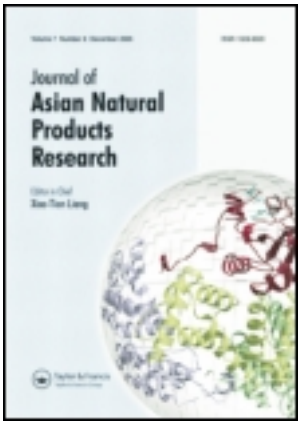

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

Ginsenjilinol, a new protopanaxatriol-type saponin with inhibitory activity on LPS-activated NO production in macrophage RAW 264.7 cells from the roots and rhizomes of Panax ginseng

Hong-Ping Wang [a], Xin-Bao Yang [b c], Xiu-Wei Yang [a], Jian-Xun Liu [b c], Wei Xu [a], You-Bo Zhang [a], Lian-Xue Zhang [d] & Ying-Ping Wang [e]

[a] State Key Laboratory of Natural and Biomimetic Drugs, Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing, 100191, China

[b] Xiyuan Hospital, China Academy of Chinese Sciences, Beijing, 100091, China

[c] Beijing University of Chinese Medicines, Beijing, 100029, China

[d] College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun, 130118, China

[e] Institute of Special Wild Economic Animals and Plants Science, Chinese Academy of Agricultural Sciences, Changchun, 130112, China

Published online: 03 May 2013.

To cite this article: Hong-Ping Wang, Xin-Bao Yang, Xiu-Wei Yang, Jian-Xun Liu, Wei Xu, You-Bo Zhang, Lian-Xue Zhang & Ying-Ping Wang (2013) Ginsenjilinol, a new protopanaxatriol-type saponin with inhibitory activity on LPS-activated NO production in macrophage RAW 264.7 cells from the roots and rhizomes of Panax ginseng, Journal of Asian Natural Products Research, 15:5, 579-587, DOI: 10.1080/10286020.2013.787992

To link to this article: http://dx.doi.org/10.1080/10286020.2013.787992

Journal of Asian Natural Products Research, 2013
Vol. 15, No. 5, 579–587, http://dx.doi.org/10.1080/10286020.2013.787992

Ginsenjilinol, a new protopanaxatriol-type saponin with inhibitory activity on LPS-activated NO production in macrophage RAW 264.7 cells from the roots and rhizomes of *Panax ginseng*

Hong-Ping Wang[a], Xin-Bao Yang[bc], Xiu-Wei Yang[a]*, Jian-Xun Liu[bc]*, Wei Xu[a], You-Bo Zhang[a], Lian-Xue Zhang[d] and Ying-Ping Wang[e]

[a]*State Key Laboratory of Natural and Biomimetic Drugs, Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China;* [b]*Xiyuan Hospital, China Academy of Chinese Sciences, Beijing 100091, China;* [c]*Beijing University of Chinese Medicines, Beijing 100029, China;* [d]*College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun 130118, China;* [e]*Institute of Special Wild Economic Animals and Plants Science, Chinese Academy of Agricultural Sciences, Changchun 130112, China*

(*Received 2 February 2013; final version received 17 March 2013*)

One new dammarane triterpene saponin named ginsenjilinol (**1**) was isolated from the roots and rhizomes of *Panax ginseng* C.A. Mey., together with two known saponins ginsenoside Rf (**2**) and ginsenoside Re_5 (= panajaponol A, **3**). Based on IR, HR–ESI–MS, and 1D as well as 2D NMR (^1H–^1H COSY, NOESY, HSQC, and HMBC) spectral data, the chemical structure of the new saponin was elucidated as 3β,12β,20*S*,26-tetrahydroxydammar-24*E*-en-6α-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside. The ability of the isolated saponins to inhibit nitric oxide production by lipopolysaccharide-activated RAW 264.7 cells was also assayed. All of the isolated saponins exhibited the significant activity in a concentration-dependent manner at concentrations of 60–200 μM with the half maximal inhibitory concentration (IC_{50}) values of 70.96 ± 2.05 μM for **1**, 74.14 ± 2.65 μM for **2**, and 79.83 ± 1.78 μM for **3**, respectively, whereas indomethacin had an IC_{50} of 63.75 ± 3.33 μM as a positive control drug.

