J Ginseng Res 40 (2016) 245–250

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

# Complete $^{1}$H-NMR and $^{13}$C-NMR spectral assignment of five malonyl ginsenosides from the fresh flower buds of *Panax ginseng*

Yu-Shuai Wang [1], Yin-Ping Jin [1], Wei Gao [1], Sheng-Yuan Xiao [1,2], Yu-Wei Zhang [1], Pei-He Zheng [1], Jia Wang [1], Jun-Xia Liu [1], Cheng-He Sun [1], Ying-Ping Wang [1,*]

[1] *Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Changchun, China*
[2] *College of Life Science of Beijing Institute of Technology, Beijing, China*

ARTICLE INFO

*Article history:*
Received 3 March 2015
Received in Revised form
5 August 2015
Accepted 6 August 2015
Available online 13 August 2015

*Keywords:*
flower buds
ginsenoside
malonyl ginsenoside Re
NMR
*Panax ginseng*

ABSTRACT

*Background:* Ginsenosides are the major effective ingredients responsible for the pharmacological effects of ginseng. Malonyl ginsenosides are natural ginsenosides that contain a malonyl group attached to a glucose unit of the corresponding neutral ginsenosides.
*Methods:* Medium-pressure liquid chromatography and semipreparative high-performance liquid chromatography were used to isolate purified compounds and their structures determined by extensive one-dimensional- and two-dimensional nuclear magnetic resonance (NMR) experiments.
*Results:* A new saponin, namely malonyl-ginsenoside Re, was isolated from the fresh flower buds of *Panax ginseng*, along with malonyl-ginsenosides Rb1, Rb2, Rc, Rd. Some assignments for previously published $^{1}$H- and $^{13}$C-NMR spectra were found to be inaccurate.
*Conclusion:* This study reports the complete NMR assignment of malonyl-ginsenoside Re, $Rb_1$, $Rb_2$, Rc, and Rd for the first time.


## 1. Introduction

*Panax ginseng* Meyer is one of the most widespread traditional drugs used in China for thousands of years to produce various pharmacological and biological effects. The most important components contributing to its multiple medicinal properties are the ginsenosides, a group of triterpenoid saponins. Up to now, > 150 ginsenosides have been isolated from *Panax* species [1]. Among these known compounds, malonyl ginsenosides (M-Rs; e.g., m-$Rb_1$, m-$Rb_2$, m-Rc, and m-Rd) are natural ginsenosides that exist in both fresh and air-dried ginseng and contain a malonyl residue attached at the 6-position of a glucosyl unit of the corresponding neutral ginsenoside [2,3]. Malonyl ginsenosides are considered an important form of ginsenoside in white ginseng, however, they are unstable and readily demalonylated or decarboxylated to their respective counterparts or acetylates by treatment with hot water or hot methanol [3–6].

Because malonyl ginsenosides are thermally unstable, their monomeric compounds are hard to obtain, although up to 20 malonyl ginsenosides have been detected by liquid chromatography/quadropole time-of-flight mass spectrometry [7]. Only six malonyl ginsenosides have been isolated and characterized [8–11]. Kitagawa et al [8] and Yamaguchi et al [9] reported the presence of malonyl ginsenosides Rb1, Rb2, Rc, and Rd in both *P. ginseng* and *P. quinquefolius* [8,9]. Sun et al [10] and Ruan et al [11] isolated malonyl notoginsenoside $R_4$ and malonyl-ginsenoside $Ra_3$ from the fresh roots of *P. ginseng*, respectively [10,11]. All previously isolated malonyl ginsenosides were derived from protopanoxadiol (PPT)-type ginsenosides [12].

In this study, we isolated five malonyl ginsenosides from the flower buds of *P. ginseng* and malonyl-ginsenoside Re (M-Re) was obtained as a PPT-type malonyl ginsenoside for the first time. Identification and characterization of ginsenosides are usually conducted using nuclear magnetic resonance (NMR) analyses , but several imperfections and/or inaccuracies existed in the published NMR data of malonyl ginsenosides given the lack of two-dimensional (2D) NMR techniques at the time of characterization. Here, with the help of modern 2D NMR techniques including

* Corresponding author. Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, 4899 Juye Street, Changchun 130112, China.
*E-mail address:* yingpingw@126.net (Y.-P. Wang).

p1226-8453 e2093-4947/$ – see front matter 
http://dx.doi.org/10.1016/j.jgr.2015.08.003

correlation spectroscopy, rotating frame nuclear Overhauser effect spectroscopy (ROESY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-band coherence (HMBC) experiments, complete NMR assignments of malonyl-ginsenosides $Rb_1$, $Rb_2$, Rc, Rd, and Re were determined for the first time.

