that the retention times were consistent with those of the authentic samples.

2.5. Cytotoxicity assay

HL-60 and MGC80-3 cells were cultured in RPMI 1640 medium, and Hep-G2 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 unit/mL penicillin plus 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cytotoxicity assay was detected by MTT method [23]. MCF-7, Hep-G2, and HL-60 cells were seeded into 96-well plates in a density of 2×10^4, 1×10^4, and 0.5×10^4 cells/well in 100 μL, respectively. After 24 h of incubation at 37°C, test compounds at various concentrations (1, 10, 50, 100, 150, and 200 μM) were added and incubated for 48 h. Continuously, each well was

Table 1
^{1}H- and ^{13}C-NMR data for Compounds **1** and **2** (C_5D_5N, 500 MHz)

No.	1		2	
	δ_C	δ_H	δ_C	δ_H
1	39.4	1.01 (1H, m), 1.72 (1H, m)	38.9	0.91(1H, m), 1.59 (1H, m)
2	28.2	1.87 (2H, m)	23.9	1.71(2H, m)
3	78.6	3.54 (1H, dd, 10.5, 6.5)	81.3	4.76 (1H, t, 9.0)
4	40.4	–	39.2	–
5	61.8	1.22 (1H, d, 10.0)	61.4	1.20 (1H, d, 10.0)
6	67.8	4.42 (1H, m)	67.4	4.35 (1H, d, 11.5)
7	47.5	1.88 (1H, m), 1.96 (1H, m)	47.3	1.86 (1H, m), 1.93 (1H, m)
8	41.3	–	41.2	–
9	50.0	1.57 (1H, m)	49.7	1.50 (1H, m)
10	39.4	–	38.6	–
11	30.8	1.61 (1H, m), 2.09 (1H, m),	30.8	1.62 (1H, m), 2.02 (1H, m)
12	70.2	4.18 (1H, m)	70.3	4.13 (1H, m)
13	49.2	2.02 (1H, m)	49.1	1.98 (1H, m)
14	51.4	–	51.4	–
15	31.0	1.06 (1H, m), 1.55 (1H, m)	30.9	0.91 (1H, m), 1.58 (1H, m)
16	26.7	1.38 (1H, m), 1.86 (1H, m)	26.7	1.35 (1H, m), 1.84 (1H, m)
17	51.6	2.58 (1H, m)	51.8	2.53 (1H, m)
18	17.7	1.12 (3H, s)	17.6	1.07 (3H, s)
19	17.5	1.03 (3H, s)	17.4	0.98 (3H, s)
20	83.5	–	83.4	–
21	22.1	1.62 (3H, s)	22.5	1.63 (3H, s)
22	36.2	1.84 (1H, m), 2.32 (1H, m)	36.1	1.82 (1H, m), 2.40 (1H, m)
23	23.0	2.29 (1H, m), 2.54 (1H, m)	23.4	2.26 (1H, m), 2.50 (1H, m)
24	126.1	5.35 (1H, t, 6.4)	126.0	5.28 (1H, m)
25	131.1	–	131.1	–
26	25.8	1.70 (3H, s)	25.8	1.62 (3H, s)
27	17.8	1.67 (3H, s)	17.9	1.60 (3H, s)
28	32.0	2.00 (3H, s)	31.4	1.65 (3H, s)
29	16.5	1.47 (3H, s)	17.0	1.33 (3H, s)
30	17.5	1.00 (3H, s)	17.4	0.95 (3H, s)
1′	98.0	5.10 (1H, d, 8.0)	98.4	5.19 (1H, d, 7.5)
2′	75.0	3.96 (1H, m)	75.3	4.02 (1H, t, 8.0)
3′	79.2	4.17 (1H, m)	79.3	4.27 (1H, t, 9.0)
4′	71.5	3.92 (1H, m)	71.6	4.21 (1H, t, 9.0)
5′	74.8	3.94 (1H, m)	78.3	3.94 (1H, m)
6′	65.8	4.68 (1H, dd, 11.5, 7.0) 5.05 (1H, br d, 11.5)	62.9	4.33 (1H, br d, 11.5) 4.50 (1H, dd, 11.5, 2.0)
1″	167.0	–	170.9	–
2″	41.7	3.71 (1H, br s), 3.72 (1H, br s)	21.3	2.11 (3H, s)
3″	167.3	–		
4″	52.3	3.67 (3H, s)		

treated with 20 μL of MTT solution (5 mg/mL), and the plates were incubated for another 4 h. Then, 100 μL of dissolving solution (containing 5% isopropanol, 10% sodium dodecyl sulfate, and 0.1% HCl, dissolved in water) was added to dissolve the formazan. The optical density was measured at 540 nm using a microplate reader (BioTeck Instruments, Vermont, US). The half inhibitory concentration (IC_{50}) value of each compound was calculated using the Origin75 software.