Keywords: *Panax ginseng*; protopanaxatriol-type triterpenoid saponin; ginsenjilinol; ginsenoside Rf; ginsenoside Re5; nitric oxide

1. Introduction

Ginseng Radix et Rhizoma, the roots and rhizomes of *Panax ginseng* C.A. Mey. (Araliaceae), is one of the most commonly used traditional Chinese medicines and is widely used in herbal diets as a tonic agent in Asian countries. In traditional medicine usage, it has several health-promoting functions, including maintaining the homeostasis of the body, anti-fatigue, anti-stress, anti-aging, improved brain function, enhanced immune system function, adjusted blood pressure, anti-oxidative effect, anti-tumor activity, anti-diabetic effects, improved menopause and sexual functions [1,2]. In regard to its chemical constituents, the major bioactive ingredients of Ginseng Radix et Rhizoma belong to a class of unique triterpenoid dammarane-type saponins called ginsenosides, which can be further classified into protopanaxadiol and protopanaxatriol (PPT) types. To date, more than 50 different ginsenosides have been isolated from the Ginseng Radix et Rhizoma and characterized. It was found that each ginsenoside has various pharmacological effects and different pharmacokinetic behavior, as well as even one single ginsenoside was demonstrated to exert multiple effects [2,3]. The material basis study of Ginseng

*Corresponding authors. Email: xwyang@bjmu.edu.cn, liujx0324@sina.com

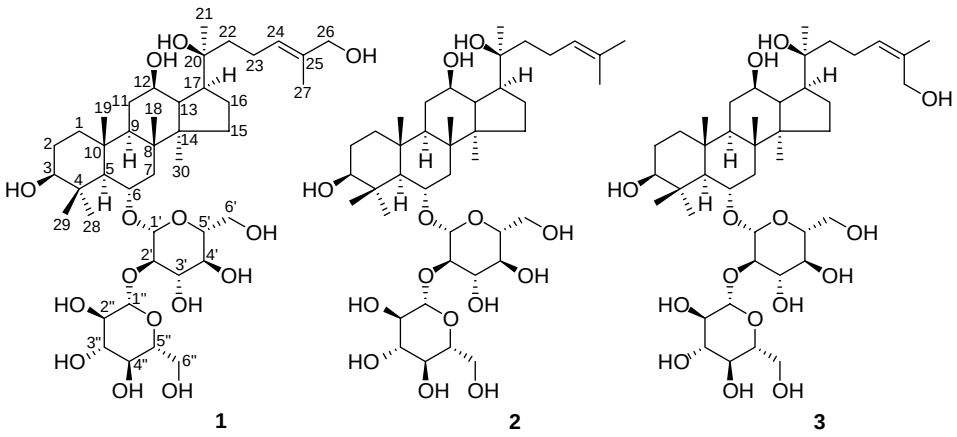

Figure 1. Structures of compounds **1**–**3**.

Radix et Rhizoma is still necessary. As part of a continuing investigation on the bioactive constituents of *P. ginseng* [4–10], we have now isolated a new PPT-type ginsenoside named ginsenjilinol (**1**), together with two known ginsenosides, namely ginsenoside Rf (**2**) [11] and ginsenoside Re_5 (**3**) [12] (= panajaponol A [13]). Herein, we report the isolation and structural elucidation of **1**–**3**. We assayed the suppressive effects of **1**–**3** on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated murine macrophage-like cells, RAW 264.7.

2. Results and discussion

The normal butanol (*n*-BuOH) soluble fraction of the 70% aqueous ethanol (EtOH) extract of Ginseng Radix et Rhizoma was separated by Amberlite XAD-4 resin and normal phase silica gel column chromatography followed by semi-preparative high-performance liquid chromatography (SP-HPLC) to provide compounds **1**–**3** (Figure 1).

