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A Novel Phenanthrene and An Undescribed Alkaloid from the Roots of *Stephania tetrandra*

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Abstract: A novel phenanthrene compound, diphenanthrin (**1**), and an undescribed alkaloid compound, *N*-chloromethylstephenanthrine (**2**), together with two known compounds (**3**-**4**) were isolated from the EtOH extract of the roots of *Stephania tetrandra*. Their structures were elucidated by 1D and 2D NMR, mass spectroscopy and comparison of NMR data with those of known compounds. In anti-inflammatory assay, compounds **3** and **4** showed the inhibitory effects on NO production, with IC_{50} values of 41.51 ± 0.20 and 23.87 ± 2.12 μM.

Keywords: *Stephania tetrandra*; phenanthrene; alkaloid; anti-inflammatory.

1. Introduction

Stephania tetrandra S. Moore, a traditional Chinese medicinal plant, mainly distributes in Jiangxi, Anhui, Zhejiang, Hubei and Hunan provinces of China. Its roots were often used to treat rheumatalgia, dropsy, dysuria, and eczema. The investigations of chemical ingredient revealed that many alkaloids, such as bisbenzylisoquinoline, aporphine, protoberberine and phenanthrene alkaloids, were isolated from the roots of *Stephania tetrandra* [1–5]. The roots of *Stephania tetrandra* exhibited a wide range of biological activities, including anti-inflammation, anti-tumor, anti-fibrosis, anti-myocardial ischemia [6-10]. In our previous work, many bisbenzylisoquinoline alkaloids with anti-inflammation activities were isolated from the roots of *Stephania tetrandra* [1]. To discover more structurally diverse compounds with anti-inflammation activities, the EtOH extract of roots of *Stephania tetrandra* was further investigated and yielded a novel phenanthrene compound (**1**), an undescribed alkaloid compound (**2**) and two known compounds (**3**-**4**). In this paper, we reported the isolation, structures elucidation and inhibitory effects against nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells of compounds **1**-**4**.

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2. Materials and Methods

2.1. Plant Material

The roots of *Stephania tetrandra* were collected from Ruichang, Jiangxi Province, China, in December 2017, and were authenticated by Professor Lin Ma, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (ID-S-2937) was preserved in the herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

2.2. General Experimental Procedures

IR spectra were recorded by a Nicolet 5700 spectrometer (Thermo Fisher Scientifific, USA). HRESIMS spectra were measured by Dionex UltiMate 3000 UHPLC equipped with Thermo Scientifific Q Exactive Focus Mass spectrometer (Thermo Fisher Scientifific, USA). The 1D and 2D NMR spectra were measured by Bruker-600 MHz spectrometer (Bruker BioSpin GmbH, Germany). Semi-preparative HPLC was carried out on a Shimadzu LC-2030C 3D and LC-20AP liquid chromatography (Shimadzu, Japan).

2.3. Extraction and Isolation

The dried and powdered roots of *Stephania tetrandra* (98 kg) were extracted with 95% EtOH (100 L × 3). The extract was concentrated under reduced pressure to give 8.0 kg residue, which was then added into 1% HCl solution with vigorous stirring. The acidic solution was filtered, and the acidic filtrate was then basified with concentrated NH_4OH to produce free alkaloids and exhaustively extracted with $CHCl_3$ to give a total alkaloid (2.1 kg).