3. Results and discussion

3.1. Structure identification

Six new (**1**, **2**, **6**–**8**, and **10**) and four known (**3**–**5** and **9**) dammarane-type saponins were isolated. The four known saponins were identified as ginsenosides F_1 (**3**) [24], Rc (**4**) [25], Re (**5**), and Rd (**9**) [19].

Compound **1** was obtained as a white amorphous powder. [α]25 D+ 25.8° (c = 0.1, MeOH). Its molecular formula was determined as $C_{40}H_{66}O_{12}$, evidenced by the High-Resolution-Electron Spray Ionization-Mass Spectra data (HR-ESI-MS) data (m/z 761.4446 $[M + Na]^+$, calculated for $C_{40}H_{66}O_{12}Na$, 761.4446). The absorption bands at 3321, 1651, and 1015 cm^{-1} due to hydroxyl, ester, and olefin groups, respectively, were shown in its IR spectrum. The acidic hydrolysis of **1** gained D-glucose. One anomeric proton (δ 5.10) in the ^{1}H-NMR spectrum and one corresponding anomeric carbon were recorded at δ 98.0 in the ^{13}C-NMR spectrum (Table 1). Furthermore, nine methyl singlets (δ_H 1.00, 1.03, 1.12, 1.47, 1.62, 1.67, 1.70, 2.00, and 3.67) were observed in the ^{1}H-NMR spectrum, and δ_H 3.67 of which was protons bearing an oxygen function. Thirty carbon signals were assigned to the 20(*S*)-PPT sapogenin [19], and a set of signals showed a β-D-glucosyl moiety in the ^{13}C-NMR spectrum. Compared with **3** [24], the presence of additional signals corresponding to two carbonyl carbons (δ_C 167.0 and 167.3), one methylene group [δ_C 41.7/δ_H 3.72, 3.71 (each 1H, br s)], and one methoxyl group [δ_C 52.3/δ_H 3.67 (3H, s)], along with the Heteronuclear Multiple Bond Correlation (HMBC) cross-peaks of δ_H 5.05, 4.68/δ_C 167.0, δ_H 3.71/δ_C 167.0, δ_H 3.71/δ_C 167.3 and δ_H 3.67/δ_C 167.3 (Fig. 1), as well as a downfield shift for C-6' (δ_C 65.8) of the glucopyranosyl unit, revealed that there was a terminal methyl-esterified malonic acid group attached to C-6′ of glucopyranosyl moiety in **1** [13,26,27]. On the basis of the aforementioned results, the structure of **1** was determined as 3β,6α,12β,20(*S*)-tetrahydroxy-dammar-24-ene-20-*O*-(6-*O*-malonyl methyl ester)-β-D-glucopyranoside and given the trivial name 6′-malonyl methyl ester ginsenoside F_1.

Compound **2** was isolated as a white amorphous powder with a molecular formula $C_{38}H_{64}O_{10}$ determined by HR-ESI-MS (m/z 703.4343 $[M + Na]^+$, calcd for $C_{38}H_{64}O_{10}Na$, 703.4391). [α]25 D+ 28.4° (c = 0.1, MeOH). Its IR spectrum showed strong absorption bands at 3324 and 1012 cm^{-1}, suggesting an oligoglycosidic structure, together with absorption band at 1649 cm^{-1} due to carbonyl group. The acid hydrolysis of **2** liberated D-glucose. The ^{1}H- and ^{13}C-NMR spectra (Table 1) of **2** were very similar to those of **3** [24], except for the signals due to an acetoxyl group at δ_C 21.3 and 170.9 and δ_H 2.11 (3H, s), which suggested that **2** was an acetyl derivative of **3** (Fig. 1). The acetoxyl group was confirmed at C-3 position of aglycone moiety on the basis of an acetylation shift effect on C-3 around δ_C 81.3 and HMBC cross-peaks of H-3 [δ_H 4.76 (1H, t, J = 9.0 Hz)] to carbonyl carbon at δ_C 170.9 and H-2″ [δ_H 2.11 (3H, s)] to C-3 (δ_C 81.3). Thus, the structure of **2** was determined as 6α,12β,20(*S*)-trihydroxy-3β-acetoxyl-dammar-24-ene-20-*O*-β-D-glucopyranoside and named as 3β-acetoxyl ginsenoside F_1.