Ginsenjilinol (**1**), positive to Liebermann–Burchard reagent, was obtained as white amorphous powder with $[\alpha]_D^{25}$ −27.6 (*c* 0.456, MeOH). The molecular formula of **1** was established as $C_{42}H_{72}O_{15}$ by its positive HR–ESI–MS showing quasi-molecular ion peaks at *m/z* 817.4952 $[M + H]^+$ and 839.4753 $[M + Na]^+$, which was one oxygen atom more than **2** and had the same molecular formula as **3**. The IR spectrum showed broad absorption bands at 3420 and 1077 cm^{-1} suggestive of oligoglycosidic structure [14]. Acid hydrolysis of **1** yielded glucose identified by silica gel thin layer chromatography (TLC) and paper chromatography (PC) with an authentic sample. The ^1H (Table 1) and ^{13}C (Table 2) NMR signals of **1** were assigned with the aid of HSQC, HMBC, ^1H–^1H COSY, and NOESY spectra and by comparison with those of both 20(*S*)-ginsenoside Rg_2 and 20(*R*)-ginsenoside Rg_2 [15], which are the PPT-type ginsenosides having an dammarane-type triterpene skeleton bearing oxygenated functions at C-3, C-6, C-12, and C-20, suggesting that **1** is a PPT-type ginsenoside. The ^1H NMR pattern of **1** is similar to that of a known PPT-type ginsenoside, ginsenoside Rf (**2**) [11], except for the signal for a hydroxymethyl group instead of a methyl group at C-25, and in **2** the Me-27 proton signal at δ_H 1.61 (3H, s) was shifted downfield to δ_H 1.82 (3H, s) and H-24 at δ_H 5.31 (1H, t, $J = 7.0$ Hz) was shifted downfield to δ_H 5.83 (1H, t, $J = 6.1$ Hz). In the ^{13}C NMR spectrum, the signal for C-26, which occurred at δ_C

Table 1. The ^{1}H NMR data of compounds **1**–**3** (in pyridine-d_5, δ_{ppm}; J in Hz).[a]

