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Megastigmane glycosides from *Urena lobata*

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ABSTRACT

Five new megastigmane glycosides, urenalobasides A–E (**1–5**), together with 11 known ones (**6–16**) were isolated from *Urena lobata*. Their structures were determined by extensive spectroscopic and spectrometric data (1D and 2D NMR, IR, and HRESIMS) and calculated electronic circular dichroism method. Compounds **1** and **2** are two unusual megastigmanes structurally containing a 6/5 fused ring system. Compound **3** exhibits inhibition of nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells with IC_{50} value of 53.7 ± 1.0 μM (positive control, dexamethasone, IC_{50} = 16.6 ± 0.8 μM).

1. Introduction

Urena lobata, belonging to the family Malvaceae, is widely distributed in Asia, South America, and Africa [1]. In south of China, the aerial part of *U. lobata* is commonly used as anti-inflammatory, antipyretic, antalgesic, and antibacterial agents for the treatment of fever, rheumatism, and diarrhea. In addition, decoctions and preparations containing *U. lobata* are also popularly used to treat gynopathies such as pathological leucorrhea and gonorrhea [2]. Previous investigations led to the isolation and structural elucidation of flavonoids, coumarins, and lignans from *U. lobata* [1,3–6]. However, as a widely used herbal medicine, the bioactive constituents of *U. lobata* remain largely unknown. Therefore, further investigations on the bioactive constituents of *U. lobata* are quite essential. In our previous reports, 24 flavonoids were identified from *U. lobata* [7,8]. As an ongoing study, five new megastigmane glycosides, urenalobasides A–E (**1–5**), together with 11 known ones (**6–16**) were obtained (Fig. 1). Herein, the isolation and structural elucidation of the new compounds as well as their inhibitory effects on nitric oxide (NO) production in LPS-stimulated RAW264.7 macrophage cells are described.

2. Experimental

2.1. General experimental procedures

Optical rotations were obtained on a Rudolph Autopol IV automatic polarimeter (NJ, USA). IR spectra were recorded on a Thermo Nicolet Nexus 470 FT-IR spectrophotometer (MA, USA) with KBr pellets. UV spectra were obtained using a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). NMR spectra were recorded on a Varian INOVA-500 spectrometer (CA, USA) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. HRESIMS was recorded on an LCMS-IT-TOF system, fitted with a Prominence UFLC system and an ESI interface (Shimadzu, Kyoto, Japan). Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), LiChroprep RP-C_{18} gel (40–63 μm, Merck, Germany), D101 macroporous adsorption resin (Qingdao Marine Chemical Inc., Qingdao, China) and Sephadex LH-20 (Pharmacia) were used for open column chromatography (CC). HPLC was performed on a Shimadzu LC-20AT pump system (Shimadzu Corporation, Tokyo, Japan), equipped with a SPD-M20A photodiode array detector monitoring at 254 nm. A semi-preparative HPLC column (YMC-Pack C_{18}, 250 × 10 mm, 5 μm) was utilized for compounds separation and purification. TLC was performed using GF_{254} plates (Qingdao Marine Chemical Inc., Qingdao, China).

2.2. Plant material

Urena lobata L. was collected in Guangxi province, China, in September 2013. The plant material was authenticated by one of the authors (S.-P. Shi), and a voucher specimen (DTH2013029) is deposited at the Modern Research Center for Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China.