## 2. Materials and methods

### 2.1. General experimental procedure

Medium-pressure liquid chromatography (MPLC) purifications were carried out on a Yamazen YFLC-AI-580 instrument (Yamazen Co., Osaka, Japan) equipped with silica gel columns (Hi-Flash columns, silica gel: 40 μm, 26 mm × 150 mm internal diameter column). Reversed-phase semipreparative high-performance liquid chromatography (HPLC) was performed on an instrument consisting of Prostar/Dynamax system control, a Varian PS-218 pump, and a Prostar 325 UV-Vis detector with a Varian Polaris C18-A semipreparative column (250 mm × 10 mm, 10 μm; Agilent Technologies, Santa Clara, CA, USA). Thin-layer chromatography (TLC) was performed using a silica gel 60 RP-18 $F_{254S}$ and Kieselgel 60 $F_{254}$, with spots detected by spraying 10% $H_2SO_4$ in ethanol followed by heating at 105oC. HPLC was carried out using an Agilent TC-C18 column (5 μm, 250 mm × 4.6 mm; Agilent Technologies) and products were eluted with a step-wise gradient at a flow rate of 1.0 mL/min using solvent A (water containing 0.0005% ammonium hydroxide and 0.02% ammonium acetate) and solvent B (acetonitrile). The elution rate using solvent B was 17.5% for 0–4 min, 17.5–28.9% for 4–9 min, 28.9–40% for 9–19 min, and 40% for 19–24 min.

The $^1$H-, $^{13}$C-, and 2D-NMR spectra were measured using a Bruker AV600 NMR spectrometer (Bruker Co., Karlsruhe, Germany; 600 MHz for $^1$H and 150 MHz for $^{13}$C) with tetramethylsilane as an internal standard. Chemical shifts ($\delta$) are expressed in ppm, with the coupling constants ($J$) reported in Hertz (Hz). The electrospray ionization mass spectrometry (ESI-MS) and high-resolution electrospray ionization mass spectrometry (HRESIMS) spectra were recorded using an Agilent 1200 HPLC with a 6300 Ion-trap liquid chromatography/mass spectrophotometry (LC/MS; Agilent Technologies; ionization mode, negative; nebulizing gas [$N_2$] pressure, 35 psi; drying gas [$N_2$] flow, 8 L/min; temp, 350oC) and Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany), respectively. For the automated MS/MS analysis, the collision energy was optimized automatically from 30% to 200% of 1.0 V and the collision time was 20 ms. Gas chromatography (GC) was performed using the Agilent 7890A GC with flame ionization detector and a HP-5 chiral capillary column (30 m × 0.25 mm; film thickness, 0.25 μm; Agilent Technologies). Column temperatures started at 170oC and increased to 200oC at 3oC/min, then increased further to 220oC at 0.8oC/min. Inlet temperature was set to 270oC, with hydrogen carrier gas and a 1/15 split, and $N_2$ was used as the carrier gas (1.0 mL/min flow rate). The infrared (IR) spectra were recorded on a Bruker Vertex 70 FT-IR spectrophotometer (Bruker Co., Ettlingen, Germany) using potassium bromide pellets.

### 2.2. Plant material

The fresh flower buds of *P. ginseng* were collected from Fu-Song, Jilin, China, in May 2014, and authenticated by one of the authors, Professor Shi-quan Xu. A voucher of the specimen collected (ZYC-RS-20131008) was deposited in the conditions of −20oC at the Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences.

### 2.3. Extraction and isolation

The fresh flower buds of *P. ginseng* (2.0 kg) were extracted five times with 80% methanol, a 6× dilution of the extracting solution was subjected to a nanofiltration membrane (ESNA1-K1-8040, Hydranautics Corporation, USA) to eliminate most of the pigment, and the filtrate (96.8 g) subjected to column chromatography on a porous polymer polystyrene resin (AB-8). After washing the column with eight column volumes of distilled water, elution was carried out with 30% and 60% aqueous ethanol, and finally with 100% ethanol. The fraction eluted with 30% ethanol (8.9 g) was loaded onto a MPLC system and eluted with $CH_2Cl_2$-MeOH-$H_2O$ (5:1:0.1-4:1:0.1-3:1:0.1) to yield six fractions (AG1-6). Fraction AG4 (2.2 g) was further separated using semipreparative reversed-phase HPLC and eluted with $CH_3CN$-$H_2O$ (1:4) at 3 mL/min to yield malonyl-ginsenoside Re (30 mg; $t_R$ 25.2 min). The fraction eluted with 60% ethanol (48.0 g) was processed on a MPLC system using a linear gradient elution (7 mL/min) of 25–45% methanol in $CH_2Cl_2$ for 250 min in order to collect fraction BG1-9. M-$Rb_1$, M-$Rb_2$, M-Rc, and M-Rd were primarily distributed within fraction BG8 through analysis by LC/MS. Fraction BG8 (7.8 g) was then applied to semipreparative reversed-phase HPLC using a linear gradient elution (3 mL/min) of 29–34% acetonitrile in water for 50 min to yield M-$Rb_1$ (21 mg; $t_R$ 19.7 min), M-$Rb_2$ (18 mg; $t_R$ 24.0 min), M-Rc (22 mg; $t_R$ 29.8 min), and M-Rd (27 mg; $t_R$ 43.9 min; Fig. 1).