No.	**1**	**2**	**3**
1β	0.95 (1H, dt, 13.6, 3.7)	0.96 (1H, dt, 13.6, 3.8)	0.96 (1H, dt, 13.6, 3.8)
1α	1.61 (1H, m)	1.61 (1H, m)	1.60 (1H, m)
2β	1.75 (1H, m)	1.75 (1H, m)	1.75 (1H, m)
2α	1.83 (1H, m)	1.83 (1H, m)	1.83 (1H, m)
3α	3.46 (1H, dt, 11.3, 4.6)	3.46 (1H, dt, 11.2, 4.9)	3.46 (1H, dt, 11.3, 4.6)
5α	1.36 (1H, d, 10.5)	1.36 (1H, d, 10.3)	1.36 (1H, d, 10.5)
6β	4.35 (1H, dt, 10.5, 3.0)	4.34 (1H, dt, 10.3, 2.4)	4.35 (1H, dt, 10.5, 3.1)
7α	2.00 (1H, dd, 12.5, 10.5)	2.01 (1H, dd, 12.7, 10.3)	2.00 (1H, overlapped)
7β	2.39 (1H, dd, 12.5, 3.0)	2.39 (1H, dd, 12.7, 2.4)	2.39 (1H, dd, 12.8, 3.1)
9α	1.51 (1H, dd, 10.2, 4.2)	1.51 (1H, dd, 10.2, 4.2)	1.50 (1H, overlapped)
11β	1.49 (1H, dd, 10.2, 9.0)	1.49 (1H, dd, 11.1, 10.0)	1.49 (1H, dd, 10.2, 9.5)
11α	2.03 (1H, dd, 10.2, 4.2)	2.05 (1H, dd, 11.1, 4.2)	2.05 (1H, dd, 10.2, 4.2)
12α	3.85 (1H, br t, 9.0)	3.85 (1H, br t, 10.0)	3.84 (1H, br t, 9.5)
13β	2.00 (1H, br dd, 10.2, 9.0)	2.01 (1H, br dd, 11.5, 10.0)	2.02 (1H, br dd, 10.2, 9.5)
15α	1.11 (1H, m)	1.11 (1H, m)	1.11 (1H, m)
15β	1.52 (1H, m)	1.53 (1H, m)	1.52 (1H, m)
16β	1.39 (1H, m)	1.40 (1H, m)	1.39 (1H, m)
16α	1.78 (1H, m)	1.78 (1H, m)	1.78 (1H, m)
17α	2.27 (1H, br dd, 12.7, 10.2)	2.27 (1H, br dd, 12.6, 11.5)	2.26 (1H, br dd, 12.7, 10.2)
18β	1.12 (3H, s)	1.13 (3H, s)	1.14 (3H, s)
19β	0.94 (3H, s)	0.93 (3H, s)	0.93 (3H, s)
21α	1.39 (3H, s)	1.38 (3H, s)	1.33 (3H, s)
22β	1.67 (1H, m)	1.65 (1H, m)	1.67 (1H, m)
22α	2.06 (1H, m)	2.01 (1H, m)	2.05 (1H, m)
23α	2.39 (1H, m)	2.59 (1H, m)	2.42 (1H, m)
23β	2.67 (1H, m)	2.27 (1H, m)	2.67 (1H, m)
24	5.83 (1H, t, 6.1)	5.31 (1H, t, 7.0)	5.46 (1H, t, 7.2)
26a	4.27 (1H, s)	1.64 (3H, s)	1.98 (3H, s)
26b	4.27 (1H, s)		
27a	1.82 (3H, s)	1.61 (3H, s)	4.54 (1H, br d, 12.1)
27b			4.46 (1H, br d, 12.1)
28α	2.07 (3H, s)	2.07 (3H, s)	2.07 (3H, s)
29β	1.45 (3H, s)	1.45 (3H, s)	1.46 (3H, s)
30α	0.78 (3H, s)	0.78 (3H, s)	0.77 (3H, s)
6-Glc			
1′	4.91 (1H, d, 7.4)	4.90 (1H, d, 7.6)	4.90 (1H, d, 7.6)
2′	4.45 (1H, dd, 8.6, 7.2)	4.45 (1H, dd, 8.5, 7.2)	4.46 (1H, dd, 8.5, 7.6)
3′	4.33 (1H, overlapped)	4.33 (1H, overlapped)	4.35 (1H, dd, 8.5, 8.2)
4′	4.11 (1H, dd, 8.6, 9.0)	4.17 (1H, dd, 8.5, 9.2)	4.11 (1H, dd, 9.0, 8.2)
5′	3.84 (1H, overlapped)	3.84 (1H, br dd, 9.2, 5.3)	3.83 (1H, br dd, 9.0, 4.1)
6′a	4.47 (1H, br d, 11.4)	4.47 (1H, br d, 11.6)	4.46 (1H, br d, 11.0)
6′b	4.29 (1H, overlapped)	4.28 (1H, dd, 11.6, 5.3)	4.28 (1H, dd, 11.0, 4.3)
2′-Glc			
1″	5.91 (1H, d, 7.5)	5.91 (1H, d, 7.2)	5.91 (1H, d, 7.2)
2″	4.18 (1H, dd, 8.4, 7.5)	4.18 (1H, dd, 8.6, 7.2)	4.18 (1H, dd, 8.5, 7.2)
3″	4.20 (1H, overlapped)	4.20 (1H, overlapped)	4.20 (1H, overlapped)
4″	4.16 (1H, dd, 8.9, 8.5)	4.16 (1H, dd, 8.6, 8.2)	4.16 (1H, dd, 8.9, 8.3)
5″	3.93 (1H, br dd, 8.6, 5.2)	3.94 (1H, br dd, 8.6, 5.0)	3.94 (1H, br dd, 8.9, 5.0)
6″a	4.46 (1H, br d, 11.6)	4.45 (1H, br d, 11.5)	4.46 (1H, br d, 11.6)
6″b	4.28 (1H, overlapped)	4.28 (1H, dd, 11.5, 5.0)	4.28 (1H, dd, 11.6, 5.0)

[a] ^{1}H NMR chemical shift values (δ ppm) followed by multiplicity. Assignments were based on ^{1}H–^{1}H COSY, NOESY, HSQC, and HMBC experiments.

Table 2. The ^{13}C NMR data of compounds **1**–**3** (in pyridine-d_5, δ_{ppm}).[a]