Compound **6** was isolated as a white amorphous powder with a molecular formula $C_{47}H_{80}O_{17}$ determined by HR-ESI-MS spectrum (m/z 939.5289 ($[M + Na]^+$, calcd for $C_{47}H_{80}O_{17}Na$, 939.5293)). [α]25 D−6.2° (c = 0.1, MeOH). Its IR spectrum showed absorption bands due to hydroxyl and olefin groups at 3412 and 1630 cm^{-1}. The ^{1}H-NMR spectrum of **6** (Table 2) displayed eight methyl singlets [δ_H 0.99, 1.10, 1.18, 1.36, 1.61, 1.62 (6H), and 2.01], a methyl doublet [δ_H 1.77 (3H, d, J = 6.2 Hz)], an olefinic [δ_H 5.26 (1H, t, J = 7.5 Hz)], and three anomeric [δ_H 6.26 (1H, br s), 5.32 (1H, d, J = 5.0 Hz), 5.20 (1H, d, J = 8.0 Hz)]. In the ^{13}C-NMR spectrum of **6**, thirty carbon signals assigned to the aglycone and three sets of signals due to one β-glucopyranosyl, one α-arabinopyranosyl, and one α-rhamnopyranosyl groups were observed. The result of acidic hydrolysis of **6** was consistent with the ^{13}C-NMR findings of sugar moiety type. Comparison of the ^{13}C-NMR chemical shifts of the aglycone moiety in **6** with those of 20(*S*)-protopanaxatriol and its glycosides [19] suggested that **6** (Fig. 1) was a 20(*S*)-PPT ginsenoside, which was confirmed by HMBC analysis. Furthermore, the HMBC correlation between anomeric proton of rhamnosyl δ_H 6.26 (1H, br s) and δ_C 77.8 of C-2′ arabinosyl showed that there was a sugar sequence of α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl in **6**. The ^{1}H- and ^{13}C-NMR data of the sugar moiety were identical to the reported data [28,29]. The linkage sites of the sugars and the aglycone were determined by HMBC correlations of H-1′ [δ_H 5.32 (1H, d, J = 5.0 Hz)] to C-6 (δ_C 75.8) and H-1‴ [δ_H 5.20 (1H, d, J = 8.0 Hz)] to C-20 (δ_C 83.3). Accordingly, the structure of **6** was determined as 6-*O*- [α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3β,6α,