### 2.4. Characterization of compounds 1–5

Compound 1 was obtained as a white amorphous powder and gave peaks at *m/z* 1,031.4 $[M-H]^-$, 987.6 $[M-H-CO_2]^-$, 945.4 $[M-COCH_2COOH]^-$, 927.8 $[M-COCH_2COOH-H_2O]^-$, 783.7 $[M-COCH_2-COOH-glu]^-$, 637.5 $[M-COCH_2COOH-rha-glu]^-$, and 475.3 $[M-CO_2-CH_3COOH-rha-2glu]^-$ in negative-mode ESI-MS, indicating its molecular weight to be 1,032. HRESIMS: *m/z* 1,055.5391 $[M+Na]^+$ (calculated for $C_{51}H_{84}NaO_{21}$, 1,055.5397). IR $\nu_{max}$ was 3,408, 2,932, 1,731, 1,636, 1,599, 1,454, 1,385, 1,075, and 1,050 $cm^{-1}$. Libermann-Buchard and Molish reactions were positive. Eight methyl groups and six quaternary carbons were identified in the analysis of the NMR spectrum (Tables 1 and 2). Molish reaction was used to proof the existence of saccharides, and test of Libermann-Buchard for steroids or triterpenes.

Compound 2 was obtained as a white amorphous powder. The molecular formula was determined as $C_{57}H_{94}O_{26}$ based on HRESIMS data at *m/z* 1,217.5921 $[M+Na]^+$ (calculated for $C_{56}H_{92}NaO_{25}$,

