SHORT REPORT

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Two Cationic Indole Alkaloids from *Ophiorrhiza japonica* and Their Xanthine Oxidase Inhibitory Activity

Yu He [1], Fan Xu [1], Chun Tian [1], Hong-Lian Ai [1], Bao-Bao Shi [1,2*] and Ji-Kai Liu [1,2*]

[1]*School of Pharmaceutical Sciences, South-Central MinZu University, Wuhan 430074, People's Republic of China*

[2] *International Cooperation Base for Active Substances in Traditional Chinese Medicine in Hubei Province, School of Pharmaceutical Sciences, South-Central Minzu University*

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Abstract: Two previously undescribed cationic indole alkaloids, named ophiorrhines L (**1**) and M (**2**), was isolated from the *Ophiorrhiza japonica*. The structures were elucidated based on spectroscopic data and quantum calculations as well as X-ray crystallographic analysis. Alkaloids **1** and **2** exhibited moderate inhibitory activities on XOD (Xanthine Oxidase) with IC_{50} values 42.5 and 22.6 μM, respectively.

Keywords: *Ophiorrhiza japonica*; indole alkaloids; XO inhibitory effect.

1. Plant Source

The whole plant of *Ophiorrhiza japonica* were collected in July 2021 in areas along Yanling County, Hubei Province, P. R. China, and identified by Dr. Hong-Lian Ai. The voucher specimen (NO. liu20210720) has been deposited at School of Pharmaceutical Sciences, South-Central MinZu University.

2. Previous Studies

Ophiorrhiza is a genus of the Rubiaceae family, including about 300 species, of which there are 70 species in China [1]. Plants from family Rubiaceae are known to contain several secondary metabolites like indole alkaloids, anthraquinones, triterpenes, and iridoids [2-4]. Some of alkaloids, such as camptothecin [5] and harmaline [6] are of major pharmaceutical importance. *Ophiorrhiza japonica* is a small shrub distributed in Southern China. Previous chemical investigations have confirmed that *O. japonica* contains alkaloids, flavonoids, and coumarins, which possess potent immunosuppressant, antioxidant, and cancer cell cytotoxicity [7-10]. As part of an ongoing search for bioactive alkaloids from the *Ophiorrhiza* plants, the 90% MeOH extract of the leaves and stems of *O. japonica* was phytochemically investigated to yield two previously undescribed cationic indole

*Corresponding author: Email: shibb0505@163.com (B.-B. Shi); liujikai@mail.scuec.edu.cn (J.-K. Liu).

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alkaloids. Here, we report the isolation, structural elucidation, and evaluation of the bioactivity of alkaloids.

3. Present Study

In this study, two alkaloids (**1** and **2**) (Figure 1) were isolated from the EtOAc extract of *O. japonica*. The structure of compound **1** was elucidated by extensive spectroscopic and X-ray crystallographic analyses. Compound **2** is a rare nitro-substituted indole alkaloid. Herein, the isolation, structural elucidation, and biological activities of the new isolates were reported.

Air dried and powdered plant (15 kg) of *O. japonica* were powdered and extracted with MeOH (25 L × 3) at room temperature for a week, the solvent was removed *in vacuo*. The extract (0.8 kg) was partitioned between 2% methanoic acid solution and ethyl acetate-petroleum ether (1:1) for three times. The acidic water layer was adjusted to pH 7-8 with 10% ammonia solution and subsequently extracted with ethyl acetate for three times. This ethyl acetate extract (72 g) was subjected to column chromatography (CC) over silica gel and eluted with gradient CHCl3- Me2CO (1:0–1:1, v/v) to afford ten fractions (I-Ⅹ). Fraction III (6.2 g) was chromatographed on a C_{18} MPLC column eluted with a gradient of MeOH-H_2O (5:95-100:0, v/v) to give the five fractions III-1~III-5. Fraction III-3 was purified a Sephadex LH-20 column eluting with 60% aqueous methanol to divide into five parts (III-3-1~ III-3-5). III-3-2 was purified by a preparative C_{18} HPLC column with a gradient of MeCN-H_2O (10:90~25:75, v/v) to obtain **1** (14.5 mg, 29.9 min). III-3-3 was purified by a preparative C_{18} HPLC column with a gradient of MeOH-H_2O (30:70~45:55, v/v) to obtain **2** (6.4 mg, 20.2 min).

