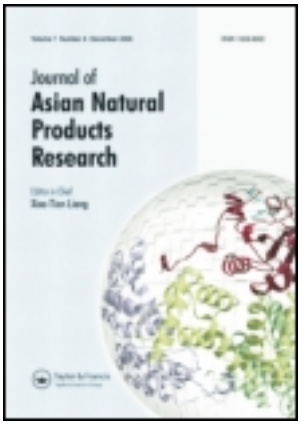

Journal of Asian Natural Products Research

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

Steroidal alkaloids from the bulbs of *Fritillaria unibracteata*

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Available online: 25 Nov 2011

To cite this article: Qing-Jian Zhang, Zhong-Fei Zheng & De-Quan Yu (2011): Steroidal alkaloids from the bulbs of *Fritillaria unibracteata* , Journal of Asian Natural Products Research, 13:12, 1098-1103

To link to this article: http://dx.doi.org/10.1080/10286020.2011.619980

Journal of Asian Natural Products Research
Vol. 13, No. 12, December 2011, 1098–1103

Steroidal alkaloids from the bulbs of *Fritillaria unibracteata*

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(*Received 13 June 2011; final version received 31 August 2011*)

Two new steroidal alkaloids peimisine-3-*O*-β-D-glucopyranoside (**1**) and puqiedinone-3-*O*-β-D-glucopyranoside (**3**), together with three known compounds peimisine (**2**), puqiedinone (**4**), and puqiedine (**5**), were isolated and characterized from the bulbs of *Fritillaria unibracteata*. Their structures were fully elucidated by spectroscopic and chemical methods. Compound **1** showed moderate protection effect on neurotoxicity of PC12 cell lines induced by rotenone.

Keywords: *Fritillaria unibracteata*; steroidal alkaloid; peimisine-3-*O*-β-D-glucopyranoside; puqiedinone-3-*O*-β-D-glucopyranoside

1. Introduction

Fritillaria unibracteata belongs to the genus *Fritillaria* of the family Liliaceae, whose bulbs, named 'Chuan Bei Mu', have been used as an antitussive, antiasthmatic, and expectorant agents in traditional Chinese medicine for hundreds of years and included in the Chinese Pharmacopoeias [1]. Previous investigation on the pharmacology and phytochemistry of the title plant suggested that the steroid alkaloids were responsible for the antitussive effects of these herbs [2]. In our phytochemical investigation on bioactive constituents in the bulbs of *F. unibracteata*, two new steroidal alkaloids peimisine-3-*O*-β-D-glucopyranoside (**1**) and puqiedinone-3-*O*-β-D-glucopyranoside (**3**), together with three known compounds peimisine (**2**), puqiedinone (**4**), and puqiedine (**5**), were isolated and characterized (Figure 1). This paper describes the isolation and structural elucidation of these alkaloids and their protective effects of PC12 cells.

2. Results and discussion

Compound **1** was isolated as a white powder with $[\alpha]_D^{20}$ −33.3 ($c = 0.19$, MeOH), showing a positive Dragendorff reaction. The HR-ESI-MS exhibited a quasi-molecular ion at *m/z* 590.3701 $[M + H]^+$, corresponding to the molecular formula $C_{33}H_{51}NO_8$, with nine degrees of unsaturation. The ESI-MS^2 exhibited a fragment ion at *m/z* 428.4 $[M + H\text{-}162]^+$, indicating the presence of a hexose moiety in compound **1**. The IR spectrum showed absorption bands of hydroxyl (3393 cm^{-1}), carbonyl (1704 cm^{-1}), the C=C double bond (1649 cm^{-1}), and the typical absorptions of a tetrahydrofuran ring at 1121, 986, and 929 cm^{-1} [3,4].

In the ^1H NMR spectrum, the signals due to two tertiary methyl groups at δ 0.51 (3H, s, H-19) and 1.69 (3H, s, H-18), two secondary methyl groups at δ 0.82 (3H, d,

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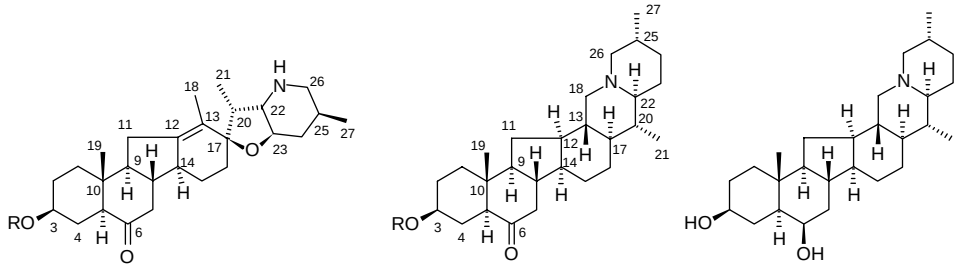
ISSN 1028-6020 print/ISSN 1477-2213 online

http://dx.doi.org/10.1080/10286020.2011.619980
http://www.tandfonline.com



1 R = β-D-glc
2 R = H
3 R = β-D-glc
4 R = H
5

Figure 1. The structures of compounds **1**–**5**.