No.	1	2	3	No.	1	2	3
1	39.4 t	39.4	39.4	23	22.6 t	23.0	22.5
2	27.7 t	27.7	27.8	24	125.7 d	126.3	128.0
3	78.6 d	78.6	78.6	25	136.1 s	130.8	136.1
4	40.2 s	40.2	40.2	26	68.1 t	25.8	21.9
5	61.4 d	61.4	61.4	27	13.9 q	17.7	60.8
6	79.8 d	79.8	79.8	28	32.0 q	32.1	32.1
7	45.0 t	45.0	45.0	29	16.8 q	16.8	16.8
8	41.1 s	41.1	41.1	30	16.7 q	16.7	16.7
9	50.1 d	50.1	50.1	6-Glc			
10	39.6 s	39.6	39.6	1′	103.9 d	103.9	103.9
11	32.0 t	32.1	32.1	2′	79.7 d	79.7	79.7
12	71.0 d	71.0	71.0	3′	79.8 d	79.9	79.9
13	48.2 d	48.3	48.2	4′	71.7 d	71.7	71.7
14	51.6 s	51.7	51.6	5′	78.4 d	78.4	78.4
15	31.2 t	31.2	31.2	6′	63.3 t	63.3	63.3
16	27.0 t	27.0	26.8	2′-Glc			
17	54.7 d	54.8	54.7	1″	103.9 d	103.8	103.8
18	17.6 q	17.6	17.6	2″	76.0 d	76.0	76.0
19	17.4 q	17.4	17.4	3″	78.1 d	78.1	78.1
20	72.9 s	73.0	72.9	4″	72.3 d	72.3	72.3
21	26.8 q	26.8	27.0	5″	77.8 d	77.8	77.8
22	35.7 t	35.8	36.1	6″	62.9 t	62.9	62.9

[a] C-multiplicities were established by a HSQC experiment; s = C, d = CH, t = CH_2, q = CH_3.

25.8 in **2**, was replaced by a signal at δ_C 68.1 in **1**, indicating that C-26 of **1** was substituted by a hydroxyl group. This conclusion was further supported by the correlations between H-24 at δ_H 5.83 and C-26 (δ_C 68.1), C-27 (δ_C 13.9) in the HMBC experiment (Figure 2). Furthermore, the signals of H-26 at δ_H 4.27 and

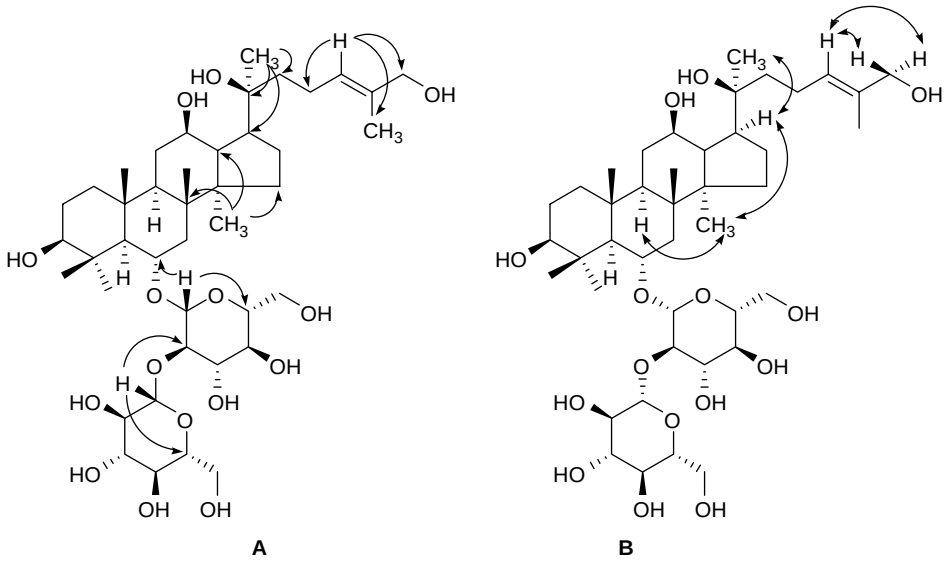

Figure 2. Key HMBC (A) and NOESY (B) correlations of **1**.

H-24 at δ_H 5.83 showed an NOE correlation in **1**, whereas the Me-26 proton signal at δ_H 1.98 (3H, s) and H-24 at δ_H 5.46 (1H, t, $J = 7.2$ Hz) showed an NOE correlation in **3**, suggesting that the double bond in **1** has an *E*-configuration. The above data evidence indicated that **1** and **3** are a pair of geometrical isomers, as in *Z*-5-methoxytrichoclin acetate and *E*-5-methoxytrichoclin acetate [16]. Furthermore, the ^{1}H (Table 1) and ^{13}C (Table 2) NMR signals attributable to the sugar unit of **1** were assigned by the 2D NMR experiments, and the data indicated that two D-glucosyl groups are in its pyranose form. In the HMBC spectrum, a correlation between H-1′ (δ_H 4.91) of glucosyl group and C-6 (δ_C 79.8) of the aglycone suggested that this glucosyl group was directly connected to C-6 of the aglycone, whereas a correlation between H-1″ (δ_H 5.91) of another glucosyl group and C-2′ (δ_C 79.7) of the above glucosyl group suggested that the second glucosyl unit was linked at C-2′ position. The values of the large coupling constant indicated β-glycosidic linkages in all cases, identical with those of **2** and **3**. Consequently, the structure of **1** was concluded to be 3β,12β,20*S*,26-tetrahydroxydammar-24*E*-en-6α-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside, trivially named ginsenjilinol.