Equipment: A Bruker spectrometer (Bruker, Germany, model AM600) was used to obtain 1D and 2D spectra. A Q Exactive HF mass spectrometer (Thermo Fisher Scientific, USA) was used to collect HRESIMS date. Applied Circular dichroism spectrometer (Chirascan, New Haven, USA) was used to record CD spectra. X-ray crystallographic analysis using Cu Kα radiation was performed on a Bruker D8 QUEST instrument (Bruker, Karlsruher, Germany). Column chromatography (CC) was performed on silica gel (Qingdao Marine Chemical Ltd., Qingdao, China), RP-18 gel (Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd., Sweden). HPLC was performed using Agilent 1260 pumps coupled with analytical semi-preparative or preparative Sunfire C_{18} columns (4.6 × 150 and 19 × 250 mm, respectively). Fractions were monitored by TLC (GF 254, Qingdao Haiyang Chemical Co., Ltd. Qingdao), and spots were visualized by Dragendorff reagent.

*Ophiorrhines L (**1**)*: pale yellow crystal; UV (MeOH) λ_{max} (log ε) = 215 (3.43), 275 (2.96), 305 (2.67); [α]24 D -69.1 (*c* 0.5, MeOH); ^{1}H NMR (600 MHz, CD_3OD): δ (ppm) = 4.33 (1H, t, *J* = 9.5 Hz, H-3), 3.16 (1H, d, m, H-5), 3.43 (1H, dd, *J* = 11.8, 10.4 Hz, H-5), 2.74 (1H, m, H-6), 2.90 (1H, m, H-6), 6.82 (1H, d, *J* = 2.4 Hz, H-9), 6.76 (1H, dd, *J* = 8.7, 2.4 Hz, H-11), 7.25 (1H, d, *J* = 8.7 Hz, H-12), 1.80 (1H, m, H-14), 2.59 (1H, m, H-14), 2.10 (1H, m, H-15), 1.77 (1H, m, H-16), 1.83 (1H, m, H-16), 3.12 (1H, m, H-17), 3.41 (1H, m, H-17), 1.01 (3H, t, *J* = 7.3 Hz, H-18), 1.61 (2H, m, H-19), 2.04 (1H, m, H-20), 2.97 (1H, m, H-21), 3.69 (1H, t, *J* = 11.5 Hz, H-21); ^{13}C NMR (150 MHz, CD_3OD): δ (ppm) = 133.3 (C, C-2), 61.9 (CH, C-3), 60.9 (CH_2, C-5), 17.9 (CH_2, C-6), 104.3 (C, C-7), 127.5 (C, C-8), 103.5 (CH, C-9), 152.2 (C, C-10), 113.6 (CH, C-11), 113.4 (CH, C-12), 130.4 (C, C-13), 26.6 (CH_2, C-14), 24.8 (CH, C-15), 26.1 (CH_2, C-16), 49.0 (CH_2, C-17), 12.0 (CH_3, C-18), 27.8 (CH_2, C-19), 37.8 (CH, C-20), 65.3 (CH_2, C-21); HRESIMS *m/z* 297.1959 $[M]^+$ (calcd for $C_{19}H_{25}N_2O$, 297.1961).

*Ophiorrhines M (**2**)*: pale yellow powder; UV (MeOH) λ_{max} (log ε) = 215 (3.38), 275 (2.88), 305 (2.54); [α]24 D -24.5 (*c* 0.5, MeOH); ^{1}H NMR (600 MHz, CD_3OD): δ (ppm) = 4.88 (1H, m, H-3), 3.45 (2H, m, H-5), 2.90 (1H, m, H-6), 3.11 (1H, m, H-6), 6.61 (1H, d, *J* = 9.0 Hz, H-11), 7.23 (1H, d, *J* = 9.0 Hz, H-12), 1.69 (2H, m, H-14), 2.24 (1H, m, H-15), 1.96 (2H, m, H-16), 3.25 (1H, m, H-17), 3.71 (1H, m, H-17), 1.04 (3H, t, *J* = 7.4 Hz, H-18), 1.91 (1H, m, H-19), 2.70 (1H, m, H-19), 2.14 (1H, m, H-20), 3.20 (1H, m, H-21), 3.77 (1H, m, H-21); ^{13}C NMR (150 MHz, CD_3OD): δ (ppm) = 130.2 (C, C-2), 61.9 (CH, C-3), 61.3 (CH_2, C-5), 20.9 (CH_2, C-6), 104.6 (C, C-7), 131.3 (C, C-8), 120.5 (C, C-9),

