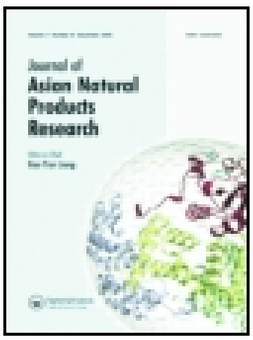

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Chemical constituents from *Bletilla striata* and their NO production suppression in RAW 264.7 macrophage cells

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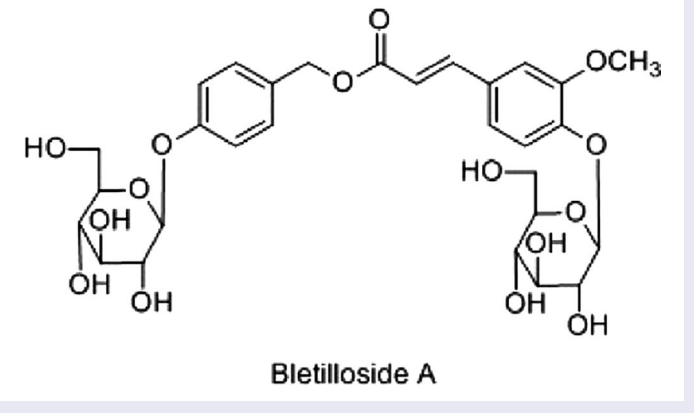
ABSTRACT

A novel glucoside bletilloside A (**1**) was isolated from the tubers of *Bletilla striata*, together with seven known compounds (**2**–**8**). Their structures were determined on the basis of extensive spectroscopic analyses. All compounds were evaluated for the inhibition on NO production effects in RAW 264.7 macrophage cells, while militarine (**4**) and dactylorhin A (**5**) exhibited moderate inhibitory effects.



Bletilloside A

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KEYWORDS
Glucosides; *Bletilla striata*; Orchidaceae; NO production

1. Introduction

The tubers of *Bletilla striata*, a traditional Chinese medicine known as "Bai-ji", have been widely used in the treatment for hematemesis, hemoptysis, traumatic bleeding, chapped skin, and ulcerative carbuncle [1]. Previous phytochemical investigations on *B. striata* resulted in the isolation of bibenzyls [2–4], phenanthrenes [5–8], triterpenoids [9,10], steroids [11,12], and glucosyloxybenzyl 2-isobutylmalates [13,14]. Their biological activities include cytotoxicity [4,11,12], antimicrobial [7], and anti-inflammation [12,14].

In our research on chemical constituents of its tubers, a new glucoside bletilloside A (**1**), together with seven known compounds shancigusin I (**2**), bleformin J (**3**), militarine (**4**), dactylorhin A (**5**), 1-(4-β-D-glucopyranosyloxybenzyl) 4-methoxyl

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(2*R*)-2-hydroxyisobutylmalate (**6**), 5-methoxybibenzyl-3, 3′-di-*O*-*β*-D-glucopyranoside (**7**), and gastrodin (**8**) (Figure 1), was observed. Herein, we report the isolation, structural elucidation, and biological activity of these compounds.

2. Results and discussion

The molecular formula ($C_{29}H_{36}O_{15}$) of **1** was determined by the HR-ESI-MS and NMR data, requiring 12 degrees of unsaturation. The IR absorption bands suggested the presence of OH group (3422 cm^{-1}) and *α*, *β*-unsaturated ester group (1701 and 1637 cm^{-1}). Analyses of NMR data revealed **1** as the analog of known compound shancigusin I (**2**), which was isolated from *Pleione yunnanensis* [15]. The distinction was attributed to the substituted benzene ring in cinnamoyl moiety, in which the ^{1}H-NMR spectrum (Table 1) showed an ABX system [δ_H 7.33 (1H, d, J = 1.7 Hz), 7.57 (1H, d, J = 8.7 Hz), 7.22 (1H, d, J = 1.7, 8.7 Hz)], and one methoxyl group at δ_H 3.78 (3H, s), establishing the benzene ring to be methoxy-substituted, which was supported by the HMBC correlations (Figure 2). The NOE correlation from H-2 (δ_H 7.33) to the methoxyl group (δ_H 3.78) and H-8 (δ_H 6.70) indicated the methoxy group was located at C-3 (Figure 3). The sugar moieties were confirmed as *β*-D-glucose by the coupling constants (7.2 and 7.2 Hz, respectively) and comparison with the authentic sugar by the acid hydrolysis. Thus, compound **1** was determined as bletilloside A.