NO is derived from L-arginine by nitric oxide synthase (NOS) in numerous mammalian cells and tissues and plays an important role in the regulation of many physiological functions, such as host defense, neurotransmission, neurotoxicity, and vasodilation [17,18]. A lot of studies have shown that NO can act as a neurotransmitter and as a messenger between our cells at low concentration, whereas overproduction of NO by NOS may have pathological consequences, such as the development of inflammation and inducing cell death [19]. It was also reported that NO is emerging as an important mediator of neurotoxicity in a variety of disorders of the nervous system under conditions of overproduction, such as multiple sclerosis, Alzheimer disease, and acquired immune deficiency syndrome dementia [20,21]. The overproduction of NO is also involved in the interrelated physiological processes of cerebral ischemia [22]. Thus, modulating the NO level in central nervous system may be a new tactics for prevention and treatment of neurodegenerative diseases. Because increased NO production is a typical phenomenon that occurs in LPS-stimulated macrophages and is used as an indicator of a typical inflammatory response, as well as Ginseng Radix et Rhizoma has effect of improved brain function and anti-inflammatory activity [1,2], the inhibition of **1**–**3** on the NO production was measured with LPS-induced activation of a murine monocyte-macrophage-like cell line RAW 264.7 as a model system in this article. The total activity showing the level of nitrite production was measured by Griess method [23]. The assayed concentrations (60–200 μM) of **1**–**3** without affecting the normal RAW 264.7 cell viability were determined before examining their inhibitory effects on the NO production by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. The viability rate of RAW 264.7 cell was above 95%. Exposure of RAW 264.7 cells to LPS increased production of NO, which was effectively inhibited by compounds **1**–**3** treatment in a concentration-dependent manner (Figure 3) with half maximal inhibitory concentration (IC_{50}) values of 70.96 ± 2.05 μM for **1**, 74.14 ± 2.65 μM for **2**, and 79.83 ± 1.78 μM for **3**, whereas indomethacin, as a positive control drug, had an IC_{50} of 63.75 ± 3.33 μM. Based on the previous results and in combination with the traditional use of Ginseng Radix et Rhizoma by Chinese doctors, **1**–**3** perhaps exerted beneficial anti-inflammatory effects and treatment of cerebral ischemia. Because macrophages can

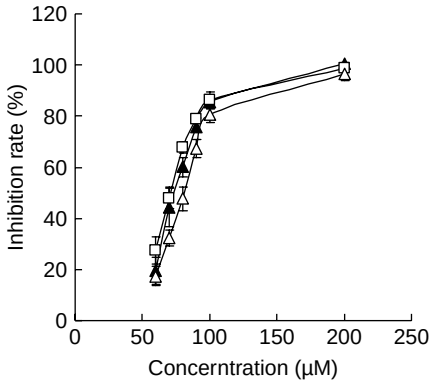

Figure 3. Inhibitory effects of ginsenjilinol (□), ginsenoside Rf (▲), and ginsenoside Re5 (Δ) against NO production in LPS-activated macrophage cell line RAW 264.7.

greatly increase NO production under inflammatory conditions, **1**–**3** have the potential to be developed into an effective anti-inflammatory agent. The inhibitory activity of compounds **1**–**3** on NO production in LPS-stimulated murine macrophage-like cells, RAW 264.7, was reported for the first time.