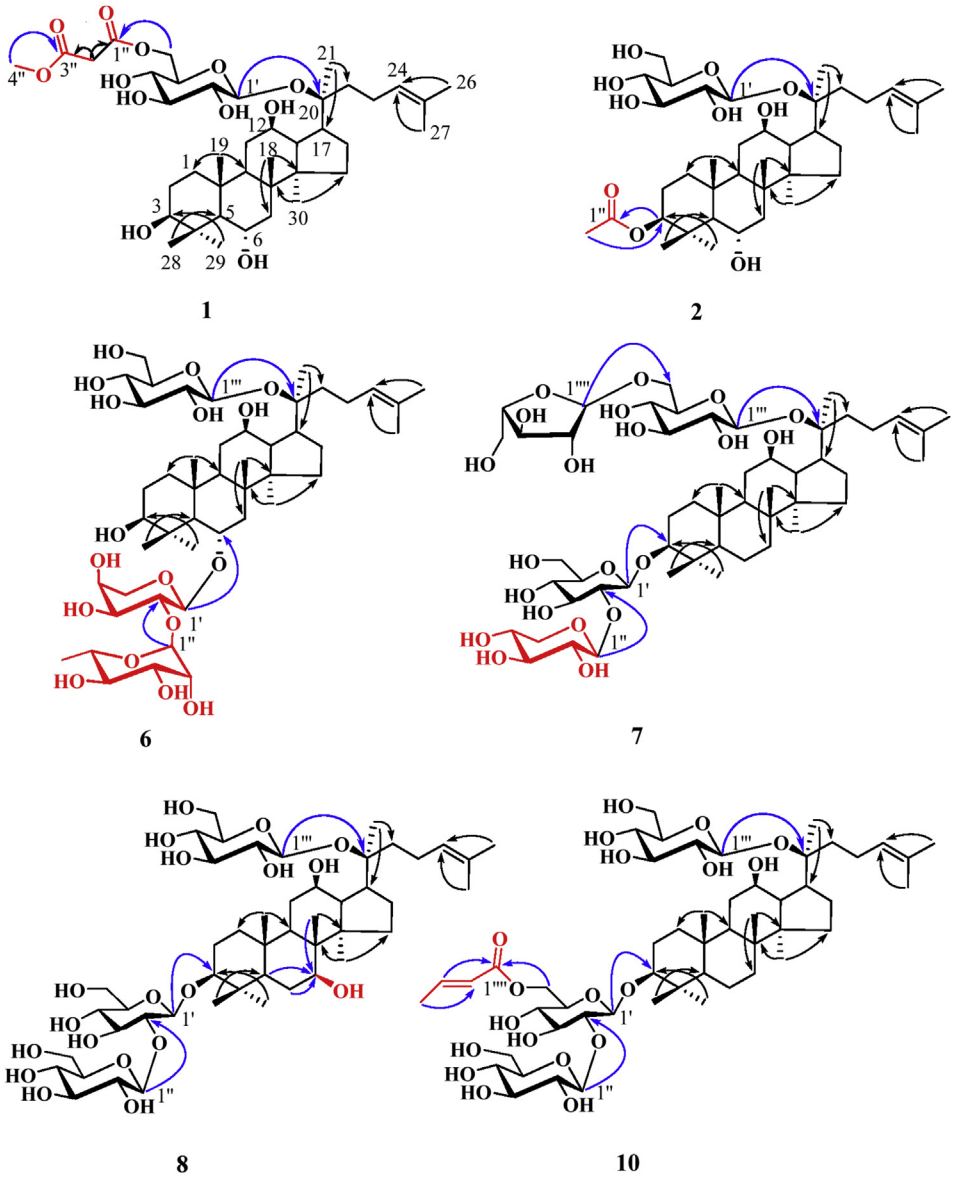

Fig. 1. Chemical structures and HMBC (H⁀C) correlations of Compounds 1, 2, 6–8, and 10. The red part of each structure shows its novelty, and the key HMBC correlations are shown by blue arrows.
HMBC, heteronuclear multiple bond correlation.

12β,20(*S*)- tetrahydroxy-dammar-24-ene-20-*O*-β-D-glucopyranoside and given the trivial name ginsenoside Rh_{24} [30]. So far as we know, the sugars and their the sequence located at C-6 resulted in a new type of sugar chain among all the known ginsenosides in *Panax* genus plants [6,7].

Compound **7** was isolated as a white amorphous powder with a molecular formula $C_{52}H_{88}O_{21}$ determined by HR-ESI-MS (*m/z* 1071.5707 $[M + Na]^+$, calcd for $C_{52}H_{88}O_{21}Na$, 1071.5716). [α]25 D+ 48.2° (*c* = 0.1, MeOH). Its IR spectrum showed absorption bands due to hydroxyl and olefin groups at 3389 and 1646 cm^{-1}. The ^{13}C-NMR spectrum gave 52 carbon signals, of which 22 were assigned to the sugar moiety and 30 to the 20(*S*)-PPD sapogenin [19]. The acidic hydrolysis of **7** obtained D-glucose, D-xylose, and L-arabinose. The ^{1}H- and ^{13}C-NMR data (Table 2) of **7** were very similar to those of **4** [25], except for a xylosyl instead of a glucosyl at C-2′. The xylosyl group at C-2′ was further confirmed by the correlation observed between anomeric proton of xylosyl δ_H 5.27 (1H, d, *J* = 7.0 Hz) and δ_C 84.1 of C-2′ glucosyl that linked at C-3 of the aglycone in the HMBC experiment (Fig. 1). Thus, the structure of **7** was determined as 3-*O*-[β-D-xylopyranoyl-(1→2)-β-D-glucopyranoyl]- 3β,12β,20(*S*)- trihydroxy-dammar-24-ene-20-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside and given the trivial name ginsenoside Rh_{25}.

Compound **8** was obtained as a white amorphous powder. It exhibited the molecular formula $C_{48}H_{82}O_{19}$ according to the HR-ESI-MS data (*m/z* 985.5349 $[M + Na]^+$, calcd for $C_{48}H_{82}O_{19}Na$, 985.5348). [α]25 D+ 51.5° (*c* = 0.1, MeOH). Its IR spectrum showed absorption bands due to hydroxyl and olefin groups at 3397 and 1604 cm^{-1}. Glucose was identified in the acid hydrolysates. Comparison of the ^{1}H- and ^{13}C-NMR data of **8** (Table 3) with those of **9** [19] revealed that some different signals due to the B-ring part of the sapogenin moiety was due to the presence of a hydroxyl group signals at δ_H 4.02/δ_C 74.6. The long-range correlations between H-5 [δ_H 0.84 (1H, br d, *J* = 12.5Hz)], H-6 [1.75 (1H, m), 1.93 (1H, m)], H-18 [δ_H 1.26 (3H, s)], and δ_C 74.6 in the HMBC experiment (Fig. 1), as well as signals for C-6 and C-7, were shifted to lower field at δ_C 29.2 and δ_C 74.6 compared to those of **9** [19], indicating that the original methylene