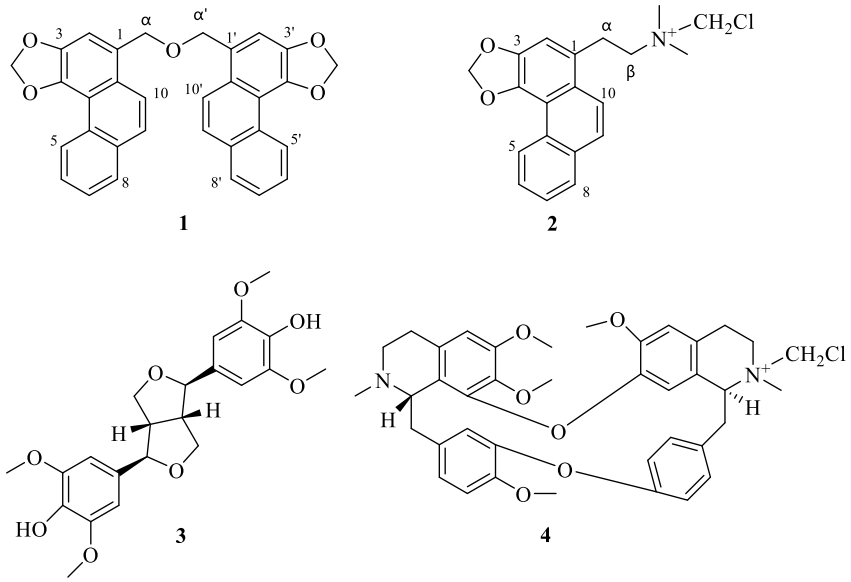

Figure 1. Structures of compounds **1**-**4**

The total alkaloid was separated on silica gel column chromatography using gradient eluted with petroleum ether-acetone and $CHCl_3$-MeOH (1:0 to 25:1 and 30:1 to 0:1), yielding seven fractions (Fr.1-Fr.7). Fr.1 (1.3 kg) was recrystallized in acetone and was subjected to a silica gel column chromatography eluted with $CHCl_3$-MeOH gradient system (1:0 to 0:1) to afford six fractions (Fr.1.1-Fr.1.6). Fr.1.3 (34.2 g) was subjected to a silica gel column chromatography eluted with petroleum ether-$CHCl_3$ (5:1) and $CHCl_3$-MeOH gradient system (30:1 to 0:1) to give five fractions (Fr.1.3.1-Fr.1.3.5). Fr.1.3.3 (512.6 mg) was chromatographed using Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1) and

semi-preparative HPLC (MeCN/H_2O, 70:30, 2 mL/min) to yield **1** (5.6 mg). Fr.1.4 (40.0 g) was separated on column chromatography of silica gel (CH_2Cl_2/MeOH, 30:1 to 0:1), Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1) and semi-preparative HPLC (MeCN/H_2O, 42:58, TFA, 0.1%, 3 mL/min) to yield **2** (2.0 mg). Fr.2 (400 g) was recrystallized in EtOH and separated by silica gel column chromatography (CH_2Cl_2-MeOH, 20:1 to10:1 and EtOAc-MeOH-NH_4OH, 20:2:1 to 10:2:1) to yield eight fractions (Fr.2.1-Fr.2.8). Fr.2.2 (15.1 g) was recrystallized in acetone and was subjected to a Sephadex LH-20 column chromatography eluted with CH_2Cl_2-MeOH (1:1) to afford three fractions (Fr.2.2.1-Fr.2.2.3). Fr.2.2.2 (0.6 g) was separated by MPLC (ODS, MeCN/H_2O, 10:90 to 100:0, 2 h, diethylamine, 0.01%) and semi-preparative HPLC (MeCN/H_2O, 42:58, TFA, 0.1%, 3 mL/min) to yield **3** (23.5 mg). Fr.2.2.1 (3.0 g) was separated on silica gel column chromatography (EtOAc-MeOH-NH_4OH, 17:2:1 and 5:2:1), MPLC (ODS, MeCN/H_2O, 40:60, TFA, 0.1%) and semi-preparative HPLC (MeCN/H_2O, 43:57, TFA, 0.1%, 3 mL/min) to yield **4** (31.8 mg).

