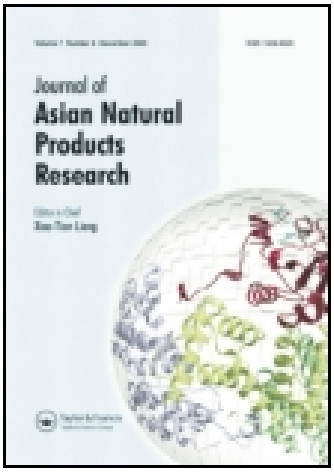

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Two new chlorophenyl glycosides from the bulbs of Lilium brownii var. viridulum

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Two new chlorophenyl glycosides from the bulbs of *Lilium brownii* var. *viridulum*

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Two rare new chlorophenyl glycosides, 2,4,6-trichlorol-3-methyl-5-methoxy-phenol 1-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**1**) and 4-chlorol-5-hydroxyl-3-methyl-phenol 1-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**2**), along with three known compounds (**3**–**5**) were isolated from the bulbs of *Lilium brownii* var. *viridulum*. The structures of the new compounds were elucidated on the basis of spectroscopic and chemical methods. All the compounds exhibited weak inhibition of NO production in LPS-stimulated RAW 264.7 cells.

Keywords: *Lilium brownii* var. *viridulum*; Liliaceae; chlorophenyl glycosides

1. Introduction

Lilium brownii Brown var. *viridulum* Baker (family Liliaceae), a bulbous perennial plant widely distributed and cultivated in northern and eastern Asia, is regularly consumed in China as food and medicine. In traditional Chinese medicine, lily bulbs have long been used for the treatment of lung ailments [1]. Previous phytochemical investigations on this species have resulted in a number of steroidal saponins, phenylpropanoid glycosides, and phenolic glycosides [2–6]. With regard to *L. brownii* var. *viridulum*, the chemical studies were largely focused on steroidal saponins [7,8]. For our interests in active constituents in the bulbs of *L. brownii* var. *viridulum*, the chemical investigation on the polar fraction of this plant resulted in the isolation of two new chlorophenyl glycosides (**1** and **2**), together with three known phenolic glycosides identified as eugenol 4-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**3**) [6], 2,6-dimethoxy-4-(prop-2-enyl)phenyl *O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**4**) [9], and 2,6-dimethoxy-4-(prop-2-enyl)phenyl *O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**5**) [10]. Herein, this paper deals with the isolation and structural elucidation of two new chlorophenyl glycosides (**1** and **2**) (Figure 1). In addition, inhibitions of NO production in LPS-stimulated RAW 264.7 cells were carried out to measure the *in vitro* anti-inflammatory activities of the isolated compounds.

2. Results and discussion

Compound **1** was obtained as white amorphous powder. It showed UV maxima at 205 and 231 nm, and IR bands at 3364, 1640, 1453, and 1380 cm^{-1}, suggesting the presence of a phenyl moiety. Its ESI-MS exhibited characteristic cluster of isotopic molecular ion peaks at *m/z* 564, 566, 568, and 570 in an approximate ratio of 27:27:9:1, indicating the presence of three chlorine atoms in the molecule [11].

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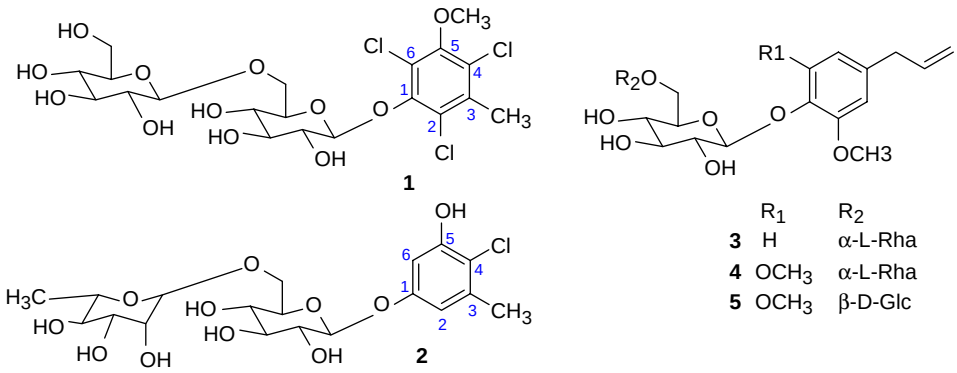

Figure 1. The structures of compounds **1**–**5**.