Table 2
^{1}H- and ^{13}C-NMR data for Compounds **6** and **7** (C_5D_5N, 500 MHz)

No.	6		7	
	δ_C	δ_H	δ_C	δ_H
1	39.4	0.97 (1H, m), 1.72 (1H, m)	39.3	0.76 (1H, m), 1.57 (1H, m)
2	27.8	1.81 (1H, m), 1.87 (1H, m)	26.7	1.37 (1H, m), 1.85 (1H, m)
3	78.4	3.45 (1H, m)	89.1	3.30 (1H, dd, 12.5, 5.0)
4	39.9	–	39.8	–
5	60.9	1.41 (1H, d, 11.0)	56.5	0.71 (1H, d, 11.0)
6	75.8	4.53 (1H, m)	18.5	1.41 (1H, m), 1.55 (1H, m)
7	45.7	2.01 (1H, m) 2.18 (1H, dd, 12.0, 3.0)	35.2	1.21 (1H, m), 1.49 (1H, m)
8	41.2	–	40.1	–
9	49.6	1.55 (1H, m)	50.3	1.40 (1H, m)
10	39.6	–	37.0	–
11	31.0	1.55 (1H, m), 2.09 (1H, m)	30.9	1.50 (1H, m), 1.99 (1H, m)
12	70.2	4.17 (1H, m)	70.3	4.15 (1H, m)
13	49.2	2.07 (1H, m)	49.5	2.01 (1H, t, 11.0)
14	51.4	–	51.5	–
15	30.8	1.02 (1H, m), 1.61 (1H, m)	30.8	1.01 (1H, m), 1.58 (1H, m)
16	26.7	1.36 (1H, m), 1.84 (1H, m)	26.9	1.37 (1H, m), 1.85 (1H, m)
17	51.6	2.56 (1H, m)	51.7	2.56 (1H, m)
18	17.6	1.18 (3H, s)	16.0	0.98 (3H, s)
19	17.3	0.99 (3H, s)	16.3	0.84 (3H, s)
20	83.3	–	83.4	–
21	22.3	1.62 (3H, s)	22.4	1.65 (3H, s)
22	36.2	1.85 (1H, m), 2.39 (1H, m)	36.2	1.85 (1H, m), 2.38 (1H, m)
23	23.2	2.25 (1H, m), 2.50 (1H, m)	23.2	2.38 (1H, m), 2.57 (1H, m)
24	126.0	5.26 (1H, t, 7.5)	126.1	5.32 (1H, t, 7.0)
25	130.9	–	131.0	–
26	25.8	1.61 (3H, s)	25.8	1.63 (3H, s)
27	17.8	1.62 (3H, s)	17.9	1.68 (3H, s)
28	32.1	2.01 (3H, s)	27.8	1.31 (3H, s)
29	17.4	1.36 (3H, s)	16.3	1.12 (3H, s)
30	17.5	1.00 (3H, s)	17.4	0.97 (3H, s)
1′	102.8	5.32 (1H, d, 5.0)	105.1	4.93 (1H, d, 7.5)
2′	77.8	4.42 (1H, m)	84.1	4.16 (1H, m)
3′	75.9	4.33 (1H, m)	78.5	4.31 (1H, m)
4′	70.8	4.22 (1H, m)	71.7	4.16 (1H, m)
5′	66.0	3.83 (1H, dd, 11.4, 8.2) 4.50 (1H, m)	78.2	3.93 (1H, dd, 11.4, 8.2)
6′			63.0	4.33 (1H, m) 4.57 (1H, br d, 11.5)
1″	101.6	6.26 (1H, br s)	107.0	5.27 (1H, d, 7.0)
2″	72.4	4.74 (1H, m)	76.6	4.13 (1H, m)
3″	72.4	4.62 (1H, dd, 9.0, 3.0)	78.2	4.16 (1H, m)
4″	74.1	4.33 (1H, m)	71.2	4.24 (1H, m)
5″	69.8	4.78 (1H, m)	67.6	3.73 (1H, t, 11.0) 4.41 (1H, dd, 11.0, 5.0)
6″	18.8	1.77 (3H, d, 6.2)		
1‴	98.3	5.20 (1H, d, 8.0)	98.2	5.15 (1H, d, 8.0)
2‴	75.2	4.01 (1H, t, 8.0)	75.1	3.98 (1H, m)
3‴	79.3	4.25 (1H, m)	79.3	4.18 (1H, m)
4‴	71.7	4.14 (1H, m)	72.2	3.99 (1H, m)
5‴	78.3	3.94 (1H, m)	76.6	4.04 (1H, m)
6‴	63.0	4.34 (1H, m), 4.50 (1H, m)	68.5	4.11 (1H, m) 4.67 (1H, dd, 11.0, 2.5)
1⁗			110.2	5.66 (1H, br s)
2⁗			83.3	4.87 (1H, m)
3⁗			78.9	4.80 (1H, m)
4⁗			86.1	4.75 (1H, m)
5⁗			62.7	4.22 (1H, m), 4.49 (1H, m)