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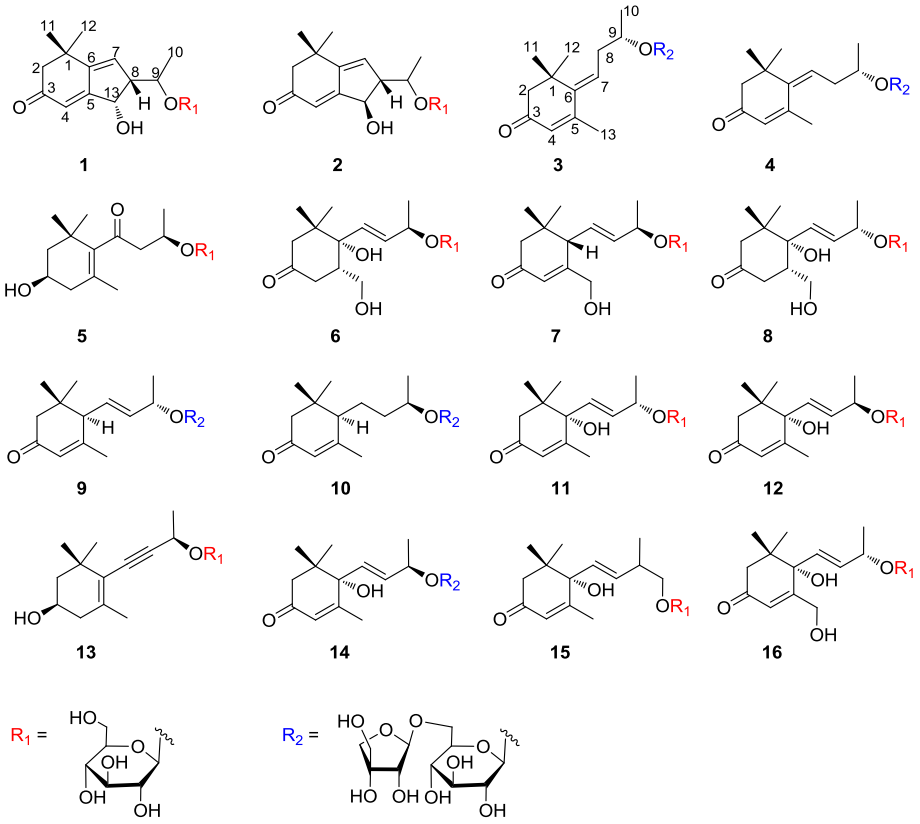

Fig. 1. Structures of compounds **1–16.**

Table 1
^{13}C NMR (125 MHz) data of compounds **1–5** (δ in ppm, in CD_3OD).

Position	1[a]	2[a]	3[a]	4[a]	5[a]
	δ_C, type	δ_C, type	δ_C, type	δ_C, type	δ_C, type
1	35.2,C	35.2, C	39.2, C	41.9, C	37.1, C
2	52.4,CH_2	52.3, CH_2	54.6, CH_2	53.7, CH_2	49.0, CH_2
3	202.3, C	202.3, C	202.3, C	201.9, C	65.0, CH
4	118.0, CH	118.5, CH	125.3, CH	128.8, CH	41.7, CH_2
5	171.5, C	171.1, C	159.6, C	160.0, C	143.7, C
6	148.9, C	149.5, C	142.6, C	144.4, C	129.2, C
7	136.1, CH	137.0, CH	135.4, CH	130.2, CH	211.7, C
8	60.5, CH	60.9, CH	38.5, CH_2	38.7, CH_2	54.4, CH_2
9	75.4, CH	75.9, CH	74.9, CH	74.9, CH	71.7, CH
10	18.1, CH_3	17.6, CH_3	20.2, CH_3	20.1, CH_3	20.2, CH_3
11	27.7, CH_3	27.8, CH_3	29.1, CH_3	28.3, CH_3	29.4, CH_3
12	28.4, CH_3	28.7, CH_3	29.2, CH_3	28.4, CH_3	29.9, CH_3
13	75.1, CH	75.5, CH	22.9, CH_3	25.1, CH_3	20.7, CH[3]
Glu-1	102.2, CH	101.9, CH	102.5, CH	102.5, CH	102.5, CH
Glu-2	75.0, CH	75.0, CH	75.9, CH	75.9, CH	75.1, CH
Glu-3	78.1, CH	77.9, CH	77.0, CH	77.0, CH	77.8, CH
Glu-4	71.8, CH	71.8, CH	71.8, CH	71.9, CH	71.6, CH
Glu-5	77.9, CH	78.1, CH	78.1, CH	78.1, CH	78.1, CH
Glu-6	63.0, CH_2	62.9, CH_2	69.1, CH_2	69.1, CH_2	62.9, CH_2
Api-1			111.0, CH	111.0, CH	
Api-2			78.0, CH	78.0, CH	
Api-3			80.4, C	80.5, C	
Api-4			75.0, CH_2	75.1, CH_2	
Api-5			65.4, CH_2	65.5, CH_2	

[a] Assignments were carried out based on HSQC and HMBC experiments.