$J = 6.3$ Hz, H-27) and 1.09 (3H, d, $J = 6.9$ Hz, H-21), and two oxygenated methine groups at δ 3.93–4.08 (overlapped, H-3) and 3.40 (m, H-23) were observed. The ^{13}C NMR spectrum (Table 1) revealed the presence of one carbonyl group at δ 209.7 (C-6); a double bond moiety at δ 128.9 (C-12) and 140.7 (C-13); three oxygenated carbons at δ 76.9 (C-3), 85.1 (C-17), and 75.6 (C-23); two nitrogenated carbons at δ 66.9 (C-22) and 54.9 (C-26); and four methyls at δ 13.4 (C-18), 12.3 (C-19), 11.2 (C-21), and 19.0 (C-27). In the ^{1}H and ^{13}C NMR spectra, the signals of a hexose moiety at δ_H 5.05 (1H, d, $J = 7.8$ Hz, H-1′), 3.93–4.08 (2H, m, H-2′,5′), 4.21–4.32 (2H, m, H-3′,4′), 4.38–4.44 (1H, m, H-6b), and 4.62 (1H, br d, $J = 11.2$ Hz, H-6a), and at δ_C 102.2 (C-1′), 75.4 (C-2′), 78.6 (C-3′), 71.8 (C-4′), 78.6 (C-5′), and 63.0 (C-6′) were assignable to a β-glucopyranosyl moiety [5].

The spectral data of aglycon moiety mentioned above were almost identical to those of peimisine (**2**), except for the obvious downfield shift of C-3 from δ_C 70.7 to δ_C 76.9 [3], suggesting the structure of **1** was peimisine glucoside, and the glucosidated site at C-3. In the HMBC spectrum (Figure 2), the long-range correlations of H-1′/C-3 was observed, which indicated that the glucose was connected to C-3. After compound **1** was hydrolyzed with β-glucosidase [5],

Table 1. The ^{13}C NMR spectral data of compounds **1**–**5**.

No	**1**[a]	**2**[b]	**3**[a]	**3**[b]	**4**[b]	**5**[b]
1	39.4	39.0	36.8	37.0	37.0	39.1
2	31.9	31.6	28.8	28.7	30.5	31.3
3	76.9	70.7	76.9	75.6	70.9	71.9
4	37.1	37.0	27.0	26.4	30.2	34.8
5	56.3	56.6	56.6	56.6	56.8	48.3
6	209.7	210.6	210.0	211.6	211.2	72.8
7	45.9	45.7	45.9	45.9	46.0	38.5
8	46.5	46.0	40.2	40.2	40.2	34.7
9	54.4	54.4	56.5	56.5	56.8	57.6
10	38.4	38.4	38.4	38.6	38.4	35.4
11	27.0	28.5	30.0	29.7	30.1	29.3
12	128.9	128.4	40.2	41.0	41.2	40.2
13	140.7	141.0	40.7	41.0	41.0	44.6
14	48.7	48.5	44.3	44.2	44.4	44.0
15	24.7	24.1	25.0	25.2	25.3	24.7
16	29.2	29.9	24.4	24.4	24.5	25.5
17	85.1	85.0	45.9	46.1	46.4	46.5
18	13.4	13.1	63.0	64.3	64.7	61.5
19	12.3	12.4	12.6	12.9	12.8	15.1
20	40.2	39.5	45.9	44.2	44.8	41.3
21	11.2	10.6	14.7	14.8	14.8	14.9
22	66.9	66.1	68.3	68.5	68.4	68.4
23	75.6	75.5	33.0	33.2	33.4	30.2
24	28.7	30.3	29.2	28.6	28.8	33.5
25	31.3	31.6	30.0	30.4	30.8	30.8
26	54.9	54.5	63.0	60.9	61.2	64.7
27	19.0	18.8	19.3	19.5	19.6	19.7
1′	102.2		102.2	101.0		
2′	75.4		75.4	73.5		
3′	78.6		78.6	78.0		
4′	71.8		71.8	70.0		
5′	78.6		78.6	76.4		
6′	63.0		63.0	61.9		

[a] C_5D_5N.
[b] $CDCl_3$.

