New dammarane-type triterpenoid saponins from *Panax notoginseng* saponins

Qian Li1,2,3, Mingrui Yuan1,2,3, Xiaohui Li1,2,3, Jinyu Li1,2,3, Ming Xu1,2,3, Di Wei1,2,3, Desong Wu1,2,3, Jinfu Wan1,2,3, Shuangxi Mei1,2,3, Tao Cui1,2,3, Jingkun Wang1,2,3,* Zhaoyun Zhu1,2,3,**

1Yunnan Institute of Materia Medica, Kunming, China
2Innovation and R&D Center, Yunnan Bai Yao Group, Kunming, China
3Yunnan Province Company Key Laboratory for TCM and Ethnic Drug of New Drug Creation, Kunming, China

1. Introduction

*Panax notoginseng* (Burk.) F.H. Chen (*P. notoginseng*), commonly called “Sanqi” or “Tianqi” in Chinese is a species of the genus *Panax*, family Araliaceae [1]. *P. notoginseng* is one of the most widely used Chinese herbal drugs for the treatment of cardiovascular diseases, such as occlusive vasculitis, coronary diseases, atherosclerosis, and cerebral infarction in China, Korea, and Russia for a long time [2]. There are about 200 chemical compositions that have been isolated from *P. notoginseng*, including saponins, flavonoids, and cyclopeptides [3]. Dammarane triterpenoidal saponins are the major bioactive ingredients of *P. notoginseng* [4].

*Panax notoginseng* saponin (PNS) is developed into the traditional Chinese medicine agents with the trademarks of Xuesaitong injection, Xuesuantong injection, and Xuesaitong tablet, which are all bestselling prescriptions used for treatment of cardiovascular and cerebrovascular diseases in China [5,6]. Notoginsenoside R1 and ginsenoside Rg1, Rd, Re, and Rb1 are regarded as the main active constituents of PNS, but a systematic research on the rare saponin compositions has not been conducted. The objective of this study was to conduct a systematic chemical study on PNS and establish the HPLC fingerprint of PNS to provide scientific evidence in quality control. In addition, the cytotoxicity of the new compounds was tested.

Methods: Pure saponins from PNS were isolated by means of many chromatographic methods, and their structures were determined by extensive analyses of NMR and HR-ESI-MS studies. The fingerprint was established by HPLC-UV method. The cytotoxicity of the compounds was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Results and Conclusion: Three new triterpenoid saponins (1–3) together with 25 known rare saponins (4–28) were isolated from PNS, except for the five main compounds (notoginsenoside R1 and ginsenoside Rg1, Rd, Re, and Rb1). In addition, the HPLC fingerprint of PNS was established, and the peaks of the isolated compounds were marked. The study of chemical constituents and fingerprint was useful for the quality control of PNS. The study on antitumor activities showed that new Compound 2 exhibited significant inhibitory activity against the tested cell lines.

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established the HPLC fingerprint and marked the compounds isolated. In addition, we tested the cytotoxic activity of the three new compounds against three human cancer cell lines.

2. Materials and methods

2.1. General experimental procedures

UV spectra: Shimadzu UV-2401A spectrophotometer (Shimadzu Instruments Co., Ltd, Tokyo, Japan); IR spectra: Nicolet FT-IR-360 spectrometer (Thermo Nicolet, Inc., Waltham, MA, USA). NMR spectra: Bruker ARX-400 spectrometers (Bruker Ltd, Karlsruhe, Germany). High resolution electrospray ionization mass spectrum (HR-ESI-MS) were taken using an Agilent G6230 instruments (Agilent Germany). High resolution electrospray ionization mass spectrum: Bruker ARX-400 spectrometer (Thermo Nicolet, Inc., Waltham, MA, USA). NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; m, multiplet. The assignment was based on DEPT, correlation spectroscopy (COSY), HSQC, and HMBC experiments.

2.2. Plant material

The PNS was provided by Yunnan Baiyao Group Co., Ltd.

