

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*-chloromethylstephananthrine (**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 IC₅₀ values of 41.51 ± 0.20 and 23.87 ± 2.12 μM.

Keywords: *Stephania tetrandra*; phenanthrene; alkaloid; anti-inflammatory. © 2023 ACG Publications. All rights reserved.

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 rheumatism, dizziness, 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 Scientific, USA). HRESIMS spectra were measured by Dionex UltiMate 3000 UHPLC equipped with Thermo Scientific Q Exactive Focus Mass spectrometer (Thermo Fisher Scientific, 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 \times 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 NH₄OH to produce free alkaloids and exhaustively extracted with CHCl₃ to give a total alkaloid (2.1 kg).

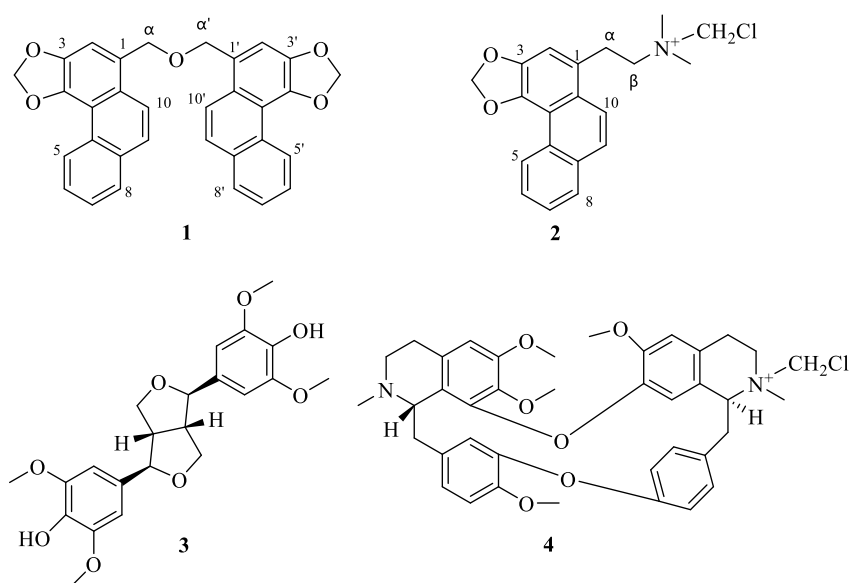


Figure 1. Structures of compounds 1-4

The total alkaloid was separated on silica gel column chromatography using gradient eluted with petroleum ether-acetone and CHCl₃-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 CHCl₃-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-CHCl₃ (5:1) and CHCl₃-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 (CH₂Cl₂/MeOH, 1:1) and semi-preparative HPLC (MeCN/H₂O, 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 (CH₂Cl₂/MeOH, 30:1 to 0:1), Sephadex LH-20 (CH₂Cl₂/MeOH, 1:1) and semi-preparative HPLC (MeCN/H₂O, 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 (CH₂Cl₂-MeOH, 20:1 to 10:1 and EtOAc-MeOH-NH₄OH, 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 CH₂Cl₂-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/H₂O, 10:90 to 100:0, 2 h, diethylamine, 0.01%) and semi-preparative HPLC (MeCN/H₂O, 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-NH₄OH, 17:2:1 and 5:2:1), MPLC (ODS, MeCN/H₂O, 40:60, TFA, 0.1%) and semi-preparative HPLC (MeCN/H₂O, 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 × 10⁴ 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% CO₂ 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 C₃₂H₂₂O₅ by HRESIMS ion peak at *m/z* 509.1357 [M+Na]⁺ (calcd. for C₃₂H₂₂O₅Na, 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*)-fengfangjine F [2], indicating the presence of the phenanthrene chromophore in **1**. The ¹H-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-OCH₂O), 5.11 (2H, s, H-α). The ¹³C-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 ¹H and ¹³C 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 positive 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 C₂₀H₂₁O₂NCl by HRESIMS ion peak at *m/z* 342.1252 [M]⁺ (calcd. for C₂₀H₂₁O₂NCl, 342.1255). The ¹H-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-OCH₂O), 5.48 (2H, s, *N*-CH₂Cl), 3.76 (2H, m,

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H- β), 3.63 (2H, m, H- α) and two methyls at δ_{H} 3.41 (6H, s, *N*-CH₃). Analysis of the ¹³C NMR spectrum revealed the presence of 20 carbon signals, including the signals corresponding to the aforementioned units and seven aromatic quaternary carbons. The ¹H and ¹³C NMR data (Table 1) of **2** were similar to those of stephananthrine[14-15], except for a methylene signal (δ_{H} 5.48, δ_{C} 69.6) in **2**. The HMBC correlations from *N*-CH₂Cl (δ_{H} 5.48) to C- β and *N*-CH₃ 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*-chloromethylstephananthrine.

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. ¹H NMR (600 MHz) and ¹³C NMR (150 MHz) data of compounds **1** and **2**

Position	1 ^a		2 ^b	
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> 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 ₂ O	6.26 s	101.4	6.30 s	102.9
(OCH ₂ O')				
<i>N</i> -CH ₃			3.41 s	50.3
<i>N</i> -CH ₂ Cl			5.48 s	69.6

^aRecorded in CDCl₃. ^bRecorded in CD₃OD.

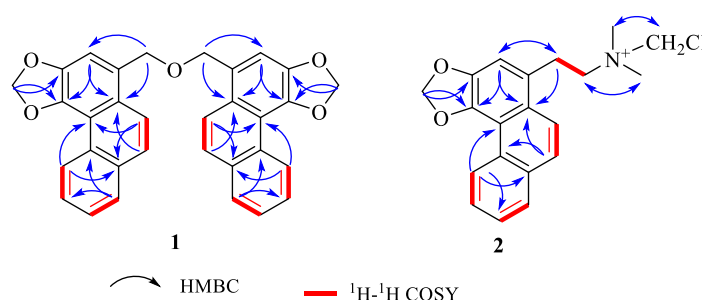


Figure 2. Key 2D NMR correlations of compounds **1** and **2**

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 $17.67 \pm 5.09\%$ and $29.05 \pm 1.77\%$ 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 $23.87 \pm 2.12 \mu\text{M}$, respectively. (Positive control DEX, $IC_{50} = 11.01 \pm 0.84 \mu\text{M}$).

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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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