The known compounds bleformin J (**3**) [16], militarine (**4**) [17], dactylorhin A (**5**) [18], 1-(4-*β*-D-glucopyranosyloxybenzyl) 4-methoxyl (2*R*)-2-isobutylmalate (**6**) [19], 5-methoxybibenzyl-3, 3′-di-*O*-*β*-D-glucopyranoside (**7**) [20], and gastrodin (**8**) [21], were identified by comparison of their spectroscopic data with reported values.

All the compounds were evaluated for their suppressive activity against the LPS-induced NO production in RAW 264.7 macrophage cells. As NO plays a pivotal role in inflammation, the steroidal anti-inflammatory drug dexamethasong (Dex) was selected as positive control. The cytotoxicity of compounds **1**–**8** (0–240 μM) showed no cell viability on RAW 264.7 macrophage over 24 h using an MTT method. Therefore, the suppressive effects of **4** and **5** on NO production could not be ascribed to cytotoxicity, and exhibited moderate inhibitory NO production effects with IC_{50} values of 48.5 and 51.7 μM, respectively (Table 2).

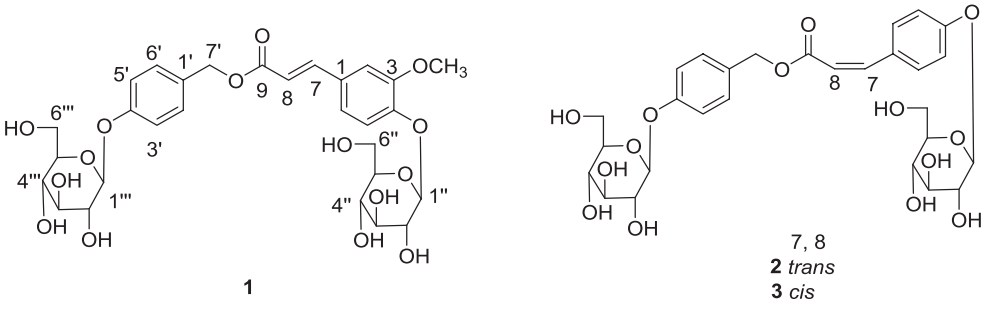

Figure 1. Structures of compounds **1**–**3**.

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1** in pyridine-d_5 (at 600 and 125 MHz, resp.).

	δ_C(ppm)	δ_H(ppm)
1	129.2, C	
2	112.1, CH	7.33, d (1.7)
3	150.4,C	
4	145.6, C	
5	116.3, CH	7.57, d (8.7)
6	123.1, CH	7.22, dd (1.7, 8.7)
7	145.6, CH	7.94, d (15.9)
8	118.0, CH	6.70, d (15.9)
9	167.4, C	
1′	130.8, C	
2′,6′	130.7, CH	7.46, d (8.7)
3′,5′	117.3,CH	7.40, d (8.7)
4′	158,9,C	
7′	66.3,CH_2	5.33, s
1″	102.1,CH	5.77, d (7.2)
2″	75.3,CH	4.36–4.38, m
3″	78.9,CH	4.35–4.37, m
4″	71.6,CH	4.34–4.36, m
5″	79.4,CH	4.13–4.15, m
6″	62.7,CH_2	4.43, m; 4.56, m
1‴	102.4,CH	5.68, d (7.2)
2‴	75.1,CH	4.36–4.38, m
3‴	78.9,CH	4.35–4.37, m
4‴	71.5,CH	4.34–4.36, m
5‴	79.3,CH	4.13–4.15, m
6‴	62.7,CH_2	4.43, m; 4.56, m
3-OCH_3	56.3, CH_3	3.78, s

Note: 1.93–1.95, m.