2.3. Extraction and isolation

The PNS (2 kg) was separated by silica gel column using a gradient of CH₂Cl₂:CH₃OH (100:1→50:1→25:1→10:1→5:1→2:1→1:1, v/v) to obtain eight fractions (Fr1→Fr8). Fr1 (10 g) was chromatographed subsequently over silica gel chromatography with CHCl₃:MeOH (30:1→20:1→10:1, v/v) to get five major compounds.

Table 1

| Position | δC | δH | Position | δC | δH | Position | δC | δH |
|----------|----|----|----------|----|----|----------|----|----|
| 1        | 39.9 | 1.70 (1H, m) | 39.7 | 1.65 (1H, m) | 40.0 | 1.71 (1H, m) |
| 2        | 28.4 | 1.94 (1H, m) | 28.2 | 1.91 (1H, m) | 28.4 | 1.95 (1H, m) |
| 3        | 79.0 | 3.55 (1H, m) | 78.7 | 3.52 (1H, m) | 79.0 | 3.55 (1H, m) |
| 4        | 61.8 | 1.46 (1H, m) | 61.7 | 1.43 (1H, m) | 61.8 | 1.46 (1H, m) |
| 5        | 80.5 | 4.46 (1H, m) | 80.2 | 4.45 (1H, m) | 80.6 | 4.45 (1H, m) |
| 6        | 45.8 | 2.59 (1H, m) | 45.6 | 2.57 (1H, m) | 45.8 | 2.55 (1H, m) |
| 7        | 41.7 | 1.59 (1H, m) | 41.5 | 1.59 (1H, m) | 41.7 | 1.59 (1H, m) |
| 8        | 51.1 | 1.59 (1H, m) | 50.9 | 1.59 (1H, m) | 51.1 | 1.58 (1H, m) |
| 9        | 40.1 | 1.50 (2H, m) | 32.8 | 1.88 (1H, m) | 31.5 | 1.98 (1H, m) |
| 10       | 11.2 | 3.51 (2H, d, J = 12.5) | 11.3 | 5.29 (2H, d, J = 12.5) | 108.4 | 5.15 (1H, s) |
| 11       | 77.0 | 4.52 (1H, m) | 91.3 | 4.71 (1H, t, J = 7.1) | 33.1 | 2.77 (1H, m) |
| 12       | 36.3 | 2.73 (1H, m) | 31.3 | 2.60 (1H, m) | 33.2 | 2.06 (1H, m) |
| 13       | 42.0 | 3.05 (1H, m) | 38.6 | 2.91 (1H, m) | 48.7 | 2.29 (1H, m) |
| 14       | 12.4 | 5.42 (1H, t, J = 6.9) | 121.1 | 5.34 (1H, t, J = 6.9) | 75.5 | 4.46 (1H, m) |
| 15       | 132.4 | 133.4 | 25.4 | 1.71 (3H, s) | 26.1 | 1.65 (3H, s) |
| 16       | 18.6 | 1.65 (3H, s) | 18.3 | 1.59 (3H, s) | 18.7 | 1.91 (3H, s) |
| 17       | 32.2 | 2.10 (3H, s) | 30.6 | 2.08 (3H, s) | 32.2 | 2.10 (3H, s) |
| 18       | 16.8 | 1.63 (3H, s) | 16.7 | 1.60 (3H, s) | 16.8 | 1.63 (3H, s) |
| 19       | 17.1 | 0.88 (3H, s) | 16.9 | 0.82 (3H, s) | 17.2 | 0.82 (3H, s) |
| 6-O-sugar | 106.5 | 5.07 (1H, d, J = 8.0) | 106.3 | 5.04 (1H, d, J = 7.8) | 106.4 | 5.05 (1H, d, J = 7.8) |
| 2        | 73.0 | 3.99 (1H, m) | 73.2 | 3.93 (1H, m) | 73.0 | 3.91 (1H, m) |
| 3        | 78.7 | 3.99 (1H, m) | 78.5 | 3.96 (1H, m) | 78.6 | 3.98 (1H, m) |
| 4        | 72.3 | 4.26 (1H, m) | 72.1 | 4.22 (1H, m) | 72.3 | 4.27 (1H, m) |
| 5        | 75.9 | 4.13 (1H, m) | 75.7 | 4.10 (1H, m) | 75.9 | 4.13 (1H, m) |
| 6        | 63.5 | 4.57 (1H, m) | 63.4 | 4.54 (1H, m) | 63.5 | 4.54 (1H, m) |

NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; m, multiplet

1) Measured in pyridine- d₅, 500 MHz for ¹H, 125 MHz for ¹³C. The assignment was based on DEPT, correlation spectroscopy (COSY), HSQC, and HMBC experiments.
fractions (Fr.1-1–Fr.1-5) based on thin-layer chromatography (TLC) analysis. Fr.1-2 was purified by ODS eluted with MeOH–H2O (40:60 → 55:45 → 70:30, v/v) to provide Compounds 2 (5.1 mg) and 4 (7.5 mg). Fr.1-3 was then separated into three major fractions (Fr.1-3a–Fr.1-3c) by silica gel chromatography with CHCl3–MeOH (15:1 and 13:1, v/v) as eluent. Fr.1-3a and Fr.1-3b were further separated by preparative HPLC (p-HPLC) eluting with MeCN–H2O (28:72, v/v). Compound 6 (3.6 mg) was prepared from Fr.1-3c with MeCN–H2O (28:72, v/v) as a solvent system, whereas Fr.2-1–Fr.2-10. Fr.2-3 was subjected to chromatography on silica gel to yield Compounds 7 (7.7 mg). Compounds 7 (5.3 mg) and 14 (3.6 mg) were isolated from Fr.2-6 by p-HPLC with MeCN–H2O (30:70, v/v), and Fr.1-3 was further fractionated by ODS eluted with MeOH–H2O (40:60 → 60:40 → 80:20, v/v) to afford five major fractions (Fr.2-4-1–Fr.2-4-5). The analysis of a combined fraction of Fr.2-4-1–Fr.2-4-5 was performed by HPLC. Compound 27 (9.5 mg) was isolated from Fr.2-4-1 by HPLC system of MeCN–H2O (28:72, v/v). Compounds 10 (8.5 mg) and 15 (3.3 mg) were prepared from Fr.2-4-2 with MeCN–H2O (20:80, v/v) as a solvent system, whereas 9 (11.2 mg) and 12 (3.5 mg) were obtained from Fr.2-4-2 with MeCN–H2O (37:63, v/v) as a solvent system. Compounds 11 (3.8 mg) and 28 (2.2 mg) were got from Fr.2-4-3 by HPLC system of MeCN–H2O (43:57 and 35:65, v/v). Fr.5 (23.5 mg) was subjected to chromatography on ODS gel to provide ten factions (Fr.5-1–Fr.5-10). Compound 5 (1115.8 mg) was purified by recrystallizing from Fr.5-1. The analysis of other compounds isolated from Fr.5 was performed by p-HPLC: Compound 22 (mg) was purified from Fr.5-2, and Compounds 17 (511 mg) and 21 (12.7 mg) were isolated from Fr.5-3 by HPLC system of MeCN–H2O (30:70, v/v). Compound 13 (13.4 mg) were obtained from Fr.5-5 HPLC system of MeCN–H2O (34:66, v/v). Compounds 1 (4.3 mg) and 26 (8.5 mg) were prepared from Fr.5-7 with MeCN–H2O (27:73, v/v) as a solvent system, whereas 23 (36.7 mg) and 24 (8.9 mg) were prepared from Fr.5-8 with MeCN–H2O (37:63, v/v) as a solvent system.