2.4. Anti-Inflammatory Assay

The RAW264.7 macrophage cell lines were inoculated into 96-well plates (1×10^4 cells/well) containing DEME medium with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum and were cultured for 24 h under a humidified atmosphere with 5% CO_2 at 37 °C. The culture medium was replaced by fresh medium containing compounds **1**-**4** with different concentrations. After incubation for 2 h, the RAW264.7 cells were then incubated with 1 μg/mL of LPS for 24 h. The culture supernatants of cells were collected and Griess reagents were added into supernatants to determine the NO level. The absorbance was measured at 540 nm with a microplate reader. Dexamethasone (DEX) was used as a positive control [11-12].

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as white amorphous powder. Its molecular formula was determined to be $C_{32}H_{22}O_5$ by HRESIMS ion peak at *m/z* 509.1357 $[M+Na]^+$ (calcd. for $C_{32}H_{22}O_5Na$, 509.1359), indicating 22 degrees of unsaturation. The IR spectrum of **1** indicated the presence of aromatic ring (3053, 1597 and 1504 cm^{-1}) functionality. The UV spectrum (λ_{max} 202, 215, 238, 248, 283, 320 and 370 nm) was like those of (*S*)-fenfangjine F [2], indicating the presence of the phenanthrene chromophore in **1**. The ^{1}H-NMR spectrum of **1** (Table 1) showed the presence of seven aromatic protons at δ_H 9.09 (1H, dd, J = 7.8, 1.2 Hz, H-5), 7.93 (1H, d, J = 9.0 Hz, H-10), 7.85 (1H, dd, J = 7.2, 1.8 Hz, H-8), 7.62 (3H, overlapped, H-9, H-6, H-7) and 7.32 (1H, s, H-2) and two singlet methylenes at δ_H 6.26 (2H, s, 3,4-OCH_2O), 5.11 (2H, s, H-α). The ^{13}C-NMR and HSQC spectra of **1** exhibited the presence of 16 carbon signals, including seven aromatic methines (δ_C 127.9, 127.4, 127.0, 126.5, 125.8, 122.6, 109.8), seven aromatic quaternary carbons (δ_C 145.0, 143.5, 132.1, 131.1, 128.7, 126.2, 117.3) and two methylenes (δ_C 101.4, 63.9). The ^{1} H and ^{13}C NMR data of compound **1** were similar to those of artapilosine B [13], except that artapilosine B showed one more methylene signal than compound **1**. In the HMBC spectrum, the HMBC correlations from H-α to C-1, C-2 and C-10a, from H-2 to C-α, C-3 and C-4 suggested that the methylene (δ_H 5.11, δ_C 63.9) was attached at C-1. The HRESIMS combined NMR data implied the existence of symmetrical unit in **1**. The fragment ion peak at *m/z* 235.0755 was also observed, which was corresponded to the left half of molecular, due to the cleavage of benzylic bond (Figure S2 and S3, Supporting Information). Meanwhile, the hydroxy group was not observed in the IR spectrum, indicating the presence of dimer form. Thus the structure of **1** was established and was named diphenanthrin.

Compound **2** was isolated as white amorphous powder. The postitive HRESIMS spectrum showed a molecular ion peak cluster at *m/z* 342.1252/344.1220 $[M]^+$ with a ratio of 3:1, indicating one chlorine atom in the molecule. Its molecular formula was determined to be $C_{20}H_{21}O_2NCl$ by HRESIMS ion peak at *m/z* 342.1252 $[M]^+$ (calcd. for $C_{20}H_{21}O_2NCl$, 342.1255). The ^{1}H-NMR spectrum of **2** (Table 1) showed the presence of seven aromatic protons at δ_H 9.12 (1H, dd, J = 7.8, 1.2 Hz, H-5), 7.90 (2H, overlapped, H-8, 10), 7.73 (1H, d, J = 9.6 Hz, H-9), 7.63 (2H, overlapped, H-6, 7),

