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The chemical constituents from the roots of *Gentiana crassicaulis* and their inhibitory effects on inflammatory mediators NO and TNF-α

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Abstract: Chemical study on the roots of *Gentiana crassicaulis* Duthie ex Burk (Gentianaceae) afforded 15 compounds, including two new iridoid glycosides, qinjiaosides B (**1**) and C (**2**). Their structures were elucidated by spectroscopic methods and chemical evidence. The isolated iridoid glycosides **1**, **4**–**6** and **8**–**11** were tested for their anti-inflammatory activity by the inhibitory effects on LPS-induced NO and TNF-α production in macrophage RAW264.7 cells. All of them showed inhibitory effects on inflammatory mediators NO at a concentration of 15 μM, while **5** and **9** displayed the most potential inhibitory effects on TNF-α with IC_{50} of 0.06 and 0.05 μM, respectively. The structure-activity relationships (SARs) of these iridoid derivatives were discussed.

Keywords: Gentiana crassicaulis, Gentianaceae, iridoid glycosides, qinjiaosides

Introduction

Rheumatoid arthritis (RA) is characterized as a chronic inflammatory disease, in which the immune system destroys synovial joints and accessory structures.[1] "Qin-Jiao" is a well-known traditional Chinese medicinal (TCM) herb commonly used for fighting RA since ancient times.[2] In the Chinese Pharmacopoeia, the roots of four plants from the genus *Gentiana* (Gentianaceae), *G. macrophylla*, *G. crassicaulis*, *G. straminea*, *G. duhurica*, are used as the original materials of "Qin-Jiao".[3] Among them, *G. macrophylla* have been chemically and biological investigation by several groups,[4–6] whose results suggested that the water extract of *G. macrophylla* could obviously resist RA.[7] The mechanism might be the inhibition of not only phagocyte to produce and release prostaglandin E2 (PGE2), but also the activity of cyclooxygenase-2 (COX-2).[8] Moreover, gentiopicroside (**5**), one of the major compounds in *G. macrophylla*, also showed inhibitory effects on inflammatory mediators NO and COX-2.[9] However, the anti-inflammatory constituents from *G. crassicaulis* have not been reported to date.

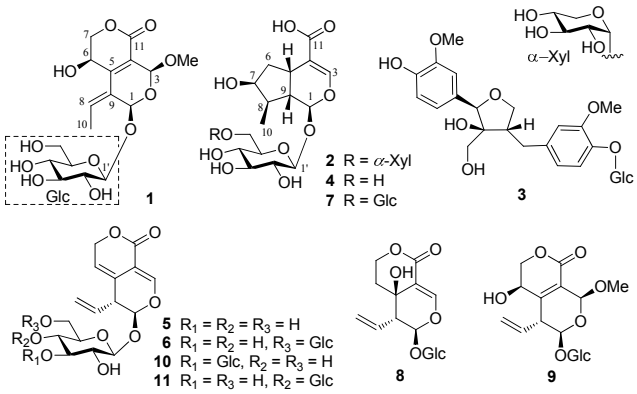

Figure 1. Compounds isolated from the roots of *Gentiana crassicaulis*

As a part of our research on new bioactive compounds from *Gentiana* medicinal plants,[10–15] the present investigation led to the isolation of two new compounds (**1** and **2**) from the roots of *G. crassicaulis*, together with 13 known ones. Their structures were elucidated by detailed 1D and 2D NMR spectroscopic analysis and chemical methods. Most of the isolates (**1**, **4**–**6** and **8**–**11**) were evaluated for their anti-inflammatory activities by inhibition of inflammatory mediators

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NO and TNF-α and their structure–activity relationships (SARs) were discussed.

Results and Discussion

Two new compounds, qinjiaosides B (**1**) and C (**2**), were isolated from the MeOH extract (909 g) of the roots (10 Kg) of *G. crassicaulis*, along with 13 known ones. The known compounds were identified as berchemol-4'-*O*-*β*-D-glucoside (**3**),[16] loganic acid (**4**),[17] gentiopicroside (**5**),[10] 6'-*O*-*β*-D-glucopyranosyl gentiopicroside (**6**),[18] 6'-*O*-*β*-D-glucopyranosyl loganic acid (**7**),[19] swertiamarin (**8**),[20] qinjiaoside A (**9**),[10] 3'-*O*-*β*-D-glucopyranosyl gentiopicroside (**10**),[21] 4'-*O*-*β*-D-glucopyranosyl gentiopicroside (**11**),[22] isoconiferinoside,[23] vanilloloside,[24] paristerone[25] and coniferin,[23] by direct comparison with authentic samples or comparison of the spectral data with those reported in literatures.