2.4. Notoginsenoside Ab1 (1)

\[3\beta,6\alpha,12\beta,22S-tetrahydroxy-dammar-20(21),24-diene-6-O-\beta-D-glucopyranoside: \text{white amorphous powder; } [\alpha]D^0 : +10.5, (c = 0.20, MeOH); IR \nu_{\text{max}} 3420, 2931, 1634, 1545, 1384, 1074, 1032 \text{ cm}^{-1}; ^1H \text{ and } ^13C \text{ NMR: see Table 1}; \text{HR-ESI-MS } m/z 659.4130 \text{ [M+Na}^+\text{] (calculated for C}_{36}\text{H}_{60}\text{O}_{10}\text{Na 659.4135).}

2.5. Notoginsenoside Ab2 (2)

\[22S-hydroperoxyl-\beta,6\alpha,12\beta,tri hydroxy-dammar-20(21),24-diene-6-O-\beta-D-glucopyranoside: \text{white amorphous powder; } [\alpha]D^0 : +11.6, (c = 0.18, MeOH); IR \nu_{\text{max}} 3422, 2933, 1637, 1452, 1384, 1075, 1031 \text{ cm}^{-1}; ^1H \text{ and } ^13C \text{ NMR: see Table 1}; \text{HR-ESI-MS } 675.4077 \text{ [M + Na}^+\text{] (calculated for C}_{36}\text{H}_{60}\text{O}_{10}\text{Na 675.4084).}

2.6. Notoginsenoside Ab3 (3)

\[3\beta,6\alpha,12\beta,24R-tetrahydroxy-dammar-20(21),25-diene-6-O-\beta-D-glucopyranoside: \text{white amorphous powder; } [\alpha]D^0 : -3.4, (c = 0.25, MeOH); IR \nu_{\text{max}} 3416, 2941, 1636, 1452, 1386, 1163, 1076, 1041 \text{ cm}^{-1}; ^1H \text{ and } ^13C \text{ NMR: see Table 1}; \text{HR-ESI-MS } 659.4132 \text{ [M+Na}^+\text{] (calculated for C}_{36}\text{H}_{60}\text{O}_{10}\text{Na 659.4135).}

2.7. Acid hydrolysis and HPLC analysis

The absolute configurations of the sugar moieties in Compounds 1–3 were determined by the method of literature reported [9]. Compounds 1–3 (2.0 mg/sample) were refluxed with 10 mL of 60% aqueous dioxane with 5% HCl for 2 h. The reaction mixture was evaporated under vacuum and then suspended in H2O and extracted with CHCl3. After drying in vacuum, the residue of aqueous layer was melted in 0.2 mL of CH3OH with 2 mg of L-cystine methyl ester hydrochloride followed by warming at 60 °C for 1 h. After that, 5 mL of o-tolylisothiocyanate is added and warmed up at 60 °C for another hour. The reaction mixture was analyzed directly by reversed-phase HPLC on a Thermo C18 column (250 × 4.6 mm, 5 μm), with 20% CH3CN at a flow rate of 1.0 mL/min at 30 °C, and the detection wavelength was 254 nm. The analysis of standard monosaccharide, D-glucose, followed the same procedure. The value of $\delta$ of the standard monosaccharide derivatives was 17.8 min, and the derivatives of 1–3 gave peaks at $\delta$ 17.7–17.9 min, respectively.

2.8. Computational studies

Conformational searches were performed with Gaussian 09W program (Gaussian Inc., USA). The geometry of each conformer in the energy window of the conformational search was optimized with Gaussian 09W in vacuum, at the B3LYP–6–31g (d,p) level. Imaginary vibrational frequency of each conformer was checked, and no such frequency indicates true energy minima. Isotropic magnetic shielding was calculated with the GIAO (gauge-independent atomic orbital) method at the B3LYP/6–31G (d,p) level by using Gaussian 09W [10,11].

2.9. Fingerprint analysis

Chromatographic conditions: Waters 1525 HPLC system (Waters Corp., Milford, Massachusetts, USA); Chromatographic column: VP ODS C18 (250 mm × 4.6 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA); volume flow: 1.0 mL/min; column temperature: 30 °C; detection wavelength: 203 nm; injection volume: 10 μL. The samples were eluted with the mixture of Solvent A (water) and Solvent B (acetonitrile). The elution rate using Solvent B was 20–45% for 0–60 min.

Preparation of samples: Accurately weighed 25 mg of powder sample was diluted with 10 mL of 70% methanol. Before injection, the samples were filtered through a 0.45-μm membrane filter.