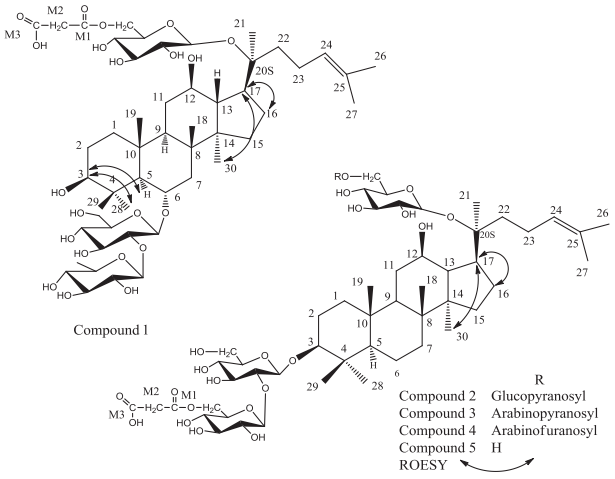


**Fig. 1.** Structures of compounds 1–5.

**Table 1**
The $^{13}$C-NMR data of compounds 1–5 (150 MHz, pyridine-$d_5$, $\delta_C$)

| C | Compound 1 | Compound 2 | Compound 3 | Compound 4 | Compound 5 |
|---|---|---|---|---|---|
| 1 | 39.859 | 39.679 | 39.654 | 39.637 | 39.645 |
| 2 | 28.202 | 27.087 | 27.105 | 27.082 | 27.239 |
| 3 | 78.880 | 89.735 | 89.707 | 89.713 | 89.716 |
| 4 | 40.448 | 40.209 | 40.187 | 40.193 | 40.190 |
| 5 | 61.297 | 56.945 | 56.927 | 56.924 | 56.922 |
| 6 | 75.122 | 18.910 | 18.898 | 18.903 | 18.905 |
| 7 | 46.388 | 35.604 | 35.596 | 35.579 | 35.599 |
| 8 | 41.652 | 40.500 | 40.485 | 40.474 | 40.487 |
| 9 | 50.027 | 50.688 | 50.666 | 50.645 | 50.649 |
| 10 | 40.117 | 37.381 | 37.370 | 37.365 | 37.367 |
| 11 | 31.372 | 31.276 | 31.229 | 31.233 | 31.335 |
| 12 | 70.593 | 70.689 | 70.657 | 70.716 | 70.683 |
| 13 | 49.520 | 49.969 | 49.926 | 49.885 | 49.914 |
| 14 | 51.937 | 52.090 | 51.858 | 51.867 | 51.890 |
| 15 | 31.166 | 31.162 | 31.154 | 31.153 | 31.227 |
| 16 | 27.153 | 27.253 | 27.244 | 27.244 | 27.110 |
| 17 | 51.873 | 51.852 | 52.130 | 52.114 | 52.117 |
| 18 | 17.742 | 16.475 | 16.463 | 16.434 | 16.427 |
| 19 | 18.087 | 16.757 | 16.747 | 16.746 | 16.760 |
| 20 | 83.925 | 83.918 | 83.954 | 83.807 | 83.783 |
| 21 | 22.483 | 22.867 | 22.788 | 22.822 | 22.851 |
| 22 | 36.513 | 36.644 | 36.617 | 36.590 | 36.571 |
| 23 | 23.468 | 23.674 | 23.668 | 23.629 | 23.691 |
| 24 | 126.501 | 126.438 | 126.406 | 126.484 | 126.408 |
| 25 | 131.495 | 131.506 | 131.552 | 131.468 | 131.376 |
| 26 | 26.257 | 26.261 | 26.246 | 26.243 | 26.225 |
| 27 | 18.272 | 18.412 | 18.333 | 18.325 | 18.232 |
| 28 | 32.662 | 28.515 | 28.510 | 28.511 | 28.512 |
| 29 | 17.951 | 16.977 | 16.977 | 16.974 | 16.978 |
| 30 | 17.769 | 17.875 | 17.847 | 17.824 | 17.828 |
| | 6-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl |
| 1’ | 102.340 | 105.350 | 105.337 | 105.342 | 105.342 |
| 2’ | 79.083 | 84.641 | 84.507 | 84.512 | 84.419 |
| 3’ | 79.850 | 78.549 | 78.523 | 78.532 | 78.501 |
| 4’ | 73.026 | 72.162 | 72.263 | 72.556 | 72.026 |
| 5’ | 78.756 | 78.268 | 78.210 | 78.215 | 78.170 |
| 6’ | 63.538 | 63.270 | 63.238 | 63.227 | 63.261 |
| | 2'-*O*-Rhamnosyl | 2'-*O*-Glucopyranosyl | 2'-*O*-Glucopyranosyl | 2'-*O*-Glucopyranosyl | 2'-*O*-Glucopyranosyl |
| 1’’ | 102.340 | 106.586 | 106.535 | 106.531 | 106.483 |
| 2’’ | 72.867 | 77.187 | 77.196 | 77.185 | 77.179 |
| 3’’ | 72.729 | 79.720 | 79.650 | 79.684 | 79.670 |
| 4’’ | 74.607 | 71.864 | 71.843 | 71.826 | 71.803 |
| 5’’ | 69.951 | 75.867 | 75.874 | 75.867 | 75.860 |
| 6’’ | 19.206 | 65.624 | 65.495 | 65.523 | 65.432 |
| | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl |
| 1’’’ | 98.471 | 98.555 | 98.592 | 98.565 | 98.734 |
| 2’’’ | 75.434 | 75.330 | 75.365 | 75.500 | 75.596 |
| 3’’’ | 79.443 | 78.862 | 78.910 | 78.906 | 78.869 |
| 4’’’ | 71.942 | 71.459 | 71.444 | 71.451 | 71.431 |
| 5’’’ | 75.389 | 77.530 | 77.196 | 77.005 | 78.729 |
| 6’’’ | 65.755 | 70.622 | 69.671 | 68.934 | 63.192 |
| | | 6’’’-*O*-Glucopyranosyl | 6’’’-*O*-Arabinopyranosyl | 6’’’-*O*-Arabinofuranosyl | |
| 1’’’’ | | 105.832 | 105.109 | 110.587 | |
| 2’’’’ | | 75.725 | 72.602 | 83.858 | |
| 3’’’’ | | 78.813 | 74.587 | 79.288 | |
| 4’’’’ | | 72.056 | 69.046 | 86.464 | |
| 5’’’’ | | 78.945 | 66.072 | 63.098 | |
| 6’’’’ | | 63.270 | | | |
| Malonyl | | | | | |
| M1 | 169.308 | 169.385 | 169.72 | 169.651 | 169.761 |
| M2 | 44.401 | 44.176 | 44.624 | 44.486 | 44.801 |
| M3 | 171.075 | 171.039 | 171.403 | 171.276 | 171.398 |

C, carbon; NMR, nuclear magnetic resonance.

1,217.5925). Negative-mode ESI-MS (*m/z*) readings: 1,193.4 $[M-H]^-$, 1,149.5 $[M-H-CO_2]^-$, 1,107.4 $[M-COCH_2COOH]^-$, 1,089.5 $[M-COCH_2COOH-H_2O]^-$, 945.5 $[M-COCH_2COOH-glu]^-$, 783.2 $[M-COCH_2COOH-2glu]^-$, 621.1 $[M-COCH_2COOH-3glu]^-$, and 459.3 $[M-COCH_2COOH-4glu]^-$. IR $\nu_{max}$: 3,383, 2,937, 1,724, 1,638, 1,454, 1,383, and 1,076. Libermann-Burchard and Molish reactions were positive. The $^{13}$C-NMR spectrum and $^1$H-NMR data are shown in Tables 1 and 2. The IR spectrum showed absorption bands for hydroxyl (3,383 $cm^{-1}$), carbonyl (1,724 $cm^{-1}$), double bond (1,638 $cm^{-1}$), methyl (1,383 $cm^{-1}$) and ether moiety (1,076 $cm^{-1}$).

Compound 3 was obtained as a white amorphous powder. The molecular formula was determined as $C_{56}H_{92}O_{25}$ based on HRESIMS data at *m/z* 1,187.5826 $[M+Na]^+$ (calculated for $C_{56}H_{92}NaO_{25}$, 1,187.5820). Negative-mode ESI-MS (*m/z*) readings: 1,163.4 $[M-H]^-$, 1,119.2 $[M-H-CO_2]^-$, 1,077.1 $[M-COCH_2COOH]^-$, 1,059.1 $[M-COCH_2COOH-H_2O]^-$, 945.2 $[M-COCH_2COOH-Ara(p)]^-$, 783.2 [M-