Compound **1**, a colorless amorphous powder, had a molecular formula $C_{17}H_{24}O_{11}$ as deduced from the HRESIMS (*m/z* 439.1011 $[M + Cl]^-$). The ^{13}C NMR and DEPT data established the presence of one methyl, one oxygen-bearing methylene, three methines linked with oxygen, one tetra- and one tri- substituted double bonds, one carbonyl, and one methoxy groups, in addition to a hexosyl moiety. In the ^1H NMR spectrum of **1** (Table 1), the signals of two methine protons [δ 5.45 (s, H-3), 6.25 (s, H-1)], a doublet methyl [δ 1.99 (d, J = 7.3 Hz, H-10)], an anomeric proton [δ 4.82 (1H, d, J = 7.8 Hz)], a methoxy proton (δ 3.59, s) and some aliphatic protons were observed. The aforementioned NMR data suggested that **1** was an analogue of compound **9**, an iridoid glucoside from the title plant and *G. macrophylla*.[10] Instead of the terminal double bond between C-10 and C-8 in **9**, compound **1** had a trisubstituted double bond (olefinic proton at δ 6.58, q, J = 7.3 Hz) linked with a methyl group (δ_C 14.3, δ_H 1.99, d, J = 7.3 Hz). In addition, obvious difference at C-8 (δ and 134.5 for **1** and **9**, respectively) was observed. The above data revealed that the terminal double bond between C-10 and C-8 in **9** was rearranged to between C-9 and C-8 in **1**. The locations of the methyl and trisubstituted double bond were further confirmed by the HMBC correlations of the methyl group (δ 1.99) with C-8 (δ 137.2) and C-9 (δ 129.0), and the trisubstituted olefinic proton (δ 6.58, H-8) with C-1 (δ 91.2) and C-5 (δ 145.5) (Figure 2). In the ROESY spectrum of **1** (Figure 2), correlations of H-3/H-1, and the broad singlet H-6 (δ 4.56, br. s) revealed the equatorial orientations of both H-3 and H-6, respectively, that is, both the C-3 methoxyl and C-6 hydroxyl groups were *β* oriented. In addition, significant ROESY correlations of the C-10 methyl protons at δ 1.99 with H-1 (δ 6.25) and the olefinic proton (δ 6.58, H-8) with H-6 (δ 4.56) were observed, revealing the configuration of the double bond between C-8 and C-9. Therefore, the structure of qinjiaoside B (**1**) was assinged as shown in Figure 1.

Compound **2** was obtained as a colorless amorphous powder and its molecular formula $C_{21}H_{32}O_{14}$ was deduced on the basis of HRESIMS (*m/z* 507.1715 $[M - H]^-$). The ^1H NMR spectrum (Table 1) displayed a doublet methyl signal (δ 1.10, d, J = 6.9 Hz), a singlet olefinic proton (δ 7.04, s), two anomeric protons (δ 4.86, d, J = 8.5 Hz; 4.97, d, J = 3.6 Hz), as well as some aliphatic protons. The ^{13}C NMR and DEPT data showed the presence of 21 cabon signals, refering to one methyl, one methylene, five methines including two oxygen-bearing ones, one trisubstituted double bond, one carbonyl, and 11 sugar carbon signals arising from a hexosyl and a pentosyl units. Acid hydrolysis of **2** afforded D-glucose and D-xylose as sugar residue, which were confirmed by GC analysis of their corresponding trimethylsilated L-cysteine adducts. The above data (Table 1) of **2** were closely related to those of loganic acid (**4**), except for the appearance of an additional *α*-xylopyranosyl moiety [anomeric C and H at δ 98.4, δ 4.97 (d, J = 3.6 Hz)]. In the HMBC spectrum of **2** (Figure 2), the xylosyl anomeric proton at δ 4.97 was

Table 1. NMR spectroscopic data [100 (^{13}C) and 400 (^1H) MHz] for compounds 1 and 2