Table 3
^{1}H- and ^{13}C-NMR data for Compounds **8** and **10** (C_5D_5N, 500 MHz)

No.	8		10	
	δ_C	δ_H	δ_C	δ_H
1	39.2	0.74 (1H, m), 1.56 (1H, m)	39.2	0.76 (1H, m), 1.58 (1H, m)
2	26.9	1.84 (1H, m), 2.23 (1H, m)	26.8	1.87 (1H, m), 2.22 (1H, m)
3	88.9	3.30 (1H, dd, 12.0, 4.5)	89.0	3.30 (1H, dd, 11.5, 4.0)
4	39.6	–	39.7	–
5	54.1	0.84 (1H, d, 12.5)	56.4	0.69 (1H, d, 11.5)
6	29.2	1.75 (1H, m), 1.93 (1H, m)	18.5	1.40 (1H, m), 1.51 (1H, m)
7	74.6	4.02 (1H, m)	35.2	1.23 (1H, m), 1.49 (1H, m)
8	45.9	–	40.1	–
9	50.0	1.39 (1H, m)	50.2	1.40 (1H, m)
10	37.0	–	36.9	–
11	31.0	1.64 (1H, m), 2.03 (1H, m)	30.7	1.50 (1H, m), 1.98 (1H, m)
12	70.2	4.15 (1H, m)	70.1	4.20 (1H, m)
13	50.5	2.07 (1H, m)	49.6	2.01 (1H, m)
14	51.6	–	51.5	–
15	34.5	1.77 (1H, m), 2.15 (1H, m)	31.0	1.03 (1H, m), 1.58 (1H, m)
16	27.2	1.46 (1H, m), 1.94 (1H, m)	26.7	1.39 (1H, m), 1.87 (1H, m)
17	50.9	2.60 (1H, m)	51.6	2.61 (1H, m)
18	10.7	1.26 (3H, s)	16.0	0.96 (3H, s)
19	16.5	0.88 (3H, s)	16.3	0.84 (3H, s)
20	83.6	–	83.5	–
21	22.4	1.66 (3H, s)	22.0	1.62 (3H, s)
22	36.3	1.90 (1H, m), 2.45 (1H, m)	36.1	1.81 (1H, m), 2.41 (1H, m)
23	23.2	2.28 (1H, m), 2.52 (1H, m)	23.0	2.31 (1H, m), 2.58 (1H, m)
24	126.0	5.27 (1H, t, 7.0)	126.1	5.30 (1H, t, 7.5)
25	131.9	–	130.9	–
26	25.8	1.61 (3H, s)	25.8	1.66 (3H, s)
27	17.8	1.62 (3H, s)	17.8	1.66 (3H, s)
28	28.1	1.29 (3H, s)	28.1	1.31 (3H, s)
29	16.8	1.13 (3H, s)	16.6	1.14 (3H, s)
30	17.4	1.17 (3H, s)	17.5	1.00 (3H, s)
1′	105.1	4.93 (1H, d, 7.5)	105.1	4.93 (1H, d, 7.5)
2′	83.6	4.24 (1H, m)	83.6	4.25 (1H, m)
3′	78.0	4.25 (1H, m)	78.0	4.26 (1H, m)
4′	71.7	4.14 (1H, m)	71.7	4.16 (1H, m)
5′	78.3	4.32 (1H, m)	75.0	4.02 (1H, m)
6′	62.9	4.36 (1H, m) 4.58 (1H, br d, 11.5)	64.4	4.75 (1H, dd, 11.5, 7.0) 5.05 (1H, dd, 11.5, 2.0)
1″	106.2	5.37 (1H, d, 7.5)	106.1	5.38 (1H, d, 7.5)
2″	77.2	4.13 (1H, m)	77.2	4.14 (1H, m)
3″	78.2	3.94 (1H, m)	78.1	3.94 (1H, m)
4″	71.7	4.33 (1H, m)	71.7	3.99 (1H, m)
5″	78.1	3.94 (1H, m)	78.3	3.94 (1H, m)
6″	62.9	4.36 (1H, m) 4.52 (1H, dd, 11.5, 2.5)	62.9	4.36 (1H, m) 4.58 (1H, dd, 12.0, 2.0)
1‴	98.3	5.23 (1H, d, 7.5)	98.1	5.14 (1H, d, 7.5)
2‴	75.2	4.04 (1H, t, 8.5)	75.0	4.02 (1H, m)
3‴	79.3	4.26 (1H, m)	79.2	4.21 (1H, m)
4‴	71.7	4.14 (1H, m)	71.7	4.16 (1H, m)
5‴	78.3	3.95 (1H, m)	78.4	3.94 (1H, m)
6‴	62.7	4.36 (1H, m), 4.46 (1H, m)	62.8	4.43 (1H, m), 4.50 (1H, m)
1⁗			166.4	–
2⁗			123.2	5.99 (1H, dt, 15.5, 1.5)
3⁗			144.8	7.07 (1H, dq, 15.5, 6.9)
4⁗			17.7	1.68 (3H, dd, 7.0, 1.5)