The HR-ESI-MS of **1** showed an accurate $[M + Na]^+$ ion at *m/z* 587.0465, corresponding to a molecular formula of $C_{20}H_{27}Cl_3O_{12}$, which was confirmed by ^1H and ^{13}C NMR experiments. In the ^{13}C NMR spectrum, signals for six aromatic carbons appeared at δ_C 151.1, 147.6, 133.9, 125.4, 124.9, and 121.6, together with one methoxy carbon (δ_C 60.5), one methyl carbon (δ_C 18.1), and a set of characteristic signals for two hexose units (see Table 1). While the ^1H NMR spectrum of **1** suggested the presence of a methoxyl signal at δ_H 3.82 (s), an aromatic methyl signal at δ_H 2.44 (s), and two anomeric protons at δ_H 5.04 (1H, d, $J = 7.5$ Hz) and 4.01 (1H, d, $J = 7.5$ Hz). Its spectroscopic data (recorded in CD_3OD), especially the ^{13}C NMR for the aglycon moiety (Table 1), were in good agreement with those of curculigine C, which indicated the same aglycon as that of curculigine C [11]. To further confirm this, the aglycon obtained by acid hydrolysis was methylated with CH_3I, to yield the same symmetrical ether as that of curculigine C by comparing its ^1H NMR spectral data with the literature values [11]. The aglycon moiety was also confirmed by the HMBC cross-peaks from the methoxy proton (δ_H 3.82) to an aromatic carbon (δ_C 151.1, C-5), from the methyl proton (δ_H 2.44) to three aromatic carbons at δ_C 133.9 (C-3), 125.4 (C-2), and 124.9 (C-4), and no ROESY correlations between the methyl resonance and the methoxy singlet, as shown in Figure 2. The sugar obtained by acid hydrolysis was identified as D-glucose, by co-TLC with authentic samples and GC-MS analysis of their chiral derivatives. A sequence of glucopyranosyl (1 → 6) glucopyranosyl linked at C-1 was unambiguously deduced by the long-range correlations of H-1′ (δ_H 5.04) to C-1 (δ_C 147.6), and from H-1″ (δ_H 4.01) to C-6′ (δ_C 67.9) on the basis of the HMBC experiment. Therefore, compound **1** was established as 2,4,6-trichlorol-3-methyl-5-methoxy-phenol 1-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside.

Compound **2** was isolated as white viscous syrup. The ESI-MS indicated the existence of one chlorine atom in **2** on the basis of the isotopic molecular ion peaks (*m/z* 466 and 468, 3:1) [11]. It had a molecular formula of $C_{19}H_{27}ClO_{11}$, as determined by the HR-ESI-MS at *m/z* 489.1131 $[M + Na]^+$. The IR and UV data of **2** were similar to those of **1**. On acid hydrolysis **2** afforded two sugars, identified as D-glucose and L-rhamnose by co-TLC with authentic samples and GC-MS analysis of their chiral derivatives. The ^1H NMR spectrum of **2** in DMSO-d_6 displayed two aromatic proton signals as an AB spin system at δ_H 6.49 (1H, d, $J = 3.0$ Hz) and 6.47 (1H, d, $J = 3.0$ Hz), one methyl group (δ_H 2.25), and two anomeric protons at δ_H 4.71 (1H, d, $J = 8.0$ Hz) and 4.55 (br s) for glucose and rhamnose, respectively.

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds **1** and **2**.