2.3. Extraction and isolation

The air-dried *U. lobata* (13 kg) was refluxed with 95% EtOH for three times (3 × 180 L, each for 1 h). After removal of solvent under reduced pressure, the residue (1.35 kg) was suspended in water (6 L), and partitioned with petroleum ether (2 × 6 L), EtOAc (5 × 6 L), and *n*-BuOH (3 × 6 L), successively. The *n*-BuOH extract (128 g) was subjected to D101 macroporous adsorption resin chromatography and eluted with H_2O–EtOH (100:0, 90:10, 50:50, 20:80, 0:100) to yield five fractions (Fr.1–5). Fr. 2 (20 g) and Fr. 3 (40 g) were combined and subjected to silica gel chromatography and eluted with a gradient of EtOAc-MeOH-H_2O from 30:2:1 to 5:2:1 to give five subfractions (Subfr. A – E). Subfr. C (6.3 g) and subfr. D (7.7 g) were combined and chromatographed on Sephadex LH-20 column eluted with MeOH to give seven subfractions (Fr. C1 – C7). Fr. C4 (4.0 g) was subjected to silica gel column eluted with a stepwise gradient of petroleum CH_2Cl_2–MeOH (20:1 → 0:1) to afford six fractions (Fr. C4a – C4f). Fr. C4e (1.5 g) was further separated using ODS C_{18} column chromatography and eluted with MeOH–H_2O (20:1, 15:1, 10:1, v/v) to obtain four fractions (Subfr. C4e1 – C4e4). Subfr. C4e1 was repeatedly separated and purified by semi-preparative HPLC (8% aqueous MeCN) to give compounds **1** (6.0 mg, t_R 45.0 min), **2** (1.4 mg, t_R 63.0 min), **5** (1.8 mg, t_R 52.5 min), **6** (2.0 mg, t_R 57.0 min), **7** (1.4 mg, t_R 23.0 min), and **8** (1.5 mg, t_R 48.5 min). Subfr. C4e2 was separated by semi-preparative HPLC (15% aqueous MeOH) to give compounds **3** (13.0 mg, t_R 32.0 min), **4** (12.4 mg, t_R 35.0 min), **9** (11.8 mg, t_R 42.5 min), and **10** (10.0 mg, t_R 45.5 min). Subfr. C4e3 was subjected to semi-preparative HPLC (12% aqueous MeCN) to yield compounds **11** (3.0 mg, t_R 35.0 min), **12** (2.4 mg, t_R 43.0 min), and **13** (1.8 mg, t_R 52.5 min). Subfr. C4e4 was separated by semi-preparative HPLC (30% aqueous MeOH) to afford compounds **14** (23.0 mg, t_R 55.0 min), **15** (2.4 mg, t_R 53.0 min), and **16** (5.8 mg, t_R 62.5 min).

Urenalobaside A (**1**): Pale yellow gum, $[\alpha]_D^{25}$: −55.4 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.09), 289 (3.55); IR (KBr) ν_{max}: 3389, 2940, 1655, 1653, 1082 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; negative-ion HRESIMS: *m/z* 419.1469 [M + Cl]$^-$ (calcd for $C_{19}H_{28}O_8Cl$, 419.1478).

Urenalobaside B (**2**): Pale yellow gum, $[\alpha]_D^{25}$: −14.1 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.08), 289 (3.55); IR (KBr) ν_{max}:

Table 2
^{1}H NMR (500 MHz) data of compounds **1–5** (δ in ppm, in CD_3OD).