7.37 (1H, s, H-2), four methylenes at δ_H 6.30 (2H, s, 3, 4-OCH_2O), 5.48 (2H, s, *N*-CH_2Cl), 3.76 (2H, m, H-*β*), 3.63 (2H, m, H-*α*) and two methyls at δ_H 3.41 (6H, s, *N*-CH_3). Analysis of the ^{13}C NMR spectrum revealed the presence of 20 carbon signals, including the signals corresponding to the afore mentioned units and seven aromatic quaternary carbons. The ^{1}H and ^{13}C NMR data (Table 1) of **2** were similar to those of stephenanthrine[14-15], except for a methylene signal (δ_H 5.48, δ_C 69.6) in **2**. The HMBC correlations from *N*-CH_2Cl (δ_H 5.48) to C-*β* and *N*-CH_3 revealed that the methylene (δ_H 5.48, δ_C 69.6) was attached at the *N* position. Therefore, compound **2** was elucidated as *N*-chloromethylstephenanthrine.

Additionally, two known compounds were identified as (–)-syringaresinol (**3**) [16] and 2'-*N*-chloromethyltetrandrine (**4**) [17] by comparison with NMR data in the literature.

Table 1. ^{1}H NMR (600 MHz) and ^{3}C NMR (150 MHz) data of compounds **1** and **2**

Position	**1**[a]		**2**[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1 (1')		131.1		126.5
2 (2')	7.32 s	109.8	7.37 s	112.3
3 (3')		145.0		146.8
4 (4')		143.5		144.7
4a (4a')		117.3		118.4
4b (4b')		128.7		129.8
5 (5')	9.09 dd (7.8, 1.8)	127.0	9.12 dd (7.8, 1.8)	128.3
6 (6')	7.62 overlapped	126.5	7.63 overlapped	128.3
7 (7')	7.62 overlapped	127.4	7.63 overlapped	127.7
8 (8')	7.85 dd (7.8, 1.8)	127.9	7.90 overlapped	128.9
8a (8a')		132.1		133.4
9 (9')	7.62 overlapped	125.8	7.73 d (9.6)	127.3
10 (10')	7.93 d (9.0)	122.6	7.90 overlapped	122.7
10a (10a')		126.2		127.4
α (α')	5.11 s	63.9	3.63 m	27.2
β (β')			3.76 m	64.3
OCH_2O (OCH_2O')	6.26 s	101.4	6.30 s	102.9
N-CH_3			3.41 s	50.3
N-CH_2Cl			5.48 s	69.6

[a]Recorded in $CDCl_3$. [b]Recorded in CD_3OD.

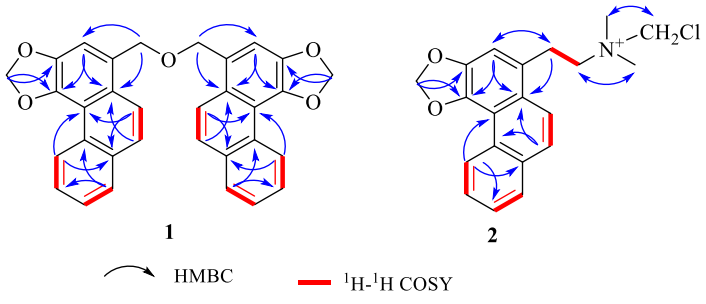

Figure 2. Key 2D NMR correlations of compounds **1** and **2**

3.2. Anti-Inflammatory Activity

The anti-inflammatory activities of compounds **1**-**4** were evaluated using an LPS-induced RAW264.7 macrophage cells model. Compounds **3** and **4** exhibited inhibitions on NO production with

17.67 ± 5.09% and 29.05 ± 1.77% at the concentration of 10 μM, but compounds **1** and **2** showed no inhibitory effects (Figure S28, Supporting Information). Compounds **3** and **4** showed the moderated anti-inflammatory activities, with IC_{50} values of 41.51 ± 0.20 and 23.87 ± 2.12 μM, respectively. (Positive control DEX, IC_{50} = 11.01 ± 0.84 μM).

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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