	1[a]		**2**[b]	
pos.	δ_C	δ_H (J in Hz.)	δ_C	δ_H (J in Hz.)
1	91.2 (CH)	6.25, s	96.0 (CH)	5.39, d (2.9)
3	95.6 (CH)	5.45, s	145.2 (CH)	7.04, s
4	120.2 (C)		119.2 (C)	
5	145.5 (C)		30.8 (CH)	3.09, dd (8.3, 3.9)
6	60.6 (CH)	4.56, br. s	40.4 (CH_2)	2.16, m; 1.79, dt (14.5, 8.3)
7	73.3 (CH)	4.46, dd (12.6, 2.1); 4.50, dd (12.6, 1.2)	75.0 (CH)	4.20, td (5.3, 1.7)
8	137.2 (CH)	6.58, q (7.3)	40.1 (CH)	1.97, m
9	129.0 (C)		45.6 (CH)	2.13, m
10	14.3 (CH_3)	1.99, d (7.3)	12.3 (CH_3)	1.10, d (6.9)
11	164.6 (C)		176.2 (C)	
OMe	58.4 (CH_3)	3.59, s		
Glc-1'	99.2 (CH)	4.82, d (7.8)	98.7 (CH)	4.86, d (8.5)
2'	74.9 (CH)	3.20, t (7.8)	72.9 (CH)	3.34, t (8.5)
3'	78.0 (CH)	3.45, m	76.0 (CH)	3.57, m
4'	71.6 (CH)	3.30, m	69.6 (CH)	3.65, m
5'	78.5 (CH)	3.40, m	76.0 (CH)	3.57, m
6'	62.9 (CH_2)	3.93, dd (11.8, 2.1); 3.69, dd (11.8, 6.1)	65.9 (CH_2)	3.98, dd (11.7, 3.4); 3.83, dd (11.7, 5.4)
Xyl-1"			98.4 (CH)	4.97, d (3.6)
2"			73.5 (CH)	3.73, m
3"			74.8 (CH)	3.72, m
4"			71.8 (CH)	3.57, m
5"			61.6 (CH_2)	3.74, m; 3.61, m

[a]Recorded in CD_3OD; [b]Recorded in D_2O

correlated with the glucosyl C-6' (δ 65.9), indicating the xylosyl moiety was linked to the glucosyl C-6' in **2**. In addition, the chemical shift of the glucosyl C-6' of **2** was down-field shifted by 4.6 ppm in the ^{13}C NMR spectrum, relative to **4**. In the ROESY spectrum of **2** (Figure 2), correlations of H-8 (δ 1.97) with H-1 (δ 5.39) and H-7 (δ 4.20), H-5 (δ 3.09) with H-9 (δ 2.13) and H-6a (δ 2.16), H-6b (δ 1.79) with H-7 and H-6a were observed, revealing the aglycone configuration of compound **2** as shown in Figure 1. Thus, the structure of **2** was established as 6'-*O*-α-D-xylopyranosyl loganic acid, namely as qinjiaoside C.

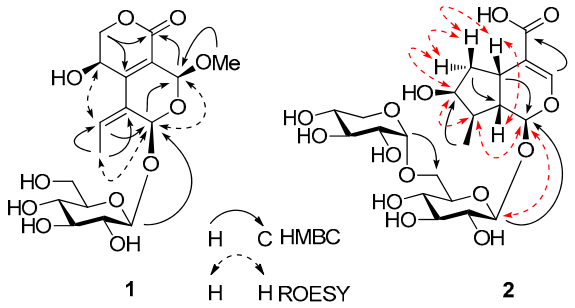

Figure 2. The key HMBC and ROESY correlations for compounds **1** and **2**

The isolated iridoid glycosides **1**, **4**–**6** and **8**–**11** were tested for their anti-inflammatory activities by inhibitory effects on LPS-induced NO and TNF-α production in macrophage RAW264.7 cells (Table 2). All of them showed inhibitory effects on inflammatory mediators NO at a concentration of 15 μM, while **5** and **9** displayed the most potential inhibitory effects on TNF-α with IC$_{50}$ of 0.06 and 0.05 μM, respectively. It is noted that the terminal double bond between C-9 and C-10 should be a key to the inhibitory effect on TNF-α. For example, compound **1** did not display activity due to the absence of the terminal double bond. In addition, compounds **6** and **10** and **11**, possessing longer sugar chain than that of **5**, had not inhibitory effects on TNF-α, revealing that the number of monosaccharide units in their sugar chains could also affect their anti-inflammatory activities.