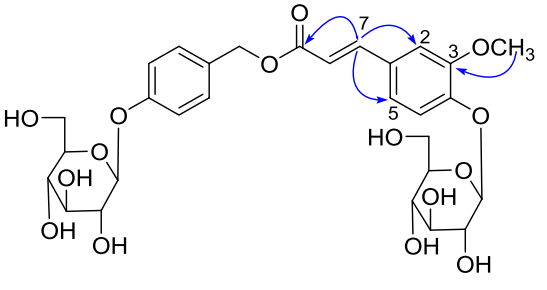

Figure 2. Key HMBC correlations of compound **1**.

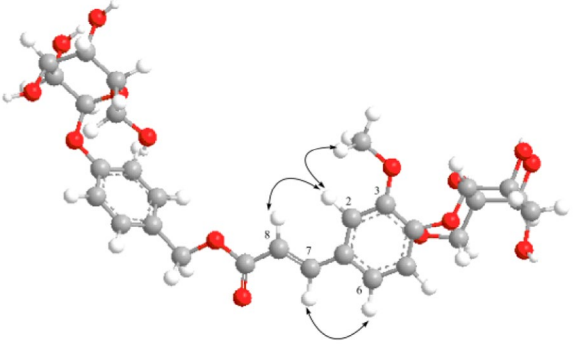

Figure 3. Key NOESY correlations of compound **1**.

Table 2. Inhibition effects of **1**–**8** on NO production.

Comp.	IC_{50} (μM)	CC_{50} (μM)
1	>70	>240
2	>70	>240
3	>70	>240
4	48.5 ± 0.7	>240
5	51.7 ± 1.1	>240
6	>70	>240
7	>70	>240
8	>70	>240
Dex	68 ± 1.2	>240

3. Experimental

3.1. General experimental procedures

Optical rotations were measured using an Autopol III automatic polarimeter (Rudolph Research Co., NJ, USA). UV spectra were recorded on Shimadzu UV2550 (Shimadzu, Kyoto, Japan). IR spectra were measured on a Thermo Nicolet Nexus 470 FT-IR spectrometer (MT, USA). The ^{1}H NMR and ^{13}C NMR spectra were recorded on JEOL ECX 600 MHz spectrometer (JEOL Ltd., Tokyo, Japan) or Bruker Avance-400FT NMR spectrometer (Bruker, Billerica, German) using TMS as an internal standard. HRESIMS were obtained on a Bruker APEX IV 70 ev FT-MS spectrometer and on a Thermo DFS spectrometer using a matrix of 3-nitrobenzyl alcohol.D101 macroporous resin (Mitsubishi Chemical Co., Japan), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and silica gel (200–300 mesh, Qingdao Marine Chemistry Co. Ltd., Qingdao, China) were used for column chromatography. And HF_{254} silica gel plates (Qingdao Marine Chemistry Co. Ltd., Qingdao, China) were used for TLC. HPLC was performed on Alltech 426 pump employing a UV detector, and the Prevail C18 column (semi-preparative, 5 μm) was purchased from Pharmacia. The murine macrophage cell line RAW 264.7 was obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco ibcogle (Invitrogen, NY, USA). 96-well plates were purchased from Corning Costar (Corning Inc., NY, USA). Dimethylsulphoxide (DMSO), LPS, and MTT were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Dexamethasong was obtained from Tianjin Jiaozuo Co. (Tianjin China), and Griess reagent was purchased from applygen (Beijing, China).

3.2. Plant materials

The tubers of *Bletilla striata* were collected from Shaoxing, Zhejiang Province and identified by Professor Qing-Lin Zhang from the Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing, China. A voucher specimen (No. BS20150824) was deposited in Beijing Institute of Radiation Medicine.