**Table 2**
The $^1$H-NMR data of compounds 1–5 (600 MHz, pyridine-$d_5$, $\delta_H$, $J$ in Hz)

| H | Compound 1 | Compound 2 | Compound 3 | Compound 4 | Compound 5 |
|---|---|---|---|---|---|
| 1 | 0.93, 1.63 | 0.73, 1.51 | 0.71, 1.52 | 0.70, 1.50 | 0.71, 1.49 |
| 2 | 1.74, 1.83 | 1.80, 2.16 | 1.81, 2.16 | 1.81, 2.16 | 1.79, 2.13 |
| 3 | 3.43 (dd, 4.6, 11.5) | 3.24 (dd, 4.3, 11.8) | 3.23 (dd, 4.3, 11.8) | 3.22 (dd, 4.3, 11.7) | 3.23 (dd, 4.3, 11.5) |
| 5 | 1.36(d, 10.7) | 0.67 | 0.66 | 0.65 | 0.66 |
| 6 | 4.65 | 1.47, 1.36 | 1.34, 1.46 | 1.46, 1.34 | 1.47, 1.36 |
| 7 | 1.96, 2.22 | 1.13, 1.45 | 1.20, 1.45 | 1.14, 1.43 | 1.14, 1.43 |
| 9 | 1.48 | 1.33 | 1.32 | 1.33 | 1.34 |
| 11 | 1.47, 2.02 | 1.53, 1.96 | 1.52, 1.95 | 1.48, 1.95 | 1.52, 1.94 |
| 12 | 4.11 | 4.30 | 4.11 | 4.16 | 4.10 |
| 13 | 1.92 | 1.97 | 1.94 | 1.95 | 1.94 |
| 15 | 0.84, 1.44 | 0.96, 1.53 | 0.96, 1.52 | 0.96, 1.34 | 0.97, 1.53 |
| 16 | 1.21, 1.74 | 1.36, 1.81 | 1.34, 1.80 | 1.34, 1.80 | 1.34, 1.79 |
| 17 | 2.46 | 2.56 | 2.54 | 2.52 | 2.52 |
| 18 | 1.15 (s) | 0.94 (s) | 0.93 (s) | 0.92 (s) | 0.93 (s) |
| 19 | 0.93 (s) | 0.81 (s) | 0.79 (s) | 0.79 (s) | 0.79 (s) |
| 21 | 1.54 (s) | 1.58 (s) | 1.59 (s) | 1.61 (s) | 1.59 (s) |
| 22 | 1.73, 2.32 | 1.81, 2.37 | 1.80, 2.35 | 1.80, 2.34 | 1.80, 2.34 |
| 23 | 2.30, 2.49 | 2.37, 2.55 | 2.35, 2.54 | 2.34, 2.53 | 2.22, 2.46 |
| 24 | 5.28 (t-like) | 5.29 (t-like ) | 5.28 (t-like) | 5.28 (t-like) | 5.21 (t, 6.9) |
| 26 | 1.62 (s) | 1.63 (s) | 1.61 (s) | 1.61 (s) | 1.57 (s) |
| 27 | 1.63(s) | 1.63 (s) | 1.63 (s) | 1.64 (s) | 1.59 (s) |
| 28 | 2.07 (s) | 1.32 (s) | 1.31 (s) | 1.31 (s) | 1.30 (s) |
| 29 | 1.33 (s) | 1.14 (s) | 1.12 (s) | 1.11 (s) | 1.11 (s) |
| 30 | 0.93 (s) | 0.92 (s) | 0.91 (s) | 0.92 (s) | 0.91 (s) |
| | 6-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl | 3-*O*-Glucopyranosyl |
| 1’ | 5.22 (d, 6.8) | 4.88 (d, 7.7) | 4.87 (d, 7.6) | 4.87 (d, 7.6) | 4.86 (d,7.6) |
| 2’ | 4.35 | 4.13 | 4.13 | 4.14 | 4.12 |
| 3’ | 4.29 | 4.15 | 4.23 | 4.16 | 4.27 |
| 4’ | 4.19 | 4.01 | 4.01 | 3.95 | 4.12 |
| 5’ | 3.92 | 3.90 | 3.86 | 4.26 | 3.85 |
| 6’ | 4.33, 4.47 | 4.33, 4.51 | 4.32, 4.51 | 4.30, 4.51 | 4.29, 4.43 |
| | 2’-*O*-Rhamnosyl | 2’-*O*-Glucopyranosyl | 2’-*O*-Glucopyranosyl | 2’-*O*-Glucopyranosyl | 2’-*O*-Glucopyranosyl |
| 1’’ | 6.46 (brs) | 5.28 (d, 7.3) | 5.27 (d, 7.63) | 5.27 (d, 7.63) | 5.27 (d, 7.6) |
| 2’’ | 4.77 (brs) | 4.02 | 4.03 | 4.07 | 4.06 |
| 3’’ | 4.64 | 4.15 | 4.13 | 4.16 | 4.15 |
| 4’’ | 4.30 | 4.20 | 4.21 | 4.26 | 4.18 |
| 5’’ | 4.90 (dt, 6.2, 9.3 ) | 4.03 | 4.02 | 4.00 | 3.98 |
| 6’’ | 1.74 (d, 6.1) | 4.97 | 4.96 | 4.95 | 4.94 |
| | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl | 20-*O*-Glucopyranosyl |
| 1’’’ | 5.04 (d, 7.7) | 5.11 (d, 7.8) | 5.10 (d, 7.7) | 5.11 (d, 7.7) | 5.16 (d, 7.7) |
| 2’’’ | 3.93 | 3.90 | 3.89 | 3.94 | 3.97 |
| 3’’’ | 4.13 | 4.27 | 4.26 | 3.87 | 3.88 |
| 4’’’ | 3.95 | 4.04 | 4.13 | 4.13 | 3.98 |
| 5’’’ | 3.96 | 4.09 | 3.98 | 4.09 | 3.88 |
| 6’’’ | 4.71 (dd, 5.6, 11.1), 4.98 (d, 5.6) | 4.19, 4.70 | 4.21, 4.65 | 4.00, 4.63 | 4.44, 4.51 |
| | | 6’’’-*O*-Glucopyranosyl | 6’’’-*O*-Arabinopyranosyl | 6’’’-*O*-Arabinofuranosyl | |
| 1’’’’ | | 5.08 (d, 7.7) | 4.96 (d,5.94) | 5.64 (d, 1.4) | |
| 2’’’’ | | 4.03 | 4.42 | 4.84 | |
| 3’’’’ | | 4.17 | 4.19 | 4.77 | |
| 4’’’’ | | 4.26 | 4.34 | 4.72 | |
| 5’’’’ | | 4.16 | 4.27,3.77 | 4.17,4.27,4.33 | |
| 6’’’’ | | 4.33, 4.50 | | | |
| Malonyl | | | | | |
| M2 | 3.75 (s) | 3.76(s) | 3.75 | 3.75(s) | 3.74(s) |