2.10. Cell line

HepG-2 (human hepatic cancer cell line), NCI-H460 (human lung cancer cell), and MCF-7 (human breast cancer cell) were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The HepG-2 and NCI-H460 cancer cells were maintained in Roswell Park Memorial Institute 1640 medium, and MCF-7 cancer cells were maintained in high-glucose Dulbecco’s minimum essential medium, supplemented with 10% fetal bovine serum. The cells grew in a 5% CO2 incubator at 37 °C. The cells were routinely digested and passaged every 3 days.

2.11. Cell viability assay

The cells were plated in 96-well plates (1 × 10^4 cells/well) overnight, then 1–3 at various concentrations of 0.01, 0.1, 1, 10, and
100 μg/mL and the positive control cisplatin at concentrations of 0.5, 1, 2, 4, and 8 μg/mL were treated in the plates for 72 h. Subsequently, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (5 mg/mL) was added to each well for 4 h, and then 100 μL of triple liquid containing 10 mg SDS (sodium dodecyl sulfate), 1.2 μL of 36–37% concentrated hydrochloric acid, and 50 μL of isobutanol were added. After the coculture for 12 h, the reduction of cell viability was determined at 570 nm using a microplate reader (Bio-Rad, USA). The cell proliferation inhibition rate was calculated according to the following formula: Inhibition rate (%) = (Acontrol – Asample)/Acontrol × 100 [12].