3. Experimental

3.1 General experimental procedures

Optical rotation was acquired on an Autopol III polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA) with methanol (MeOH) as solvent. IR spectrum was recorded on a Nexus-470 FT-IR spectrometer (Thermo Nicolet, Inc., Madison, WI, USA) with KBr disks. Mass spectra were recorded on an API QSTAR (Applied Biosystems/MDS Sciex., Foster City, CA, USA) for ESI-TOF-MS and an APEX IV Fourier transform ICR high-resolution mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA) for HR-ESI-MS. 1D and 2D NMR spectra were measured on a Bruker AVANCE III 400 spectrometer (Bruker BioSpin AG Facilities, Fällanden, Switzerland; 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) with trimethylsilane as an internal standard and pyridine-d_5 as a solvent. The chemical shifts are reported in parts per million (ppm), and the coupling constants are given in hertz (Hz). The SP-HPLC was performed on a Beijing CXTH 3000 system (Beijing Chuang Xin Tong Heng Sci. Technol. Co. Ltd, Beijing, China) including two P3050 pumps, UV3000 ultraviolet-visible detector, A1359 liquid handler, and Daisogel C_{18} column (250 × 30 mm, 10 μm), as well as the entire utlraviolet (UV) detection was set up at 203 nm with a flow rate of 15 ml/min.

Open column chromatographic (CC) separation was performed with silica gel (200–300 mesh; Qingdao Marine Chemical Co., Qingdao, China) and Amberlite XAD-4 resin (20–60 mesh; Sigma Chemical Co., St Louis, MO, USA) as stationary phase. TLC was conducted on silica gel GF_{254} plates (Merck, Darmstadt, Germany). All other chemicals including ethanol (EtOH), *n*-BuOH, and chloroform ($CHCl_3$) were of analytical reagent grade and purchased from Beijing Chemical Works (Beijing, China). Acetonitrile (MeCN) and MeOH were of chromatographic grade and purchased from Tianjin Xihua Special Type Reagent Factory (Tianjin, China). Water (H_2O) was milli-Q grade.

Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 5% H_3PO_4), LPS, MTT, β-D-glucopyranose, and indomethacin were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco™ (Grand Island, NY, USA). MTT assay was carried out in an MCO-15 AC carbon dioxide (CO_2) incubator (Sanyo Electric Co. Ltd, Osaka, Japan) and detected with a Multiskan MK 3 Automated Microplate Reader (Thermo-Labsystems, Franklin, MA, USA). The 96-well plates were obtained from Corning Costar (Corning Costar #3599, Cambridge, MA, USA).

The murine macrophage cell line RAW 264.7 was purchased from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China).

3.2 Plant material

The roots and rhizomes of *P. ginseng* were collected from 'The National GAP Base of Chinese Materia Medica for *P. ginseng* C.A. Mey.' at Changbai County, Jilin Province of China in September 2012 and identified by Prof. Xiu-Wei Yang, who is from the State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, China. A voucher specimen (no. 201209JLRS) has been deposited in the State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University (Beijing, China).

3.3 Extraction and isolation

The dried and powdered Ginseng Radix et Rhizoma (6.0 kg) was extracted for six times (the first time for 2 h, and later each time for 1 h) with 48 liters of 70% EtOH aqueous by reflux to give ethanolic extract (1307 g; yield 21.8%). The extract was suspended into 3 liters of H_2O and partitioned with 3 liters of *n*-BuOH saturated with H_2O to obtain an *n*-BuOH extract (300 g; yield 5%) and H_2O-soluble moiety. The *n*-BuOH extract (300 g) was dissolved in H_2O and then subjected to Amberlite XAD-4 resin CC and eluted with a gradient solvent system of H_2O, 60% and 95% EtOH aqueous solutions to give three fractions (Frs 1–3) monitored by TLC. The Fr. 2 (189.0 g) was further subjected to a silica gel CC and eluted with a gradient solvent system of $CHCl_3$–MeOH–H_2O (5:1:0.1 → 4:1:0.1 → 3:1:0.1 → 2:1:0.1 → 1:1:0.1) to yield nine fractions, namely F1 (5.6 g), F2 (4.4 g), F3 (3.3 g), F4 (34.5 g), F5 (18.3 g), F6 (26.4 g), F7 (31.2 g), F8 (50.0 g), and F9 (5.0 g).