brs, broad singlet; dd, double doublet; H, hydrogen; m, multiplet; NMR, nuclear magnetic resonance; s, singlet; t, triplet; t-like, triplet-like.

$COCH_2COOH$-Ara(p)-glu]$^-$, 621.0 [M-$COCH_2COOH$-Ara(p) -2glu]$^-$, and 459.4 [M-$COCH_2COOH$-Ara(p)- 3glu]$^-$. IR $\nu_{max}$: 3,392, 2,943, 1,729, 1,638, 1,452, 1,385, and 1,077. Libermann-Burchard and Molish reactions were positive. Molish reaction was used to proof the existence of saccharides, and test of Libermann-Buchard for steroids or triterpenes.

Compound 4 was obtained as a white amorphous powder. The molecular formula was determined as $C_{56}H_{92}O_{25}$ based on HRE-SIMS data at *m/z* 1,187.5822 [M+Na]$^+$ (calculated for $C_{56}H_{92}NaO_{25}$, 1,187.5820). Negative-mode ESI-MS (*m/z*) readings: 1,163.4 [M-H]$^-$, 1,119.7 [M-H-$CO_2$]$^-$, 1,077.4 [M-$COCH_2COOH$]$^-$, 1,059.3 [M-$COCH_2$-$COOH$-$H_2O$]$^-$, 945.2 [M- $COCH_2COOH$-Ara(f)]$^-$, 783.3 [M-$COCH_2$-$COOH$-Ara(f)-glu]$^-$, 621.2 [M-$COCH_2COOH$-Ara(f)- 2glu]$^-$, and 459.1 [M-$COCH_2COOH$-Ara(f)- 3glu]$^-$. IR $\nu_{max}$: 3,387, 2,942, 1,728, 1,636, 1,452, 1,388, and 1,076. Libermann-Burchard and Molish reactions were positive. The IR spectrum showed absorption bands for hydroxyl (3,387 cm$^{-1}$), carbonyl (1,728 cm$^{-1}$), double bond (1,636 cm$^{-1}$), methyl (1,388 cm$^{-1}$) and ether moiety (1,076 cm$^{-1}$). Molish reaction was used to proof the existence of saccharides, and test of Libermann-Buchard for steroids or triterpenes.

Compound 5 was obtained as a white amorphous powder. The molecular formula was determined as $C_{51}H_{84}O_{21}$ based on HRE-SIMS data at *m/z* 1,055.5400 [M+Na]$^+$ (calculated for $C_{56}H_{92}NaO_{25}$, 1,055.5397). Negative-mode ESI-MS (*m/z*) readings: 1,031.5 [M-H]$^-$, 987.4 [M-H-$CO_2$]$^-$, 945.4 [M-$COCH_2COOH$]$^-$, 927.3 [M-$COCH_2$-$COOH$-$H_2O$]$^-$, 783.3 [M- $COCH_2COOH$-glu]$^-$, 621.2 [M-

$COCH_2COOH$-2glu]$^-$, and 459.0 [M-$COCH_2COOH$-3glu]$^-$. IR $\nu_{max}$: 3,392, 2,944, 1,731, 1,634, 1,453, 1,388, and 1,076. Libermann-Burchard and Molish reactions were positive. Molish reaction was used to proof the existence of saccharides, and test of Libermann-Buchard for steroids or triterpenes.