	1			**2**	
No.	δ_C (CD_3OD)	δ_C (DMSO-d_6)	δ_H (multi, J in Hz) (DMSO-d_6)	δ_C (DMSO-d_6)	δ_H (multi, J in Hz) (DMSO-d_6)
Aglycon					
1	149.3	147.6		155.7	
2	127.3	125.4		108.6	6.47 (d, 3.0)
3	135.9	133.9		136.6	
4	127.3	124.9		113.0	
5	153.2	151.1		153.1	
6	123.9	121.6		102.4	6.49 (d, 3.0)
3-CH_3	18.5	18.1	2.44 (s)	19.9	2.25 (s)
5-OCH_3	61.3	60.5	3.82 (s)		
Glc′				Glc′	
1′	104.7	102.8	5.04 (d, 7.5)	100.3	4.71 (d, 8.0)
2′	75.2	73.9	3.35 (m)	72.7	3.19 (m)
3′	78.4	77.1	3.32 (m)	76.1	3.26 (m)
4′	71.8	70.0	3.08 (m)	69.4	3.11 (m)
5′	78.1	76.6	2.88 (m)	75.2	3.41 (m)
6′	69.8	67.9	3.88 (dd, 11.5, 2.0) 3.56 (dd, 11.5, 7.0)	65.9	3.85 (dd, 11.0, 1.5) 3.42 (m)
Glc″				Rha″	
1″	104.2	102.7	4.01 (d, 7.5)	100.2	4.55 (br s)
2″	75.9	73.3	2.82 (m)	70.3	3.45 (m)
3″	77.9	76.5	2.88 (m)	70.0	3.62 (br s)
4″	71.8	69.9	2.99 (m)	71.7	3.18 (m)
5″	77.9	76.2	3.23 (m)	67.9	3.42 (m)
6″	62.9	60.9	3.62 (dd, 11.5, 5.5) 3.41 (dd, 11.5, 5.5)	17.4	1.11 (d, 6.0)

Note: Assignments were made by HSQC, HMBC, and ROESY data.

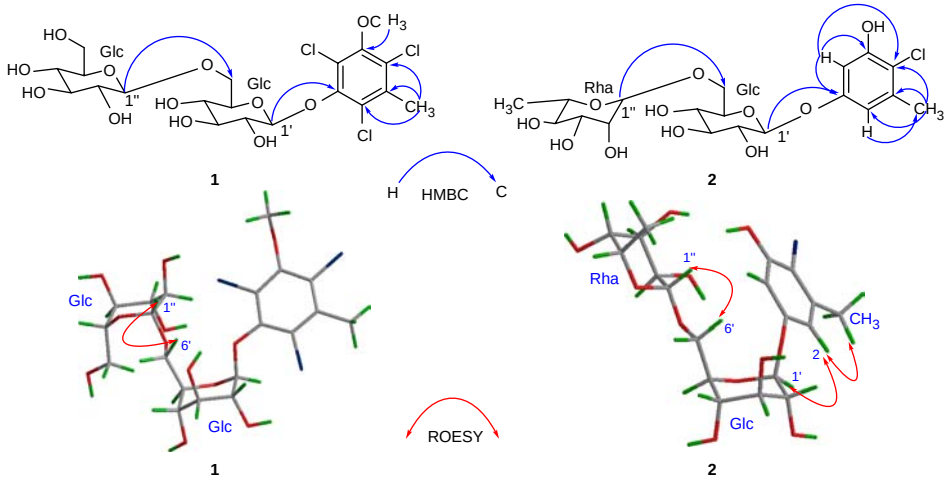

Figure 2. The key HMBC and ROESY correlations of compounds **1** and **2**.