3. Result and discussion

Compound 1 was obtained as white amorphous powder. The molecular formula of 1 was deduced to be C_{36}H_{60}O_{9} by positive
mass spectrometry (HR-ESI-MS) data at m/z 659.4130 [M+Na]+ (calculated for C_{36}H_{60}O_{9}Na, 659.4135). The $^{13}$C NMR (Table 1) showed 36 carbon signals. The distortionless enhancement by polarization transfer (DEPT) spectrum exhibited 7 methyls, 9 methylenes, 14 methines, and 6 quaternary carbons signals. Four olefinic carbon signals at $\delta_{C}$ 160.2, 132.8, 122.4, and 111.2 ppm suggested two double bonds in the molecule. The $^{1}H$ NMR showed signals of seven methyl groups at $\delta_{H}$ 0.88 (3H, s), 1.04 (3H, s), 1.25 (3H, s), 1.63 (3H, s), 1.65 (3H, s), 1.71 (3H, s), and 2.10 (3H, s); four oxygen substituted protons at $\delta_{H}$ 3.55 (1H, m), 4.29 (1H, m), 4.46 (1H, m), and 4.52 (1H, m); and one anemic proton at $\delta_{H}$ 5.07 (1H, d, $J = 8.0$). The $^{1}H$ and $^{13}C$ signals were fully assigned according to heteronuclear signal quantum correlation (HSQC) spectra (Table 1). Methylene carbon signal at $\delta_{C}$ 111.2 ppm showed correlation spots with protons at $\delta_{H}$ 5.18 (H-21) and 4.52 (H-21) ppm in HSQC spectrum. These two proton signals showed connections with carbon signals at $\delta_{C}$ 160.2 (C-20), 77.0 (C-22), and 40.2 (C-17) ppm in heteronuclear multiple bond correlation (HMBC) spectrum, and $\delta_{H}$ 4.52 (H-22) showed connection with $\delta_{C}$ 111.2 (C-20), 122.4 (C-24), and 40.2 (C-17) (Fig. 2). Thus, the signals at $\delta_{C}$ 160.2, 111.2, and 77.0 ppm were assigned to be the signals of C-20, C-21, and C-22, respectively. $\delta_{H}$ 5.42 (H-24) showed connections with $\delta_{C}$ 132.8 (C-25), 26.4 (C-26), and 18.6 (C-27). Therefore, it was concluded that the two double bonds were at $\Delta 20(21)$ and $\Delta 24(25)$. The signals of Compound 1 were quiet similar to those of ginsenoside Rk3, except for the chemical shift of C-20, C-22, and C-23, which were at $\delta_{C}$ 160.2, 77.0, and 36.3 of Compound 1 but were at $\delta_{C}$ 155.4, 33.7, and 27.0 of ginsenoside Rk3, respectively [13].
downfield of C-20 (−4.8 ppm), C-22 (−43.3 ppm), and C-23 (−9.3 ppm) indicated that C-22 of 1 was linked to hydroxyl. In addition, the β configuration was prompted by the large coupling constant observed for the anomeric proton δH 5.07 (1H, d, J = 8.0). The absolute configurations of sugar was elucidated as D-glucose through acid hydrolysis and HPLC analysis. Moreover, the linkages between H-1' (δH 5.07) and C-6 (δC 80.5) were determined by HMBC correlations. The configuration of OH at C-6 was α based on the correlations between H-6 with H-18i, 19β in rotating frame overhauser effect (ROESY) spectrum. In addition, H-17 was deduced as α-forms by correlations between H-17 and Me-30 in the ROESY spectrum. The configuration of C-22 was identified by the comparison of the calculated and experimental chemical shifts of 13C. The calculated chemical shifts of C-22 about (22S-hydroperoxyl-3-saccharide was determined to be D-glucose by HPLC analysis of notoginsenoside Ab1. The conformation of C-22 was determined as notoginsenoside Ab1. The compound 2 was isolated as white amorphous powder. The HR-ESI-MS spectrum showed [M+Na]+ at m/z 657.4077 (calcd. for C35H46O12Na 675.4135). It was proposed to possess a hydroperoxyl group at the result of positive response to 5-(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The n-ginsenoside ST2 (22S)-3 and (22S)-1 were δC 72.0 and δC 77.8, whereas the experimental result was δC 77.0. Therefore, the configuration of C-22 was identified as S. On the basis of the aforementioned analyses, Compound 1 could be deduced to be 3β,6α,12β,22S-tetrahydroxy-dammar-20(21),24-diene-6-O-β-D-glucopyranoside and named as notoginsenoside Ab1. The compound 2 was isolated as white amorphous powder. The HR-ESI-MS spectrum showed [M+Na]+ at m/z 657.4077 (calcd. for C35H46O12Na 675.4135). It was proposed to possess a hydroperoxyl group at the result of positive response to 5-(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The n-ginsenoside ST2 (22S)-3 and (22S)-1 were δC 72.0 and δC 77.8, whereas the experimental result was δC 77.0. Therefore, the configuration of C-22 was identified as S. On the basis of the aforementioned analyses, Compound 1 could be deduced to be 3β,6α,12β,22S-tetrahydroxy-dammar-20(21),24-diene-6-O-β-D-glucopyranoside and named as notoginsenoside Ab1. The compound 2 was isolated as white amorphous powder. The HR-ESI-MS spectrum showed [M+Na]+ at m/z 657.4077 (calcd. for C35H46O12Na 675.4135). It was proposed to possess a hydroperoxyl group at the result of positive response to 5-(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The n-ginsenoside ST2 (22S)-3 and (22S)-1 were δC 72.0 and δC 77.8, whereas the experimental result was δC 77.0. Therefore, the configuration of C-22 was identified as S. On the basis of the aforementioned analyses, Compound 1 could be deduced to be 3β,6α,12β,22S-tetrahydroxy-dammar-20(21),24-diene-6-O-β-D-glucopyranoside and named as notoginsenoside Ab1. The compound 2 was isolated as white amorphous powder. The HR-ESI-MS spectrum showed [M+Na]+ at m/z 657.4077 (calcd. for C35H46O12Na 675.4135). It was proposed to possess a hydroperoxyl group at the result of positive response to 5-(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The n-ginsenoside ST2 (22S)-3 and (22S)-1 were δC 72.0 and δC 77.8, whereas the experimental result was δC 77.0. Therefore, the configuration of C-22 was identified as S. On the basis of the aforementioned analyses, Compound 1 could be deduced to be 3β,6α,12β,22S-tetrahydroxy-dammar-20(21),24-diene-6-O-β-D-glucopyranoside and named as notoginsenoside Ab1.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.12.001.

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