### 2.5. Acid hydrolysis of compound 1

Compound 1 (5.0 mg) was hydrolyzed with 3.0N HCl (5 mL) at 100oC for 2 h. The reaction mixture was extracted with chloroform to afford the aglycone, and the aqueous layer was repeatedly evaporated to dryness with methanol until neutral. The sample was then analyzed by TLC over a silica gel with n-BuOH-AcOH-$H_2O$ (9:4:2) as the developing solvent. The sample spots were detected by spraying diphenylamine-aniline-phosphoric acid reagent (2% aniline in acetone: 2% diphenyl in acetone: 85% phosphoric acid = 5:5:1) and heating at 100oC [13]. The chromogenic agent was used to react with monosaccharides and appear coloration through heating. β-D-Glucose and α-L-rhamnose were used as authentic samples. Furthermore, the aqueous layer residues mentioned above were dissolved in anhydrous pyridine (2 mL) and stirred with L-cysteine methyl (1.5 mg) for 1 h at 60oC, then 1.2 mL of hexamethyldisilazane:trimethylchlorosiane (3:1) was added and the mixture was stirred at 60oC for another 30 min. The precipitate was centrifuged and the supernatant dried under $N_2$ steam at room temperature [14]. The residue was partitioned between hexane and water, and the hexane layer was analyzed by GC. Identification of D-glucose and L-rhamnose was carried out for compound 1, giving peaks at 9.90 min and 17.08 min, respectively.

## 3. Results

Compound 1 was obtained as a white amorphous powder. The molecular formula was determined as $C_{51}H_{84}O_{21}$ based on HRESIMS data at *m/z* 1,055.5391 $[M+Na]^+$ (calculated for $C_{51}H_{84}NaO_{21}$, 1,055.5397). The IR spectrum showed absorption bands for hydroxyl (3,408 $cm^{-1}$), carbonyl (1,731 $cm^{-1}$), and methyl (1,385 $cm^{-1}$) groups, as well as double bond (1,636 $cm^{-1}$), and ether moieties (1,075 $cm^{-1}$). The $^1$H-NMR spectrum (Table 2) showed eight methyl groups [$\delta_H$ 0.93 (6H, s), H-19, H-30; 1.15 (3H, s), H-18; 1.33 (3H, s), H-29; 1.63 (3H, s), H-27; 1.54 (3H, s), H-21; 1.62 (3H, s), H-26; 2.07 (3H, s), H-28], one olefinic proton [$\delta_H$ 5.28 (1H, t), H-24], one oxygen-substituted proton [$\delta_H$ 4.65 (1H, m), H-6], and three anomeric protons [$\delta_H$ 5.22 (1H, d, $J$ = 6.8), H-1′; 6.46 (1H, brs), H-1″; 5.04 (1H, d, $J$ = 7.7), H-1‴]. The $^{13}$C-NMR (Table 2) spectrum showed 51 carbon signals, including a pair of olefinic carbons at C-24 ($\delta$c 126.501) and C-25 ($\delta$c 131.495), two oxygen-substituted carbons at C-6 ($\delta$c 75.122) and C-20 ($\delta$c 83.925), and two carbonyl-group signals at C-M1 ($\delta$c 169.308) and C-M3 ($\delta$c 171.075). These data suggest that compound 1 was a dammarane-type triterpene glycoside with a double bond and a malonyl group [15–18]. The chemical structure of compound 1 was further elucidated by a HMBC (Figure 2) experiment in which correlations were observed between H-1′ ( $\delta_H$ 5.22, d, $J$ = 6.8 Hz ) and the carbon resonance signal at C-6 ( $\delta_c$ 75.122 ), H-1″ ( $\delta_H$ 6.46, brs ) and C-2′ ( $\delta_c$ 79.083 ), and H-1‴ ( $\delta_H$ 5.04, d, J = 7.7 Hz ) and C-20 ( $\delta_c$ 83.925 ), which indicated that the C-$1_{Glc'}$, C-$1_{Glc''}$, and C-$1_{Rha'''}$ were linked to C-6, C-2′, and C-20, respectively. The malonyl group was assigned to the C20-glc-C-6‴ position based on the correlations of C20-glc-H-6‴ with C-M1 and C-4‴, as shown in Figure 3.

The $^1$H- and $^{13}$C-NMR spectroscopic data for compound 1 were similar to those of ginsenoside Re, except for the data attributed to a malonyl group ($\delta_H$ 3.75, $\delta_C$ 169.308, $\delta_C$ 44.401, $\delta_C$ 171.075). Other carbon shifts included an upfield shift of C-5‴ ($\delta_C$ 75.389) and a downfield shift of C-6‴ ($\delta_C$ 65.755), as compared to ginsenoside Re [15,16]. H-5‴ yielded a peak at 3.96, and H-6‴ at 4.98 and 4.71, based on the HSQC spectrum. Acid hydrolysis of compound 1 yielded ginsenoside Re. The absolute configurations of the sugar moieties were further determined to be β-D-glucose and α-L-rhamnose by chiral GC analysis. The relative configuration of 1 was established through analysis of the ROESY experiment. As shown in Figure 1, correlations of H-3 to H-28 and H-5 indicated β-orientation for the 3-OH group. H-17 showed ROESY correlations with H-30 and H-16α, therefore, the side chain at C-17 was β-oriented. ROESY correlation between H-17 and H-21 confirmed assignment of the C-20(*S*) configuration. Moreover, the chemical shifts of C-17, C-21, and C-22 were 51.873, 22.483, and 36.513, respectively, which corresponded to the NMR data of 20(*S*)-ginsenoside Re [15–17].