The ^{13}C NMR spectrum of **2** showed 19 carbon signals. Among them, six signals at δ_C 155.7, 153.1, 136.6, 113.0, 108.6, and 102.4 attributed to aromatic ring, and a signal at δ_C 19.9 belonged to a methyl group, while the remaining 12 carbon resonances were consistent with the sugar moieties of α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl (see Table 1), which were confirmed by the HMBC correlation (Figure 2) between the H-1″ (δ_H 4.55) and C-6′ (δ_C 65.9). Careful comparison of the spectral data with those of the known 2-chloro-3,5-dihydroxytoluene, indicated compound **2** was to be a disaccharide glycoside of 2-chloro-3,5-dihydroxytoluene [12]. Acid hydrolysis of **2** provided a 2-chlorol substituted derivative of the aglycon with the same reaction condition of 2 M HCl–MeOH (tube, 6 h, 50°C) as **1**, which was consistent with the known 4,6-dichloro-5-methylresorcinol by comparing its ESI-MS and NMR data with those reported in literature [13], suggested the aglycon of **2** to be the same structure as 2-chloro-3,5-dihydroxytoluene. This was also supported unambiguously by the 2D NMR experiment (Figure 2). The HMBC spectrum of **2** showed correlations from the methyl proton (δ_H 2.25) to three aromatic carbons [δ_C 136.6 (C-3), 113.0 (C-4), and 108.6 (C-2)], from H-2 (δ_H 6.47) to C-1 (δ_C 155.7) and the methyl carbon (δ_C 19.9), from H-6 (δ_H 6.49) to C-1 at δ_C 155.7, C-5 at δ_C 153.1, and C-4 at δ_C 113.0, indicating the methyl group at C-3, the chlorine group at C-4, and C-5 substituted by a hydroxyl group. Moreover, the HMBC correlation of H-1′ (δ_H 4.71) with C-1 (δ_C 155.7) and the ROESY correlation of H-2 (δ_H 6.47) with H-1′ (δ_H 4.71), confirmed the connection of the sugar chain with C-1. On the basis of above spectral and chemical evidence, compound **2** was deduced as 4-chlorol-5-hydroxyl-3-methyl-phenol 1-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside.

Chlorine-containing compounds are rare in terrestrial plants. Previously, three chlorophenyl glycosides were reported from *Curculigo orchioides* (Hypoxidaceae) [11]. This is the first report of chlorophenyl glycosides from the *Lilium* species, though the chlorinated orcinol derivatives had been isolated from *L. maximowiczii* [12].

Table 2. NO inhibition rate of the compounds in LPS-stimulated RAW 264.7 cells ($n = 3$).

Compounds	Inhibition (%) (100 μM)
1	27.5 ± 0.37
2	21.8 ± 1.15
3	22.7 ± 3.29
4	28.3 ± 0.25
5	25.4 ± 0.94
N-Monomethyl-L-arginine[a]	90.7 ± 1.5
MeOH extract[b]	87.21 ± 0.51

[a] *N*-Monomethyl-L-arginine as a positive control.
[b] The inhibition (%) of MeOH extract with concentration at 100 μg/ml.

In our preliminary screening, the MeOH extract of *L. brownii* var. *viridulum* showed inhibitory effects on the LPS-induced NO production in RAW 264.7 cells (see Table 2). Thus, all the compounds were also assayed by inhibiting NO formation in LPS-induced macrophage RAW 264.7 cells. As shown in Table 2, the tested compounds exhibited weak inhibitory effects on NO production, with the inhibition rate in the range of 21.8–28.3% at the concentration of 100 μM.

3. Experimental

3.1 General experimental procedures

Optical rotations were determined on a JASCO P-1020 digital polarimeter (JASCO, Tokyo, Japan). IR spectra were measured on a Bruker Tensor 27 spectrometer with KBr-disks (Bruker, Karlsruhe, Germany). UV spectra were recorded on a UV-2450 UV/vis spectrophotometer (Shimadzu, Tokyo, Japan). ESI-MS data were obtained on an Agilent 1100 Series LC/MSD Trap mass spectrometer and HR-ESI-MS data were obtained on an Agilent 6520B Q-TOF spectrometer (Agilent, Santa Clara, CA, USA). NMR spectra were recorded on a