The F4 (34.5 g) was subjected to a silica gel CC and eluted with a gradient solvent system of $CHCl_3$–MeOH–H_2O (3:1:0.1 → 2:1:0.1) to obtained six fractions (F4-1–F4-6). The F4-3 was chromatographed on an SP-HPLC (70% MeOH aqueous solution) to obtain two fractions (F4-3-1 and F4-3-2). The F4-3-2 was further purified by SP-HPLC (30% MeCN aqueous solution) to give compound **2** (1378 mg). The F5 (18.3 g) was subjected to a silica gel CC and eluted with a gradient solvent system of $CHCl_3$–MeOH–H_2O (4.5:1.5:0.2 → 2:1:0.2) to afford five fractions (F5-1–F5-5). The F5-4 (8.2 g) was separated by SP-HPLC (75% MeOH aqueous solution) to give three fractions (F5-4-1–F5-4-3). The F5-4-1 (5.4 g) was then chromatographed on SP–HPLC (50% MeOH aqueous solution) to obtain four fractions (F5-4-1-1–F5-4-1-4). The F5-4-1-1 (0.2 g) was further purified by SP-HPLC (20% MeCN aqueous solution) to obtain compound **1** (13.2 mg). At the same time, the F5-4-1-2 (0.5 g) was purified by SP-HPLC (19% MeCN aqueous solution) to afford compound **3** (12.6 mg).

3.3.1 *Ginsenjilinol (**1**)*

A white amorphous powder; $[\alpha]_D^{25}$ −27.6 (*c* 0.456, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3420, 1633, 1077, 1030; For ^1H and ^{13}C NMR spectral data, see Tables 1 and 2; positive ESI-MS: *m/z* 839.5 $[M + Na]^+$; positive HR-ESI-MS: *m/z* 817.4952 $[M + H]^+$ (calcd for $C_{42}H_{73}O_{15}$, 817.4950), 839.4753 $[M + Na]^+$ (calcd for $C_{42}H_{72}NaO_{15}$, 839.4769).

3.4 Acid hydrolysis of 1

Compound **1**, 2 mg in a mixture of 8% HCl (1 ml) and MeOH (10 ml), was heated under reflux for 2 h. The reaction mixture was evaporated to dryness *in vacuo* and dissolved in MeOH (0.5 ml), and then subjected to TLC (eluent: EtOAc/MeOH/

H_2O/HOAc, 6:2:1:1) and PC (eluent: *n*-BuOH/HOAc/H_2O, 4:1:5) together with authentic β-D-glucopyranose. The chromatograms were sprayed with aniline hydrogen phthalate followed by heating at 100°C. As a result, the hydrolysate was coincident with authentic sample. The R_f value was 0.36 for TLC and 0.18 for PC.

3.5 Biological assays

3.5.1 Assay for cell viability

Cell viability was assessed using the MTT assay as described in a previous report [24]. In brief, RAW 264.7 cells were seeded into a 96-well plastic plate at a concentration of 1.5×10^6 cells per well and incubated at 37°C in a humidified air with 5% CO_2 for 12 h. The cells were then treated with various concentrations of assayed compound dissolved in new serum-free DMEM. After additional 20 h incubation at 37°C, aliquots of 100 μl of supernatant were removed carefully from the wells. Then, 20 μl of MTT stock solution (5 mg/ml) supplemented with 100 μl of serum-free medium was added into each well, and the incubation continued for another 4 h. In the end of incubation, the precipitated formazan crystals were dissolved thoroughly with a 100 μl of dissolving solution (10% sodium dodecyl sulfate, 5% isopropanol, and 0.012 M HCl), and the plates were kept on orbital shaker for 12 h at 37°C. The resulting color was assayed at 492 nm using an automated microplate reader. The cell growth was represented with absorbance at a wavelength of 492 nm.

3.5.2 Assay for inhibition of cellular NO production

The cell culture was described in a previous report [25]. Briefly, the cells were seeded in 96-well culture plates with a volume of 100 μl and a concentration of 1.5×10^5 cells/ml, and incubated for 12 h. Then, the cells were treated with 50 μl of LPS (1 μg/ml) and 50 μl of various concentrations (60–200 μM) of assayed compound, and the plates were again incubated for 20 h at 37°C. Aliquots of 100 μl of supernatant were carefully pipetted into 0.1 ml of Griess reagent solution, which were incubated at 37°C for 15 min to form a purple azodye. The absorption at 540 nm was determined by an automated microplate reader. The IC_{50} values were calculated by the software Origin 7.5. The cell viability (>95%) was determined with the MTT assay. Indomethacin was used as a positive control drug.

Acknowledgments

This research was financially supported by the National Key Technology R&D Program of China (2011BAI03B01; 2011BAI07B08), the National Natural Science Foundation of China (30830118), and the "Major New Medicine Project" in Mega-projects of Science Research of China (2009ZX09301-005-028).

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