at C-7 was transformed to a hydroxyl group. In the rotating frame overhause effect spectroscopy (ROESY) experiment, rotating frame overhause effect (ROE) correlations were observed between H-7 [δ_H 4.02 (1H, m)], H-9 [δ_H 1.39 (1H, m)], and H-7 and H-30 [δ_H 1.17 (3H, s)] (Fig. 2). Thus, the hydroxyl at C-7 was β configuration. Consequently, the structure of **8** was determined as 3-*O*-[β-D- glucopyranoyl-(1→2)-β-D-glucopyranoyl]-3β,7β,12β,20(*S*)-tetrahydroxy-dammar-24-ene-20-*O*-β-D-glucopyranoside and given the trivial name 7β-hydroxyl ginsenoside Rd.

Compound **10** was obtained as a white amorphous powder. It possessed the molecular formula $C_{52}H_{86}O_{19}$, which was determined by the HR-ESI-MS data (*m/z* 1037.5646 $[M + Na]^+$, calcd for $C_{52}H_{86}O_{19}Na$, 1037.5661). [α]25 D+ 40.4° (*c* = 0.1, MeOH). The IR spectrum of **10** showed absorption bands at 1728 and 1655 cm^{-1} ascribed to an α,β-unsaturated ester and olefin and strong absorption bands at 3337 and 1073 cm^{-1} suggestive of an oligoglycosidic structure. Glucose was identified in the acid hydrolyzates. The ^{1}H- and ^{13}C-NMR data (Table 3) of **10** were very similar to those of **9** [19], except for the additional signals, i.e., a methyl group δ_C 17.7/δ_H 1.68, a double bond δ_C 144.8, 123.2/δ_H 7.07, 5.99, and a carbonyl carbon δ_C 166.4. Further analysis confirmed that these signals were assigned to a crotonic group. Based on the typical *AB*-system coupling constant (*J* = 15.5 Hz) of δ_H 7.07 and δ_H 5.99, the configuration of the crotonic unit was determined as (*E*). By comparing the ^{13}C-NMR

