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Research article

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

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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

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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 (δ) 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 ν_{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 $C_{56}H_{92}NaO_{25}$,

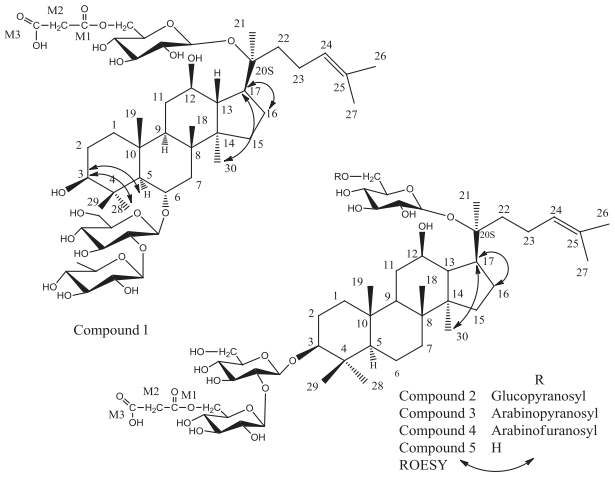

Fig. 1. Structures of compounds 1–5.