Bruker AVANCE III-500 NMR spectrometer (Bruker). Preparative HPLC was carried out on an Agilent 1100 Series with Shimpak RP-C_{18} column (20 × 200 mm, i.d.) and 1100 Series Multiple Wavelength detector. Analytical HPLC was carried out on an Agilent 1260 Infinity Chromatograph equipped with an Agilent ZORBAX SB-C_{18} column (5 μm, 4.6 mm × 250 mm). GC-MS was carried out on a Varian CP-3800 Gas Chromatograph (Varian, Palo Alto, CA, USA) with a Saturn 2200 Mass detector. Column chromatography (CC) was taken on a silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd, Qingdao, China), macroporous resin D101 (pore size B 13–14 nm, H&E Co., Beijing, China), MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical, Tokyo, Japan), ODS-C_{18} (40–63 μm, Fuji Silysia Chemical Ltd, Aichi, Japan), and Sephadex LH-20 (20–100 μm, Pharmacia, Uppsala, Sweden). Fractions were monitored by TLC with silica gel GF_{254} (Qingdao Haiyang Chemical Co. Ltd). Detection of spots was done by heating silica gel plates sprayed with 1% vanillin–10% H_2SO_4 in EtOH.

3.2 Plant material

The air-dried bulbs of *L. brownii* var. *viridulum* were purchased from Anhui Bozhou Crude Drug Market in November 2010 and were identified by Prof. Mian Zhang, from the Department of Medicinal Plants, China Pharmaceutical University. A voucher specimen (No. L20101101) has been deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

3.3 Extraction and isolation

The dried bulbs of *L. brownii* var. *viridulum* (6 kg) were extracted three times with 95% EtOH under reflux. After evaporation, the residue was dissolved in H_2O. The H_2O solution was passed through a macroporous resin D101 column, eluted with EtOH–H_2O (0:100, 30:70, 70:30, 95:5, v/v), successively. Then, the 30% ethanol elution fraction (22 g) was subjected to MCI column using a MeOH–H_2O gradient (0:100, 10:90, 30:70, 50:50, v/v) to afford four fractions A–D. Fraction C (4 g) was applied on ODS-C_{18} column (MeOH–H_2O, 30:70, v/v) to give three fractions C1–C3. Fraction C3 was separated by sephadex LH-20 (MeOH) and silica gel column chromatography eluted with $CHCl_3$–MeOH (4:1, v/v) to give compound **1** (13.1 mg). Fraction C2 was successively purified over sephadex LH-20 (MeOH) and preparative HPLC (ODS column: 20 × 200 mm, i.d., 5 μm; flow rate: 10 ml/min) using MeOH–H_2O (40:60, v/v) to afford compounds **2** (9.2 mg, $t_R = 11.7$ min), **3** (2.6 mg, $t_R = 11.4$ min), **4** (4.3 mg, $t_R = 9.9$ min), and **5** (3.8 mg, $t_R = 10.9$ min).

3.3.1 Compound *1*

White amorphous powder; $[\alpha]_D^{17}$ −13.8 ($c = 0.12$, MeOH); UV (MeOH) λ_{max} (nm) (log ε): 205 (4.28), 231 (3.49); IR ν_{max} (KBr) cm^{-1}: 3364, 2914, 1640, 1453, 1380, 1074, 771; for ^1H and ^{13}C NMR spectral data, see Table 1; ESI-MS *m/z* 562.8 $[M - H]^-$, 238.7 $[M - H\text{-}162\text{-}162]^-$; HR-ESI-MS *m/z* 587.0465 $[M + Na]^+$ (calcd for $C_{20}H_{27}Cl_3O_{12}Na$, 587.0460).