No.	1[a]	2[a]	3[a]	4[a]	5[a]
2	2.26, d, (16.0) 2.38, d, (16.0)	2.27, d, (16.0) 2.38, d, (16.0)	2.34, s	2.30, s	1.68, dd, (12.5, 2.5) 1.41, t, (12.5)
3					3.94, m
4	5.91, s	5.92, s	5.87, s	5.89, s	2.29, dd, (17.5, 6.0) 1.93, m
7	6.37, br s	6.25, br s	6.36, t, (6.5)	5.99, t, (7.0)	
8	2.83, m	2.91, m	2.68, dd, (12.0, 6.5)	2.63, m 2.53, m	3.19, dd, (19.0, 6.5) 2.65, dd, (19.0, 6.5)
9	4.07, m	4.03, m	3.16, dd, (12.0, 6.0)	3.14, dd, (12.0, 6.0)	4.42, dd, (12.5, 6.5)
10	1.31, d, (6.0)	1.26, br s	1.26, d, (6.0)	1.23, d, (6.0)	1.26, d, (6.5)
11	1.27, s	1.27, s	1.30, br s	1.19, br s	1.18, s
12	1.16, s	1.16, s	1.30, br s	1.18, br s	1.03, s
13	4.80, m	4.80[b]	2.14, s	2.27, s	1.64, s
Glu-1	4.36, d, (8.0)	4.38, d, (8.0)	4.34, d, (8.0)	4.33, d, (8.0)	4.36, d, (7.5)
Glu-2	3.14, dd, (10.0, 8.0)	3.11, dd, (9.0, 8.0)	3.99, m	3.99, m	3.11, dd, (12.0, 7.5)
Glu-3	3.36, m	3.35, m	3.42, m	3.40, m	3.35, m
Glu-4	3.27, m	3.26, m	3.25, t, (9.5)	3.25, t, (9.0)	3.26, m
Glu-5	3.27, m	3.26, m	3.36, m	3.35, m	3.26, m
Glu-6	3.65, dd, (12.0, 6.0) 3.88, dd, (12.0, 2.0)	3.65, dd, (12.5, 4.5) 3.87, d, (12.5)	3.99, m 3.59, dd, (11.0, 6.5)	3.97, m 3.58, dd, (11.5, 6.5)	3.84, dd, (12.0, 1.0) 3.65, dd, (12.0, 5.5)
Api-1			4.99, d, (2.5)	4.99, d, (2.5)	
Api-2			3.88, d, (2.5) 3.95, d, (9.5)	3.88, d, (2.0) 3.95, m	
Api-4			3.75, dd, (9.5, 2.0)	3.75, d, (9.5)	
Api-5			3.55, m	3.55, m	

[a] Assignments were carried out based on HSQC and HMBC experiments.
[b] Overlapped with H_2O signal.

3400, 2935, 1653, 1650, 1076 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; negative-ion HRESIMS: *m/z* 419.1483 $[M + Cl]^-$ (calcd for $C_{19}H_{28}O_8Cl$, 419.1478).

Urenalobaside C (**3**): Colorless gum, $[\alpha]_D^{25}$: −52.4 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.66), 229 (3.48), 282 (3.78); IR (KBr) ν_{max}: 3410, 2935, 1653, 1650, 1056 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; negative-ion HRESIMS: *m/z* 537.2092 $[M + Cl]^-$ (calcd for $C_{24}H_{38}O_{11}Cl$, 537.2108).

Urenalobaside D (**4**): Colorless gum, $[\alpha]_D^{25}$: −54.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 206 (3.52), 230 (3.33), 288 (3.62); IR (KBr) ν_{max}: 3375, 2930, 1655, 1650, 1082 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; negative-ion HRESIMS: *m/z* 537.2106 $[M + Cl]^-$(calcd for $C_{24}H_{38}O_{11}Cl$, 537.2108).

Urenalobaside E (**5**): Colorless gum, $[\alpha]_D^{25}$: −24.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 202 (2.86), 227 (2.84); IR (KBr) ν_{max}: 3397, 2935, 1653, 1650, 1056 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; negative-ion HRESIMS: *m/z* 423.1770 $[M + Cl]^-$ (calcd for $C_{19}H_{32}O_8Cl$, 423.1791).

2.4. Biological assays

The murine macrophage RAW264.7 cells were purchased from Peking Union Medical College (PUMC) Cell Bank (Beijing, China). Cell maintenance, experimental procedures, and data determination for the inhibition of NO production and the viability assay are the same as previously described [9–11]. The IC_{50} values were determined using GraphPad Prism 5 software from experiments performed in triplicate (GraphPad Software, Inc., San Diego, CA, USA). Dexamethasone was used as a positive control (16.6 ± 0.8 μM). All the compounds were prepared as stock solutions in DMSO (final solvent concentration less than 0.2% in all assays).

2.5. Acid hydrolysis of compounds *1*, *3*, and *4*

Each compound (**1**, **3**, and **4**) 2.0 mg was dissolved in 1 M HCl (3.0 mL) and heated at 90 °C for 3 h. The reaction mixture was cooled to room temperature and extracted with EtOAc. The aqueous layer was analyzed using silica plate developed with EtOAc − MeOH − H_2O (7:5:1). Glucose was detected from the hydrolysate of **1**, Glucose and apiose were detected from the hydrolysates of **3** and **4**.