164.1 (C, C-10), 122.0 (CH, C-11), 120.3 (CH, C-12), 129.3 (C, C-13), 27.9 (CH_2, C-14), 25.2 (CH, C-15), 26.2 (CH_2, C-16), 49.1 (CH_2, C-17), 12.0 (CH_3, C-18), 26.9 (CH_2, C-19), 38.1 (CH, C-20), 65.3 (CH_2, C-21), 170.3 (C, C-22); HRESIMS *m/z* 342.1810 $[M]^+$ (calcd for $C_{19}H_{24}N_3O_3$, 342.1812).

Inhibitory Activity Assays of Xanthine Oxidase: The inhibitory effects on XO were evaluated using the spectrophotometric method in 96-well plates as described in the literature [11-12]. In brief, the reagents were 0.2 mol/L phosphate buffer pH 7.5, 400 μmol/L xanthine solution dissolved in phosphate buffer (pH 7.5), and 2.5 U/L XO solution (diluted from a stock enzyme solution into the buffer solution). The test samples were initially prepared in dimethyl sulfoxide (DMSO), followed by dilution with the buffer. A mixture consisting of 20 μL of phosphate buffer solution, 20 μL of working solution, and 40 μL of XO solution (2.5 U/L) was pre-incubated at 25 °C for 15 min. The reaction only took place when 40 μL xanthine solution (400 μmol/L) was added and incubated for 10 min at 25 °C. The uric acid production was calculated according to the increasing absorbance at 290 nm on a microplate reader (Tecan spark 10 M). The blank of each sample was prepared in the same way, but in the absence of XO solution. The negative control (0.5% DMSO) in the absence of an inhibitor and the positive control (allopurinol) were run simultaneously. The XO inhibitory activity was measured at doses of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 μmol/mL and estimated as the half-maximal inhibitory concentration (IC_{50}), calculated by the program Prism 8.0. All experiments were performed in triplicate. Inhibition rate (%) of XO = $[1-(D_2–D_3)/(D_1–D_0)] \times 100\%$, D_0: blank group, D_1: enzyme reaction group, D_2: inhibitor group, D_3: negative control group.

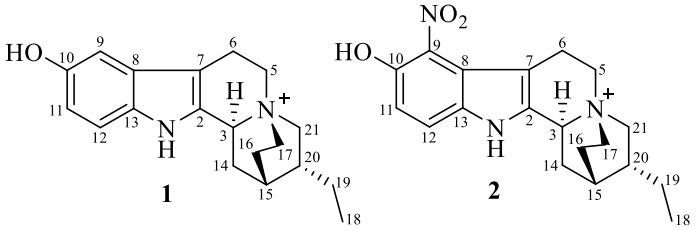

Figure 1. The chemical structures of compounds **1** and **2**

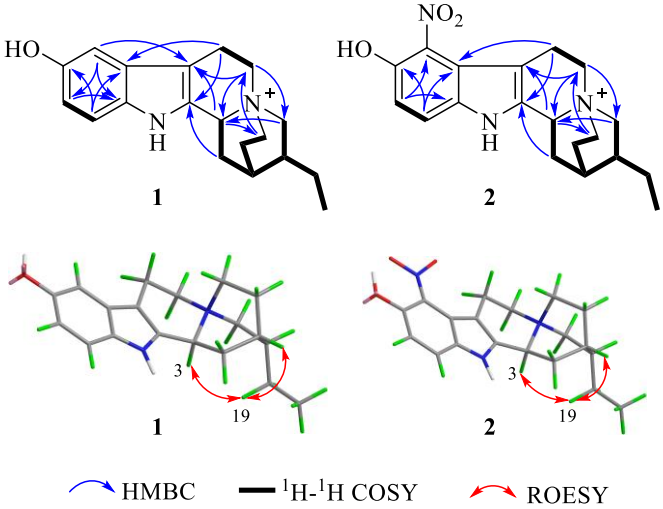

Figure 2. Key HMBC, ^{1}H-^{1}H COSY and ROESY correlations of compound **1**.