3.3.2 Compound *2*

White viscous syrup; $[\alpha]_D^{17}$ −47.3 ($c = 0.14$, MeOH); UV (MeOH) λ_{max} (nm) (log ε): 201 (4.39), 223 (3.75); IR ν_{max} (KBr) cm^{-1}: 3417, 2925, 1677, 1591, 1452, 1340, 1050, 757; for ^1H and ^{13}C NMR spectral data, see Table 1; ESI-MS *m/z* 465.2 $[M - H]^-$, 156.8 $[M - H\text{-}146\text{-}162]^-$; HR-ESI-MS *m/z* 489.1131 $[M + Na]^+$ (calcd for $C_{19}H_{27}ClO_{11}Na$, 489.1134).

3.4 Acid hydrolysis

Each of compounds **1** and **2** (5 mg) dissolved in MeOH (3 ml) was treated with 2 M HCl–MeOH (tube, 6 h, 50°C) [14,15]. The hydrolysate was concentrated to dryness, then partitioned with H_2O and $CHCl_3$. The

$CHCl_3$ part was evaporated to yield an aglycon. The aqueous layer was examined by co-TLC with Me_2CO–BuOH–H_2O as the solvent system comparing with the authentic samples (Glc, $R_f = 0.36$; Rha, $R_f = 0.45$). Then, each remaining aqueous layer was concentrated to dryness to give a residue, which was dissolved in pyridine (2 ml), reacted with L-cysteine methyl ester hydrochloride (2 mg) at 60°C for 2 h. Then, trimethylchlorosilane (0.5 ml) was added to the reaction mixture and kept at 60°C for 30 min. The solution was concentrated to dryness and diluted with H_2O and extracted with *n*-hexane (1 ml × 3). The organic layer was subjected to GC-MS analysis. As a result, D-glucose was identified in the acid hydrolysate of **1**, while D-glucose and L-rhamnose detected from that of **2** by comparing the retention time with the standard sugar derivatives (D-glucose, 15.49 min; L-rhamnose, 14.18 min).

3.5 Methylation of the aglycon of 1

The aglycon of **1** obtained by acid hydrolysis with 2 M HCl–MeOH (tube, 6 h, 50°C) was dissolved in CH_3COCH_3. Then, K_2CO_3 and CH_3I were added to the sample solution. After being kept at 25°C for 2 h, the reaction mixture was evaporated under reduced pressure to give almost pure 2,4,6-trichloro-3,5-dimethoxytoluene: ^{1}H NMR (500 MHz, $CDCl_3$), δ_H 2.47 (3H, s, CH_3), 3.88 (6H, s, $OCH_3 \times 2$).

3.6 2-Chlorol substituted derivative of the aglycon of 2

On acid hydrolysis with 2 M HCl–MeOH (tube, 6 h, 50°C), compound **2** afforded the 2-chlorol substituted derivative of the aglycon of **2**, confirmed by its ESI-MS and NMR data. [ESI-MS (*m/z*): 192 $[M]^+$, 194 $[M + 2]^+$, 196 $[M + 4]^+$, 9:6:1; ^{1}H NMR (500 MHz, acetone-d_6): δ_H 6.61 (1H, Ar*H*, s), 2.41 (3H, CH_3, s), 8.65 (2H, OH × 2, br s); ^{13}C NMR (125 MHz, acetone-d_6): δ_C 18.6, 103.5, 113.9 (two carbons), 153.7 (two carbons).]

3.7 Inhibition of NO production

All these compounds were *in vitro* evaluated for their anti-inflammatory activity on the inhibition of NO production in LPS-stimulated RAW 264.7 cells, according to a reported method [16]. In briefly, RAW 264.7 cells (1×10^5 cells/well) were plated in 96-well microtiter plates with DMEM medium supplemented with 10% FBS, and allowed to adhere overnight at 37°C in a humidified atmosphere containing 5% CO_2. Then, the cells were treated with the tested compound at different concentrations for 2 h, followed by incubation with 100 ng/ml LPS for 24 h. And the amount of NO in cell-free culture medium was determined on the Griess reaction. The absorbance was measured at 540 nm, using an ELISA reader. And *N*-monomethyl-L-arginine was used as a positive control.

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