3.3. Extraction and isolation

The dried and powdered tubers of *B. striata* (1.5 kg) were refluxed with 95% EtOH (15 L × 3 times, 3 h for each). The extract was evaporated to dryness under vacuum to afford a crude residue (50 g), which was subjected to D101 macroporous resin column chromatography

(CC) eluted with aqueous EtOH (0, 20, 40, and 60%) to afford four fractions (*FA–FD*). *FB* was further purified on a Sephadex LH-20 column to give **8** (980.2 mg). *FC* (15.2 g) was further fractionated on an ODS column (3 × 30 cm), eluted with a constant gradient of MeOH/H_2O (20%), to obtain five portions (*FC1–FC5*). *FC1* (1.2 g) was further fractionated on an ODS column with a constant gradient of MeOH /H_2O (30%), followed by preparative RP-C18 HPLC, to give **5** (30.3 mg). *FC2* (2.5 g) was further fractionated on an ODS column with a constant gradient of MeOH /H_2O (32%), followed by preparative RP-C18 HPLC (flow rate 2 ml/min, UV detection at λ = 300 nm), to give **1** (5.8 mg, t_R = 22.3 min) and **3** (34.0 mg, t_R = 31.5 min). *FC3* (450 mg) was further fractionated on an ODS column with a gradient of MeOH/H_2O (from 30 to 50%), followed by preparative RP-C18 HPLC, to give **2** (6.8 mg, t_R = 19.6 min) and **7** (6.8 mg, t_R = 21.5 min). *FC4* (4.5 g) was further purified on a Sephadex LH-20 column to give **4** (1.9 g) and **6** (10.5 mg).

3.3.1. Bletilloside A (1)

Amorphous power; $[\alpha]_D^{25} - 40.8$ (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.26) 304 (4.24) nm; IR (KBr) ν_{max} 3422, 2923, 1701, 1637, 1509, 1458, 1384, 1258, 1234, 1162, 1072 cm^{-1}; ^1H and ^{13}C NMR spectral data, see Table 1; HRESIMS: *m/z* 647.1939 $[M + Na]^+$ (calcd for $C_{29}H_{36}O_{15}Na$, 647.1952).

3.4. Acid hydrolysis of 1

Compound **1** (1.0 mg) was heated in 2 mol/L trifluoroacetic acid (TFA) (1 ml) for 2 h in screen-cap vial. After cooling, 1 ml of EtOAc was added into the hydrolysate. The aqueous layer was evaporated to dryness with ethanol under vacuum until TFA was removed. The residues were determined by comparison with D-glucose using TLC ($CHCl_3$: MeOH: H_2O = 3: 2:0.2, visualization with ethanol-5% H_2SO_4 spraying). In addition, the specific rotation of sugar derived from **1** ($[\alpha]_D^{25} + 79.8$) was in accordance with that of D-glucose ($[\alpha]_D^{25} + 96.8$) and opposite to that of L-glucose $[\alpha]_D^{25} - 79.8$), indicating the sugar of **1** to be in the D-form.

3.5. Cell viability

Cell viability was evaluated by MTT assay. RAW 264.7 macrophage cell was seeded at 2×10^5 cells/ml in 96-well plates containing 100 µl of DMEM (Dulbecco's modified Eagle's medium) (supplemented with 10% FBS (Fetal bovine serum), 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate), and cultured in a 37 °C humidified atmosphere of 5% CO_2. After overnight incubation, the cells were treated with LPS (1 µg/ml) in the absence or presence of the test compounds at different concentrations and the plates were incubated for 24 h. The cells were then incubated with an MTT solution for 4 h at 37 °C under 5% CO_2. The medium was removed and DMSO (150 µl) was added to each well. The absorbance was measured at 492 nm by a Microplate Reader (Multiskan MK3, Thermo).

3.6. NO inhibition assay

Using the Griess method, the nitrite concentration was determined in the culture supernatants. The cells were planted at 2×10^5 cells/ml in 96-well plates and incubated overnight.

After treatment with various concentrations of tested compounds for 1 h, cells were treated with LPS (1 μg/ml) for 24 h. Dex served as a positive control. The culture supernatant (100 μl) was moved into another 96-well plate, where nitrite reacted with the standard Griess reagent (100 μl) for 15 min. The optical density of each well was measured at 492 nm by a Microplate Reader.

Disclosure statement

No potential conflict of interest was reported by the authors.

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