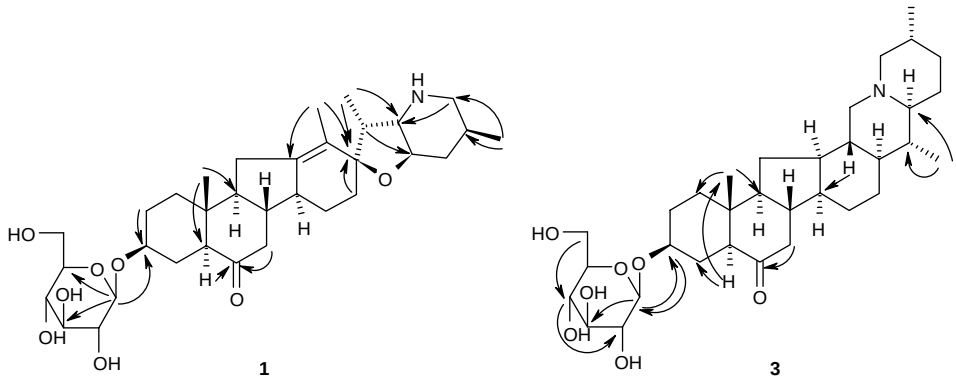


Figure 2. Key HMBC correlations of compounds **1** and **3**.

both of peimisine and D-glucose were detected. This further supported the above result. Thus, the structure of **1** was elucidated as peimisine-3-*O*-β-D-glucopyranoside (Figure 1).

Compound **3** was obtained as a white powder with $[\alpha]_D^{20}$ −41.6 ($c = 0.08$, MeOH) and showed a positive Dragendorff reaction. The molecular formula $C_{33}H_{53}NO_7$ was assigned on the basis of the quasi-molecular ion peak at *m/z* 576.3907 $[M + H]^+$ in its HR-ESI-MS. The ion at *m/z* 414 $[M - 162 + H]^+$ indicated the presence of a hexose moiety. The IR spectrum of **3** displayed the absorption bands of hydroxyl (3398 cm^{-1}), carbonyl (1704 cm^{-1}), and *trans*-quinolizidine (2750 cm^{-1}) moieties [6].

The ^1H NMR (500 MHz, C_5D_5N) spectrum disclosed three methyl signals at δ 0.69 (3H, br s, H-19), 0.78 (6H, br d, $J = 5.5$ Hz, H-21, 27). An oxy-substituted methine at δ 3.94 (1H, m, H-3) with $W_{1/2} > 25$ Hz indicated a β-configuration of RO-3 [7]. The signals of a β-glucopyranosyl moiety at δ 5.02 (1H, d, $J = 7.5$ Hz, H-1′), 4.02–4.10 (1H, m, H-2′), 4.20–4.30 (2H, m, H-3′,4′), 3.95–4.01 (1H, m, H-5′), 4.60 (1H, d, $J = 12.0$ Hz, H-6′), and 4.40 (1H, dd, $J = 6.0, 12.0$ Hz, H-6′) were observed. ^{13}C NMR (125 MHz, C_5D_5N) spectrum (Table 1) disclosed the presence of three methyl groups at δ_C 12.6 (C-19), 14.7 (C-21), and 19.3 (C-27), one carbonyl group at δ 210.0 (C-6), besides the six carbon signals at δ_C 102.2, 75.4, 78.6, 71.8, 78.6, and 63.0 assignable to the β-glucopyranosyl moiety. To compare the carbon signals of aglycon with those of known compounds, the ^{13}C NMR spectrum of **3** was tested in the commonly used solvent $CDCl_3$, in which the signals of aglycon were similar to those of puqiedinone (**4**), except for the obvious downfield shift of C-3 from δ_C 70.9 to δ_C 75.6 [6]. The spectral data suggested that compound **3** was puqiedinone glucoside and the glycosidated site at C-3. In the HMBC spectrum, the long-range correlations of H-1′/C-3 and H-3/C-1′ confirmed that the glucose located at C-3 (Figure 2). After the hydrolysis of compound **3** in the same way as **1**, D-glucose and puqiedinone were detected. Thus, the structure of compound **3** was elucidated as puqiedinone-3-*O*-β-D-glucopyranoside (Figure 1).