2.6. Enzymatic hydrolysis of compounds *1*, *3*, and *4*

Each compound (**1**, **3**, and **4**) 2.0 mg was dissolved in citric acid buffer solution (pH 4.5, 60 mL), and incubated with 4.5 mg cellulase at 37 °C for 16 h. After stopped the reaction by the addition of MeOH, the reaction mixture was extracted with EtOAc. Residue obtained from the organic layer was purified by HPLC (35% MeCN) to obtain the aglycons **A1** (0.3 mg, t_R 13.0 min), **A3** (0.8 mg, t_R 12.0 min), and **A4** (0.6 mg, t_R 14.0 min).

2.7. ECD calculations

A preliminary conformational analysis was performed with the SYBYL-X 2.0 software package using the random search method with the MMFF94s force field. The conformers were further optimized by using the TDDFT method at the B3LYP/6-31G(d) level, and the frequency was calculated at the same level of theory. The stable conformers without imaginary frequencies were subjected to ECD calculation by the TDDFT method at the B3LYP/6-31 + G(d) level with the CPCM model in MeOH. ECD spectra of different conformers were simulated using SpecDis v1.51with a half-band width of 0.3 eV, and the final ECD spectra were obtained according to the Boltzmann calculated contribution of each conformer (S38). All calculations were performed with the Gaussian 09 program package [12,13].

3. Results and discussion

The 95% EtOH extract of the *U. lobata* was suspended in H_2O and extracted successively with petroleum ether, EtOAc, and *n*-BuOH. The *n*-BuOH soluble fraction was fractionized by D101 macroporous adsorption resin, and repeatedly separated by silica gel and Sephadex LH-20 column chromatography, and purified by semi-preparative HPLC to afford five new megastigmane glycosides (**1–5**) together with 11 known

ones (Fig. 1).

Compound **1** was obtained as a pale yellow gum, $[\alpha]_D^{25}$–55.4 (*c* 0.05, MeOH). Its molecular formula was assigned as $C_{19}H_{28}O_8$ by the $[M + Cl]^-$ ion peak at *m/z* 419.1469 in the HRESIMS spectrum, which was supported by the ^{13}C NMR data (Table 1). The ^1H NMR spectrum of **1** showed the presence of three methyls at δ_H 1.31 (3H, d, *J* = 6.0 Hz, H_3-10), 1.27 (3H, s, H_3-11), and 1.16 (3H, s, H_3-12), one methylene signals at δ_H 2.38 (1H, d, *J* = 16.0 Hz, H-2), 2.26 (1H, d, *J* = 16.0 Hz, H-2), two oxygen-bearing methines at δ_H 4.80 (1H, m, H-13) and 4.07 (1H, m, H-9), one methine at δ_H 2.83 (1H, m, H-8), and two olefinic protons at δ_H 6.37 (1H, br s, H-7) and δ_H 5.91 (1H, s, H-4), respectively. In addition, protons tentatively assigned to a glucopyranosyl moiety were observed in the ^1H NMR spectrum of **1**, the anomeric proton was presented at δ_H 4.36 (1H, d, *J* = 8.0 Hz), corresponding to the carbon presented at δ_C 102.2 in the HSQC spectrum. Acid hydrolysis of **1** with 1 M HCl afforded glucose, revealing the occurrence of a glucopyranosyl moiety in **1.** Irrespective of the carbons due to the glycopyranosyl moiety, the ^{13}C NMR spectrum of **1** showed the presence of 13 carbons including four conjugated olefinic carbons, one carbonyl carbon, three methyls, three methines, one methylene, and one quaternary carbon, which suggested that the aglycon of **1** might be a megastigmane-type sesquiterpene. The molecular formula ($C_{19}H_{28}O_8$) of **1** revealed that the index of hydrogen deficiency (IHD) of **1** was six, four of which were contributed to two double bonds, one carbonyl group, and one glucopyranosyl moiety. The remaining two IHD suggested the presence of two carbocyclic rings in **1**. All the protons and carbons were unambiguously assigned (Tables 1 and 2) by 2D NMR experiments, including ^1H-^1H COSY, HSQC, HMBC, and NOESY. In the ^1H-^1H COSY spectrum, the correlation between H-8 and H-13 strongly suggested that a C-C bond was formed between C-8 and C-13, which was confirmed by HMBC correlations of H-7/C-13 and H-9/C-13 (Fig. 2). The linkage of the aglycon and the glucopyranosyl moiety was established by the long range correlation between the anomeric proton Glu-H-1 and C-9, which was further confirmed by the NOESY correlation between H-9 and Glu-H-1.The relatively large coupling constant of the anomeric proton (*J* = 8.0 Hz) suggested that the anomeric carbon of the glucopyranoyl moiety was in *β* configuration.