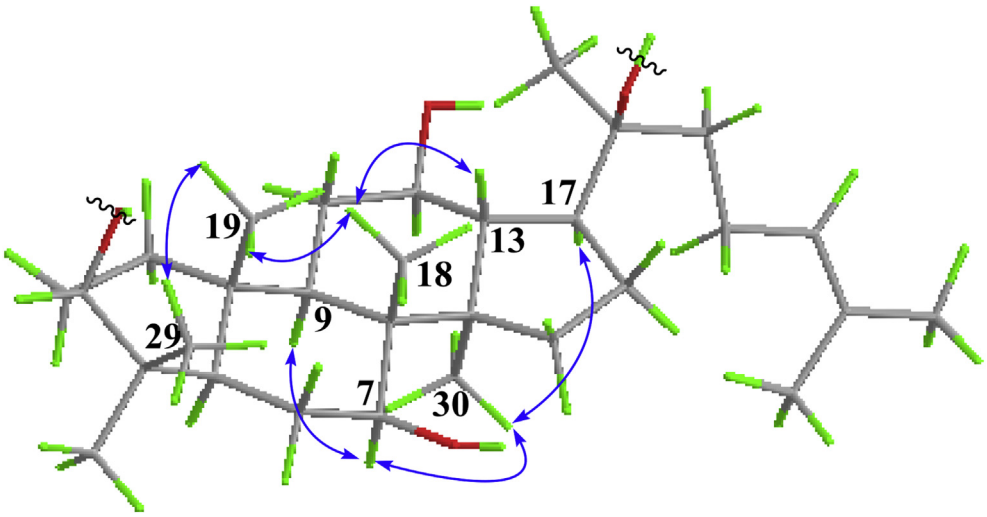

Fig. 2. Selected key ROESY correlations of the sapogenin of Compound **8**.

Table 4
Cytotoxicity of Compounds **1**–**10** against three human cancer cell lines (IC_{50}, μM)

Compounds	HL-60	MGC80-3	Hep-G2
1	16.74 ± 2.65	29.51 ± 0.82	20.48 ± 1.83
2	58.31 ± 3.32	42.18 ± 2.49	25.62 ± 0.87
3	65.36 ± 1.35	87.58 ± 1.76	82.14 ± 3.88
4	>200	>200	>200
5	>200	>200	>200
6	174.12 ± 1.93	168.14 ± 3.36	>200
7	>200	>200	>200
8	122.63 ± 2.46	158.83 ± 2.05	>200
9	>200	>200	>200
10	105.51 ± 4.84	82.37 ± 4.58	156.74 ± 2.61
Vinorelbine	9.36 ± 2.77	25.24 ± 3.18	18.86 ± 1.19

Data were expressed as mean ± SD ($n = 3$). Vinorelbine was used as a positive drug.
IC_{50}, half inhibitory concentration; SD, standard deviation.

data with those of **9** [19], the terminal C-6′ resonance was deshielded, whereas that of C-5′ was shielded. The malonylation site at 6′-OH was confirmed by the HMBC cross-peaks of H-6′ [δ_H 4.75 (1H, dd, $J = 11.5$, 7.0 Hz) and δ_H 5.05 (1H, dd, $J = 11.5$, 2.0 Hz)] to the carbonyl carbon at δ_C 166.4 (Fig. 1). The structure of **10** was thus defined as 3-*O*-{6-*O*-[(*E*)-but-2-enoyl]-*β*-D-glucopyranoyl-(1→2)-*β*-D-glucopyranoyl}-3*β*,12*β*, 20(*S*)-trihydroxy-dammar-24-ene-20-*O*-*β*-D-glucopyranoside and given the trivial name ginsenoside Rh_{26}. Its analogs with C-6″ crotonyl group have been isolated from the roots of *P. ginseng* and *P. quinquefolium* [26,31]. To the best of our knowledge, **10** that possesses crotonyl group is the first ginsenoside isolated from *P. ginseng* flower buds.

3.2. MTT assay

Ginsenosides have attracted increasing interest because of its promotion of tumor apoptosis and prevention of tumor invasion and metastasis [32–34]. To find more potent antitumor agents from *P. ginseng* flower buds, in this study, we evaluate the antitumor activity of the isolated compounds against three human cancer cell lines including human leukemia cell line HL-60, human gastric cancer cell line MGC80-3, and human hepatoma cell line Hep-G2 in an MTT assay, using vinorelbine as a positive control.

As can be seen from the data expressed in the IC_{50} values in Table 4, Compound **7** did not affect the cell proliferation of all the three cell lines even at the highest test concentration of 200 μM, whereas the other new compounds displayed antiproliferative activity at wide different concentrations. The direct substitution of malonyl or acetyl at C-6′ or C-3 position to Compounds **1**, **10,** or **2** could increase the antiproliferative activity, by comparing Compounds **1** and **2** with **3**, **9** and **10**. The IC_{50} value of Compound **1** was about threefold to fourfold higher than that of Compound **3** on the three cell lines, whereas Compound **10** showed minor cytotoxicity. Additional 7*β*-hydroxyl at C-7 position did not significantly increase antiproliferative potency, as shown in Compounds **8** and **9**. Comparing with Compounds **1**–**3** with one sugar moiety, the antiproliferative effects of Compounds **4**–**10** with 3 or 4 sugar moieties were increased with the decreasing number of sugar moieties in a ginsenoside molecule, which was consistent with the literature [1]. And, the changes in the sugar type of the sugar moieties had a slight effect on the antitumor activity, which can be seen between Compounds **4** and **7** and between **5** and **6**.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.12.008.

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