Compounds **2**, **4**, and **5** were deduced to be peimisine (**2**) [3], puqiedinone (**4**) [6], and puqiedine (**5**) [8] by comparison of their spectral data with those reported in the literature.

The effect of compounds **1**–**4** on neurotoxicity of PC12 cell lines induced by rotenone was evaluated (Table 2). Compound **1** showed moderate protection effect with cell viability rate 46.3 ± 0.6% ($p < 0.01$) at 10 μM.

Table 2. The effect of compounds **1**–**4** on neurotoxicity of PC12 cell lines induced by rotenone (means ± SD, $n = 3$).

	Cell survival rate (%)
Control	100.0 ± 5.4
Model	42.3 ± 1.6$^{\#\#\#}$
1	46.3 ± 0.6**
2	40.8 ± 2.6
3	45.0 ± 2.7
4	39.9 ± 1.8

Note: $^{\#\#\#}p < 0.001$ vs. control; $^{**}p < 0.01$ vs. model.

3. Experimental

3.1 General experimental procedures

Optical rotations were obtained on a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Waltham, MA, USA). IR spectra were recorded on a Nicolet IMPACT-400 spectrophotometer (Thermo Electron, Madison, WI, USA) as KBr disks. Mass spectra were obtained on the AutoSpec Ultima-TOF instruments (Micromass, Manchester, UK). NMR spectra were recorded with Mercury-300 and INOVA-500 spectrophotometer (Varian, Palo Alto, CA, USA). Silica gel (200–300 mesh) for column chromatography and silica gel GF254 for TLC were obtained from Qingdao Marine Chemical Factory (Qingdao, China). Size-exclusion chromatography was carried out using Sephadex LH-20 (Pharmacia, Uppsala, Sweden). HPLC were carried out on a Lumtech KT-501 chromatograph (Knauer, Berlin, German) with YMC ODS-A C-18 column (20 × 250 mm, YMC, Kyoto, Japan) and an Alltech 2000 ELSD detector (Alltech, Deerfield, IL, USA). GC was conducted on an Agilent 7890A instrument (Agilent, Waldbronn, Germany).

3.2 Plant material

The bulbs of *F. unibracteata* were collected in July 2007 from the Ruo-Er-Gai County, Sichuan Province, China, which were identified by Prof. Shi-Lin Chen, the Institute of Medicine Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (ID-S-2371) has been deposited at the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

3.3 Extraction and isolation

The dried plant materials (4.9 kg) were powdered and refluxed with MeOH for three times under reflux to give 230 g of an extract, which was dissolved in 1% HCl. After filtered, the acidic solution was extracted with petroleum ether. Then, it was basified with ammonia to pH > 11 and extracted with chloroform to give the crude total alkaloid 1.45 g, which was further fractionated by column chromatography on silica gel with petroleum ether–acetone–Et_3N (200:20:1 to 40:20:1) to yield **4** (15 mg) and seven fractions. Fraction 5 (27 mg) and fraction 6 (57 mg) were subjected to a silica gel H column (ϕ 1.5 × 20 cm) and eluted with petroleum ether–acetone–Et_3N (30:10:1) to provide compounds **5** (2 mg) and **2** (20 mg), respectively. Fraction 7 (1.17 g) was isolated with HPLC to yield **1** (10 mg, R_t 27.3 min) and **3** (6 mg, R_t 53.7 min). The HPLC was carried out with YMC column (C-18, 20 × 250 mm), mobile phase acetonitrile-0.2% Et_3N/H_2O (acetonitrile increased from 10% to 100% in 60 min, 5 ml/min), and the Alltech 2000 ELSD detector (3.1 l/min velocity of air flow and 110°C of the drift tube).

3.3.1 Peimisine-3-O-β-D-glucoside (*1*)

White powder, $[\alpha]_D^{20}$ −33.3 ($c = 0.19$, MeOH), positive Dragendorff reaction. IR (FT-IR microscope transmission) ν_{max}: 3393, 2925, 2856, 1704, 1649, 1121, 1077, 1035, 986, 929 cm^{-1}. ^1H NMR (300 MHz, C_5D_5N): δ 0.51 (3H, s, H-19), 0.82 (3H, d, $J = 6.3$ Hz, H-27), 1.09 (3H, d, $J = 6.9$ Hz, H-21), 1.69 (3H, s, H-18), 3.93–4.08

(overlapped, H-3), 3.40 (m, H-24) 5.05(1H, d, $J = 7.8$ Hz, H-1′), 3.93–4.08 (2H, m, H-2′,5′), 4.21–4.32 (2H, m, H-3′,4′), 4.38–4.44 (1H, m, H-6b), 4.62 (1H, br d, $J = 11.2$ Hz, H-6a). ^{13}C NMR spectral data (125 MHz, C_5D_5N), see Table 1. ESI-MS: *m/z* 612.5 $[M + Na]^+$, 590.5 $[M + H]^+$. ESI-MS^2: *m/z* 428.4 $[M + H-162]^+$. HR-ESI-MS: *m/z* 590.3701 $[M + H]^+$ (calcd for $C_{33}H_{52}NO_8$, 590.3687).