In order to resolve the absolute configuration of **1**, the electronic circular dichroism (ECD) spectra of the aglycon of **1** (**A1**) were calculated using TDDFT at the B3LYP/6-31G level with the CPCM model in MeOH. Analysis of the calculated ECD spectra of eight isomers (S38) revealed that the ECD spectrum of **A1** was crucially depended on the absolute configuration of C-13 but little concern with the configurations of C-8 and C-9. When the configuration of C-13 was *R*, positive Cotton effect could be found at 295 nm in the calculated ECD spectrum of **A1**. In contrast, negative Cotton effect was presented at 295 nm, corresponding to the *S* configuration of C-13. To obtain the experimental ECD spectrum of **A1**, we have to prepare the aglycon by hydrolysis of **1**. However, analysis of the hydrolysate by LC-MS revealed that acid hydrolysis produced a degradation product but not the expected aglycon **A1**. Therefore, enzymatic hydrolysis of **1** by cellulase was performed, leading to the generation of the proto-aglycon **A1** with a molecular formula of $C_{13}H_{18}O_3$ assigned by HRESIMS (S8). After purification of **A1** by HPLC, the experimental ECD spectra of **A1** and **1** were collected (Fig.3). The superposable ECD spectra of **1** and **A1** revealed the

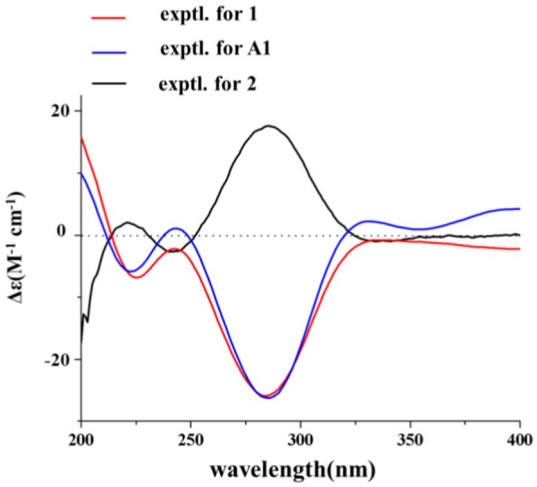

Fig. 3. Exprimental and calculated ECD spectra of **1**, **A1**, and **2** (in MeOH).

invalidity of the glycosyl moiety to the ECD absorption. The presence of a typically negative Cotton effect at 295 nm in the experimental ECD spectrum of **A1** allowed establishing the *S* configuration of C-13. In the NOESY spectrum of **1**, the NOE correlations of H-8/H-13 revealed a *cis*-orientation of H-8 and H-13. Accordingly, the absolute configuration of C-8 was assigned as *R*. The absolute configuration of C-9 should be resolved using Mosher's method. Unfortunately, the preparation of (*R*/*S*)-MTPA esters were failed due to limited amount of **A1**. Therefore, the structure of **1** was elucidated as shown in Fig. 1, named as urenalobaside A. As far as our knowledge, megastigmanes with a 6/5 fused ring system have never been reported.

Compound **2** was obtained as a pale yellow gum, $[\alpha]_D^{25}$ –14.1 (*c* 0.05, MeOH). Its molecular formula was assigned as $C_{19}H_{28}O_8$ by the $[M + Cl]^-$ ion peak at *m/z* 419.1483 in the HRESIMS spectrum. Comparison of the NMR data of **2** with those of **1** revealed that compound **2** shared a very similar skeleton with that of **1**. Unambiguous assignment of the protons and carbons of **2** by 2D NMR experiments allowed establishing the planar structure of **2**, which was completely same to that of **1**. However, the quite different retention time (S9) suggested that **1** and **2** may be stereoisomers. In the experimental ECD spectrum of **2**, the presence of a positive Cotton effect at 295 nm suggested that the absolute configuration of C-13 was *R*. Considering that the NOE correlation of H-8/H-13 could not be found in the NOESY spectrum of **2**, *trans*-oritentation of H-8 and H-13 was arbitrarily assigned, resulting in a tentatively determination of the absolute configuration of C-8 as *R*. Therefore, the structure of **2** was elucidated as shown in Fig. 1, named as urenalobaside B.