The structures of compounds 2–5 were identified based on their spectroscopy data and by comparison of their data with literature sources [8,18]. The NMR spectroscopic data of the malonyl group in the present study showed significant differences with values reported in the literature. The chemical shifts of the methylenes between the two carboxyls of the malonyl group were ~44.176–44.801, which represented different values than those cited [8–11]. In the $^1$H-NMR spectrum, the chemical shifts of H-5″ and H-6″ were ~3.98–4.03 and ~4.94–4.97, respectively, which are reported here for the first time.

By normal-phase silica gel TLC (n-BuOH-$CH_3COOH$-$H_2O$ = 4:1:5), $R_f$ values were 0.32 for M-Re (1), 0.17 for M-$Rb_1$ (2), 0.18 for M-$Rb_2$ (3), 0.20 for M-Rc (4), and 0.27 for M-Rd (5). Reverse-phase ODS TLC (MeOH-$H_2O$ = 2:1) yielded $R_f$ values of 0.72, 0.36, 0.30, 0.35, and 0.27, respectively. Each compound was light purple on TLC when sprayed with 10% $H_2SO_4$ in ethanol followed by heating at 105oC. HPLC retention times were 12.8 min for M-Re (1),

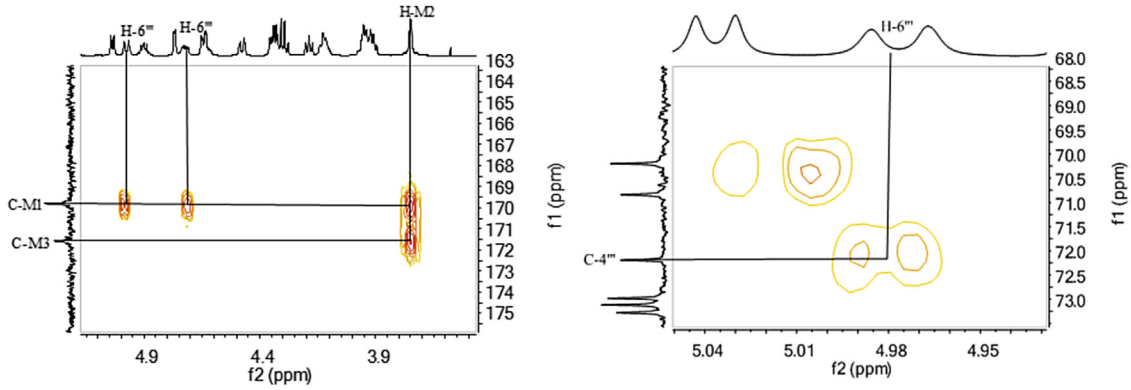


**Fig. 2.** Partially enlarged heteronuclear multiple-bond connectivity spectrum of compound 1.

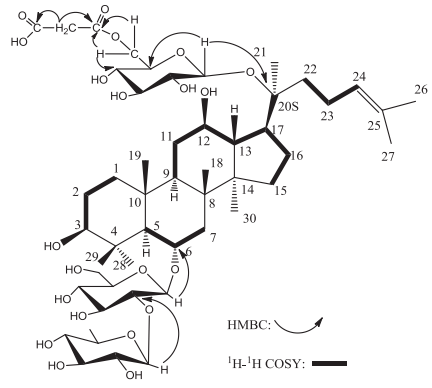


**Fig. 3.** Key HMBC and $^{1}$H-$^{1}$H COSY correlations for compound 1. COSY, correlated spectroscopy; HMBC, heteronuclear multiplebond correlation.

18.0 min for M-$Rb_1$ (2), 19.3 min for M-$Rb_2$ (3), 18.7 min for M-Rc (4), and 20.9 min for M-Rd (5).

## 4. Discussion

A phytochemical investigation of the fresh flowers of *P. ginseng* led to the isolation of a new saponin (20*S*)-Protopanaxatriol-6-[*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-(6-*O*-malonyl)-β-D-glucopyranoside, along with malonyl-ginsenosides $Rb_1$, $Rb_2$, Rc, and Rd. The complete $^{1}$H-NMR data of the malonyl ginsenosides were assigned for the first time.

Malonyl ginsenosides are unstable, not readily available, making them more difficult to analyze by HPLC than their neutral counterparts, and not used as indices for evaluation and quality control of ginseng. However, they are reported to be present in significant quantities in ginseng species, so the conventional evaluation index may not comprehensively reflect all ginseng properties or processed products. This study reports a simple and efficient way to prepare malonyl ginsenosides, as well as their physicochemical and NMR data, which provided scientific basis for the preparation of the standard substance.

## Conflicts of interest

All contributing authors declare no conflicts of interest.

## Acknowledgments

We are grateful to Gao-Fei Hu (College of Science, Beijing University of Chemical Technology) for measuring the NMR spectra. This work was financially supported by the National Key Technology Research and Development Program of the Ministry of Science and Technology of China (no. 2011BAI03B01 ).

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