3.3.2 *Puqiedinone-3-O-β-D-glucopyranoside (**3**)*

White powder, $[\alpha]_D^{20}$ −41.6 ($c = 0.08$, MeOH), positive Dragendorff reaction. IR (FT-IR microscope transmission) ν_{max}: 3398, 2915, 2749, 1704, 1455, 1373, 1078, 1034 cm^{-1}. ^{1}H NMR (500 MHz, C_5D_5N): δ 0.69 (3H, br s, H-19), 0.78 (6H, br d, $J = 5.5$ Hz, H-21 and H-27), 5.02 (1H, d, $J = 7.5$ Hz, H-1′), 4.02–4.10 (1H, m, H-2′), 4.20–4.30 (2H, m, H-3′,4′), 3.95–4.01 (1H, m, H-5′), 4.60 (1H, d, $J = 12.0$ Hz, H-6′), 4.40 (1H, dd, $J = 6.0$, 12.0 Hz, H-6′), 3.94 (1H, m, $W_{1/2} > 25$ Hz, H-3). ^{13}C NMR (125 MHz) spectral data, see Table 1. ESI-MS: *m/z* 598.4 $[M + Na]^+$, 576.5 $[M + H]^+$, 414.3 $[M-162 + H]^+$. HR-ESI-MS: *m/z* 576.3907 $[M + H]^+$ (calcd for $C_{33}H_{54}NO_7$, 576.3895).

3.3.3 *The hydrolysis of **1** and **3** with β-glucosidase*

Compound **1** (2 mg) was dissolved in 100 μl DMSO, and 0.5 mg β-glucosidase (from almonds, TCI Corp., Tokyo, Japan) was added. The mixture was diluted to 1 ml, and hydrolyzed at 40°C for 10 h. The mixture was separated through the ODS solid phase extraction eluted with water and MeOH, respectively. Peimisine was detected in the MeOH-eluted fraction by TLC analysis. The water-eluted fraction was evaporated under vacuum, and the residue was dissolved in anhydrous pyridine (1 ml), to which L-cysteine methyl ester hydrochloride (2 mg) was added and stirred at 60°C for 3 h. After the mixture evaporated *in vacuo* to dryness, 0.2 ml of *N*-trimethylsilylimidazole was added and kept at 60°C for another 3 h. The reaction mixture was partitioned between *n*-hexane and H_2O, and the *n*-hexane extract analyzed by GC under the following conditions: capillary column, HP-5 (30 m × 0.25 mm, with a 0.25 μm film, Dikma, Lake Forest, CA, USA); detection, Flame ionization detector; detector temperature, 280°C; injection temperature, 250°C; initial temperature 160°C, then raised to 280 at 5°C/min, final temperature maintained for 10 min; carrier, N_2 gas. From the hydrolysate of **1**, D-glucose was confirmed by comparison of the retention time of its derivative with that of authentic sugar derivatized in a similar way, which showed the retention time of 19.1 min. Compound **3** was hydrolyzed and detected in the same method as compound **1**, and puqiedinone and D-glucose were identified in the MeOH and water fractions, respectively.

3.4 *PC12 cells protective assay*

PC12 cells at a density of 5×10^3 cells per well in 96-well plates were cultured in Dulbecco's modified eagle medium media supplemented with 5% fetal bovine serum and 5% horse serum, and L-glutamine (2 mM). Cultures were maintained at 37°C in 5% CO_2 in a humidified incubator. On the second day after plating, compounds at concentrations of 10 and 4 μM rotenone were added to the cells. After incubation for another 48 h, 10 μl of the 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and maintained for 4 h. Absorbance was measured at 570 nm using an Ultramark microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was evaluated.

Acknowledgements

This research program was financially supported by National Science and Technology

Project of China (No. 2009ZX09311-004), and the Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Peking Union Medical College, Ministry of Education. Our gratitude is extended to Prof. Jin-Feng Hu at the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College by bioassays.

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