Compound **3** was obtained as a colorless gum, $[\alpha]_D^{25}$–52.4 (*c* 0.05, MeOH). Its molecular formula was assigned as $C_{24}H_{38}O_{11}$ by the $[M + Cl]^-$ ion peak at *m/z* 537.2092 in the HRESIMS spectrum. The ^1H NMR spectrum exhibited the presence of two quaternary methyls at δ_H1.30 (6H, br s, H_3-11, H_3-12), a vinyl methyl at δ_H 2.14 (3H, s, H_3-13), a secondary methyl at δ_H 1.26 (1H, d, *J* = 6.0 Hz, H_3-10), two methylenes at δ_H 2.34 (2H, s, H_2–2) and 2.68 (2H, dd, *J* = 12.0, 6.5 Hz, H_2-8), one oxygen-bearing methines at δ_H 3.16 (1H, dd, *J* = 12.0,

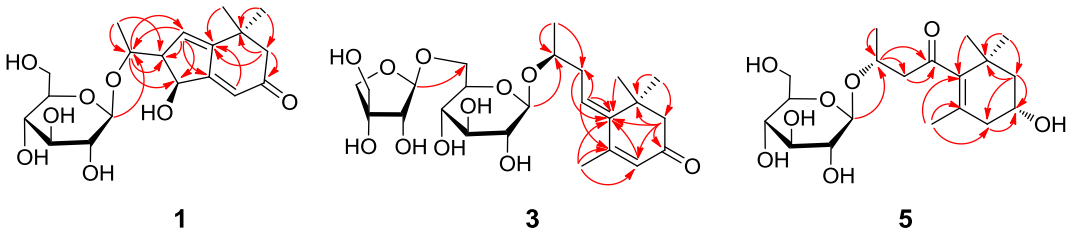

Fig. 2. Key HMBC correlations of compounds**1**, **3**, and **5**.

6.0 Hz, H-9), and two olefinic protons at δ_H 5.87 (1H, s, H-4), 6.36 (1H, t, J = 6.5 Hz, H-7). In addition, two anomeric protons were observed at δ_H 4.34 (1H, d, J = 8.0 Hz) and 4.99 (1H, d, J = 2.5 Hz), corresponding to the carbons at δ_C 102.5 and 111.0 in the HSQC spectrum, respectively. Acid hydrolysis of **3** with 1 M HCl afforded glucose and apiose. The ^{13}C NMR spectrum showed the presence of 24 carbons, 13 of which were assigned to the aglycon, and the other 11 carbons were assigned to glucopyranosyl and apiofuranosyl moieties. All the protons and carbons were unambiguously assigned by analysis of the 1D and 2D NMR spectra. In the HMBC spectrum, the correlations of H-4/C-6, H-7/C-5, C-6, C-8, and H_3-13/C-4, C-5, C-6 established the $\Delta^{4,5}$ and $\Delta^{6,7}$ conjugated double bonds, the HMBC correlations of H-2/C-3 determined the carbonyl group was at C-3. Additionally, the HMBC correlation of Glu-H-1/C-9 established that the glucopyranosyl moiety was linked at C-9 of the aglycon, the HMBC correlation of Api-H-1/Glu-C-6 established that the apiofuranosyl miety was linked at C-6 of the glucopyranosyl moiety (Fig. 2). In the NOESY spectrum, the correlation of H-7/H-13 suggested a *E* configuration of $\Delta^{6,7}$ double bond. Enzymatic hydrolysis of **3** by cellulase afforded the known compound (6*E*,9*S*)-9-hydroxy-4,6-megastigmadien-3-one (S22–23) [14,15]. Accordingly, the structure of **3** was determined as shown in Fig. 1, named as urenalobaside C.