In conclusion, 15 compounds including two new iridoid glycosides (**1** and **2**) were identified. The iridoid glycosides **1**, **4**–**6**, and **8**–**11** showed inhibitory effects on inflammatory mediators NO, while only **4**, **5**, **8** and **9** displayed potential inhibitory effects on TNF-α. This study provided valuable information for "Qin-Jiao" as a TCM herb traditionally used for fighting RA. Further chemical and biological investigations on the iridoid scaffolds from *Gentiana* are currently underway, in order to explore the therapeutic potential of this important class of natural products as anti-inflammatory leads for drug discovery.

Table 2. Effect of compounds 1, 4–6, 8–11 on NO and TNF-α production

compounds	% of inhibition NO (15 μM)	IC$_{50}$ of inhibition TNF-α (μM)
1	27.0 ± 0.4	> 50
4	10.3 ± 4.0	11.84
5	19.0 ± 2.9	0.06
6	27.6 ± 2.2	> 50
8	15.3 ± 2.4	48.4
9	33.9 ± 2.1	0.05
10	26.6 ± 0.6	> 50
11	32.1 ± 1.4	> 50
control*	10.1 ± 0.4	**

*NG methyl L-arginie acetate

**Cell activated with LPS without compound was used as control.

Experimental Section

General Experimental Procedures. Optical rotations were performed on a P-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra were measured on a Bruker Tensor 27 spectrometer with KBr pellets. 1D and 2D NMR spectra were run on Bruker AM-400 and DRX-500 instruments operating at 400 and 500 MHz for ^{1}H, and 100 and 125 MHz for ^{13}C, respectively. Coupling constants are expressed in Hertz and chemical shifts are given on a ppm scale with tetramethylsilane as internal standard. The MS data were recorded on a VG Auto Spec-3000 spectrometer (VG, Manchester, U.K.) with glycerol as the matrix. HRESIMS were recorded on an API Qstar Pulsa LC/TOF spectrometer. GC analysis was run on a Shimadzu GC-14C gas chromatograph.

Column chromatography was performed with Diaion 101 resin (Tianjin Haiguang Chemical Co., Ltd. Tianjin, China), silica gel (200–300 mesh, Qingdao Makall Group Co., Ltd. Qingdao, China), MCI gel CHP 20P (Mitsubishi Chemical Co. Tokyo, Japan), Rp-8 gel, Rp-18 gel (40–60 μm, Merck, Darmstadt, Germany) and a 250 × 9.4 mm, i.d., 5μm Zorbax SB-C$_{18}$ column (Agilent, California, USA). Thin-layer chromatography (TLC) was carried out on silica gel H-precoated plates (Qingdao Makall Group Co., Ltd.) with $CHCl_3$/MeOH/H_2O (8.5:1.5:0.1, 8:2:0.2 or 7:3:0.5, v/v), RP-8 and RP-18 precoated plates (Merck) with MeOH/H_2O (7:3 or 8:2, v/v). Spots were detected by spraying with 10% H_2SO_4 in EtOH followed by heating.

Plant Material. The roots of *Gentiana crassicaulis* Duthie ex Burk. were collected in Lijiang, Yunnan province, China, on June 2007, and identified by Professor Chong-Ren Yang. A voucher specimen (KUN_550904) has been deposited in Herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences (CAS).

Extraction and Isolation. Dried, powdered roots of *G. crassicaulis* (10 Kg) were extracted under reflux with methanol (1000 mL) for 3 times, each for 3 h. The MeOH extract (909 g) afforded after evaporation of the solvent. Then the extract (909 g) was suspended in H_2O (2000 mL) and extracted with chloroform (2000 mL × 3).The aqueous layer (707 g) was subjected to a column of Diaion HP20SS, eluting with MeOH/H_2O (0:1–1:0) to give five fractions (Fr. 1–5). Fr. 1 (42.6 g) was subjected to Chromatorex ODS with MeOH/H_2O (10% to 35% with a 5% increment) to afford **4** (9.4 g), **7** (7 mg) and vanilloloside (5 mg). Fr. 3 was repeatedly chromatographed over MCI-gel CHP-20P (MeOH/H_2O, 5% to 45% with a 5% increment), silica gel ($CHCl_3$/MeOH/H_2O, 9:1:0.1 to 6:4:1) and Rp-18 (MeOH/H_2O, 35% to 55% with a 5% increment) to yield **2** (13 mg), **5** (12.4 g), **6** (2.7 g), **8** (223 mg),

10 (10 mg), **11** (28 mg), isoconiferinoside (9 mg), paristerone (3 mg), and coniferin (5 mg). Compounds **1** (28 mg), **3** (10 mg), and **9** (11 mg) were obtained from Fr. 4 (52.8 g) by repeated MCI-gel CHP-20P ($MeOH/H_2O$, 10% to 35% with a 5% increment), silica gel ($CHCl_3/MeOH/H_2O$, 9:1:0.1 to 6:4:1) and Rp-18 ($MeOH/H_2O$, 40% to 60% with a 5% increment) column chromatographes.