Compound **4** was obtained as a colorless gum, $[\alpha]_D^{25}$–54.0 (*c* 0.05, MeOH). The HRESIMS spectrum showed the presence of a quasimolecular ion peak $[M + Cl]^-$ at *m/z* 537.2106, consistent with an empirical molecular formula of $C_{24}H_{38}O_{11}$. Comparison of the NMR data of **4** with those of **3** revealed that **4** shared a similar skeleton to **3**. The only difference was that the *E* configuration of $\Delta^{6,7}$ double bound in **3** was changed into *Z* configuration in **4**, which was confirmed by the NOESY correlation of H-7/H-11. Enzymatic hydrolysis of **4** by cellulase afforded the known compound (6*Z*,9*S*)-9-hydroxy-4,6-megastigmadien-3-one (S30–31) [14,15]. Accordingly, the structure of **3** was determined as shown in Fig. 1, named as urenalobaside D.

Compound **5** was obtained as a colorless gum, $[\alpha]_D^{25}$–24.0 (*c* 0.05, MeOH). Its molecular formula was assigned as $C_{19}H_{32}O_8$ by the $[M + Cl]^-$ ion peak at *m/z* 423.1770 in the HRESIMS spectrum. The ^{1}H and ^{13}C NMR spectrum of **5** showed the presence of four methyls at δ_H 1.64 (3H, s, H_3-13), 1.26 (3H, d, J = 6.5 Hz, H_3-10), 1.18 (3H, s, H_3-11), and 1.03 (3H, s, H_3-12), three methylenes at δ_H 3.19 (1H, dd, J = 19.0, 6.5 Hz, H-8), 2.65 (1H, dd, J = 19.0, 6.5 Hz, H-8), 2.29 (1H, dd, J = 17.5, 6.0 Hz, H-4), δ_H 1.93 (1H, m, H-4), 1.68 (1H, dd, J = 12.5, 2.5 Hz, H-2), and δ_H 1.41 (1H, t, J = 12.5 Hz, H-2), two oxygen bearing methines at δ_H 4.42 (1H, dd, J = 12.5, 6.5 Hz, H-9), and 3.94 (1H, m H-3), and multiple protons due to a glucopyranoyl moiety in the range of δ_H 3.0–4.5. The ^{13}C NMR showed the presence of 19 carbons, 13 of which was assigned to the aglycon. Comparison of the NMR data of **5** with those of the known compound **13** revealed that signals due to the acetylenic bond in **13** disappeared [16]. In contrast, signals assigned to a methlylene and a carbonyl group was observed. In the HMBC spectrum, the long range correlations (Fig. 2) of H-2/C-3, H-4/C-3, H-8/C-7, and H-9/C-7 demonstrated that the hydroxyl group and the carbonyl group were at C-3 and C-7, respectively. Additionally, the cross peak between the anomeric proton Glu-H-1 and C-9 of the aglycon confirmed that the glucopyranosyl moiety was linked at C-9. Limited by the small amount of **5**, further determination of the absolute configuration of **5** was reluctantly abandoned. Considering that **5** may be biogenetically synthesized from the known compound **13**, the absolute configuration of **5** was elucidated as shown in Fig. 1, named as urenalobaside E.

By comparison of their NMR, CD spectra, and specific rotation data with those of the known compounds, the other 11 compounds were identified as lauroside B (**6**) [17], inamoside (**7**) [18], lauroside C (**8**) [17], 3-oxo-*α*-ionyl-9-*O*-*β*-D-apiofuranosyl-(1 → 6)-*β*-D-glucopyranoside (**9**) [19], guettardionoside (**10**) [20], (6*S*,9*S*)-roseoside (**11**) [21], vomifoliol-9-*O*-*β*-D-glucopyranoside (**12**) [22], (3*R*,9*R*)-3-hydroxy-7,8-dehydro-ionol-9-*O*-*β*-D-glucopyranosides (**13**) [16], vomifoliol-9-*O*-*β*-D-apiofuranosyl-(1 → 6)-*β*-D-glucopyranoside (**14**) [19], (6*S*,7*E*,9*ξ*)-6,9,10-trihydroxy-4,7-megastigmadien-3-one10-*O*-*β*-D-glucopyranoside (**15**) [23], bridelionoside A (**16**) [24], respectively.

Compounds **1**, **3–16** were evaluated for their inhibitory effects on the NO production in LPS-stimulated RAW264.7 macrophage cells. Compounds **3** exhibited weak inhibition of NO production with IC_{50} value of 53.7 ± 1.0 μM.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

The NMR and HRESIMS spectra for compounds **1–5**, the aglycon of compounds **3** and **4** (**A3**, **A4**), and the calculated ECD spectra of the eight stereoisomers of **1** are available in the online version. Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.fitote.2018.02.017

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