Qinjiaoside B (1): colorless amorphous powder; $[\alpha]_D^{20}$ – 48.9 (*c* 0.012 MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.66) and 273 (3.96) nm; IR (KBr)ν_{max}: 3423 (OH), 2927, 1704 (C=O), 1640 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) see Table 1; ESIMS (negative ion) *m/z* 439 [M + $Cl]^-$; HRESIMS *m/z* 439.1011 (calcd for $C_{17}H_{24}O_{11}Cl$, 439.1007).

Qinjiaoside C (2): colorless amorphous powder; $[\alpha]_D^{20}$ – 12.7 (*c* 0.010, H_2O); UV (D_2O) λ_{max} (log ε): 193 (3.94), 225 (3.73) nm; IR (KBr)ν_{max}: 3424 (OH), 1644, 1412 cm^{-1}; ^1H NMR (400 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O) see Table 1; ESIMS (negative ion) *m/z* 507 $[M - H]^-$; HRESIMS *m/z* 507.1715 (calcd for $C_{21}H_{31}O_{14}$, 507.1713).

Acidic Hydrolysis of Compounds 1 and 2. Compounds **1** (4 mg) and **2** (6 mg) in 1M HCl-dioxane (1:1, v/v, 4 mL) were heated at 90 ˚C on water bath for 6 h, respectively. The reaction mixture was partitioned between $CHCl_3$ and H_2O four times. The aqueous layer was passed through an Amberlite IRA-401 (OH^- form), and eluate was concentrated to dryness to give a saccharide mixture. TLC analysis indicated the presence of glucose in the water layer for compound **1** (isopropanol-MeOH-H_2O, 25:1:2, R_f 0.6), while the presence of glucose and xylose in the water layer for the compound **2** [isopropanol-MeOH-H_2O, 25:1:2, R_f 0.60 (glucose); R_f 0.45 (xylose)] were observed. The solution of the sugar residue of compounds **1** and **2** together with the standard D/L glucose and D/L xylose in 1.5 mL pyridine was added to L-cysteine methyl ester hydrochloride and kept at 60 ˚C for 1h, respectively. The trimethylsilylimidazole was added to the reaction mixture and kept again at 60˚C for 30 min, and then the supernatants (4 L) were analyzed by GC, respectively. By comparison of the retention times with those of the standard sugars' derivatives, the monosaccharides of compounds **1** and **2** were determined to be D-glucose for **1**, and D-glucose and D-xylose for **2**, respectively.

Cell Culture.[26] The RAW264.7 murine macrophage cell line (ATCC TIB-71; American Type Culture Collection, Manassas,VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovineserum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a 37 °C incubator with 5% CO_2. For all experiments, the cells were grown to 80–90% confluence, with no more than 20 passages. Cells were stimulated by LPS (0.5 μg mL^{-1}) in the presence or absence of compounds for the measurement of the production of pro-inflammatory cytokines (TNF-α) and accumulation of nitric oxide (NO).

Measurement of NO release.[26] RAW264.7 cells were pretreated by tested compounds for 1 h and stimulated by LPS (0.5 μg mL^{-1}) after 18 h of incubation. NO production was estimated from the amount of stable nitrite produced in the cell culture supernatants measured photometrically by the Griess assay against a standard curve obtained with different concentrations of sodium nitrite. Each experiment was performed three times in duplicate.

Measurement of TNF-α production.[26] RAW264.7 cells were cultured in 96-well plates (1×10^4 cell mL^{-1}) and pre-incubated with compounds for 1 h, followed by a further 18 h treatment with LPS for measurement of TNF-α. Contents of TNF-α in the culture medium were measured by ELISA using anti-mouse TNF-α antibodies and a biotinylated secondary antibody, according to the manufacturer's instructions. The optical density of each well was measured at 450 nm with an ELISA reader (Molecular Devices 5, Menlo Park, CA, USA).

Statistical Analysis.[26] Results are expressed as the mean ± SD of three experiments. Statistical significance was evaluated by one-way ANOVA followed by Student's *t* test for paired populations. P values < 0.05 are considered statistically significant.

Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/ 10.1007/s13659-012-0067-3 and is accessible for authorized users.

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