Ophiorrhines L (**1**) was obtained as colorless crystals, and its molecular formula was determined as $C_{19}H_{25}N_2O$ based on HRESIMS data of *m/z* 297.1959 $[M]^+$ (calcd. for $C_{19}H_{25}N_2O$, 297.1961), requiring nine degrees of unsaturation. The ^{1}H NMR data indicated a monosubstituted indole ring with signals at δ_H 6.82 (1H, d, *J* = 2.4 Hz), 6.76 (1H, dd, *J* = 8.7, 2.4 Hz), 7.25 (1H, d, *J* = 8.7 Hz), and one methyl signal at δ_H 1.01 (3H, t, *J* = 7.3 Hz). The ^{13}C NMR and DEPT data revealed the presence of five quaternary carbons (δ_C 133.3, 104.3, 127.5, 152.2, 130.4), six methines (δ_C 61.9, 103.5, 113.6, 113.4, 24.8, 37.8), seven methylenes (δ_C 60.9, 17.9, 26.6, 49.0, 26.1, 27.8, 65.3), and one methyl group

(δ_C 12.0). The ^{1}D NMR spectroscopic data of **1** were similar to those of Ophiorrhizine [13] except that the hydroxyl group at C-11 in Ophiorrhizine is absent and additional hydroxyl group is present at C-10 in **1**. The HMBC correlations from δ_H 7.25 (H-12) to δ_C 152.2 (C-10), δ_C 127.5 (C-8), from δ_H 6.76 (H-11) to δ_C 103.5 (C-9), δ_C 130.4 (C-13), and from δ_H 6.82 (H-9) to δ_C 113.6 (C-11), δ_C 130.4 (C-13), δ_C 104.3 (C-7) confirmed this result (Figure 2). Thus, the planar structure of compound **1** was determined. The structure with the absolute configuration of **1** was further determined by the single crystal X-ray diffraction as shown in figure 3 (Flack parameter = 0.00(5), CCDC: 2366847). Consequently, the stereochemistry of **1** was proved to be 3*S, 4R, 15S, 16R*.

Ophiorrhines M (**2**) was isolated as white powder. The positive HR-ESI-MS at m/z 342.1810 ([M]$^+$, calcd. 342.1812) corresponded to the molecular formula $C_{19}H_{24}N_3O_3$, with ten degrees of unsaturation. The ^{13}C NMR and DEPT data of **2** suggested that **2** had one methyl, seven methylenes, five methines including two sp2 aromatic carbons, and six quaternary carbon. Comparison of ^{13}C NMR data showed that compound **2** and **1** possessed nearly identical core structures, except for the absence of an olefinic methine (δ_C 103.5) and the presence of an additional quaternary carbon (δ_C 120.5), revealing that compound **2** was a disubstituted indole ring derivative of **1**. The resulting substructure encompassed all of the required hydrogens and carbons, and one of the oxygen, leaving only one nitrogen atom and two oxygen atoms unaccounted for. The HMBC spectrum showed correlations of H-11 with C-9 (δ_C 38.7) and C-13 (δ_C 131.5); H-12 with C-10 (δ_C 38.7) and C-8 (δ_C 131.5) indicating a nitro groups are substituted at C-9 of the indole ring (Figure 2). Considering the biogenetic relationship between **1** and **2**, the stereochemical structures of the two compounds were predicted to be identical. Therefore, the absolute configuration of **2** was defined as 3*S*,15*S*,20*R*. Furthermore, the identity of the measured and calculated ECD spectra of **2** was supportive for the above conclusion (Figure 4).

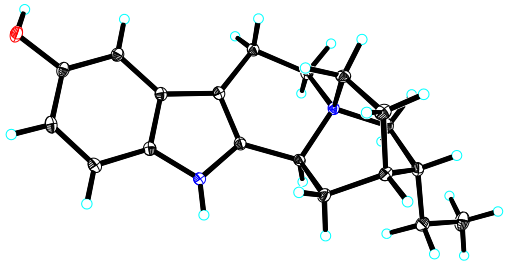

Figure 3. X-ray structure of compound **1**.

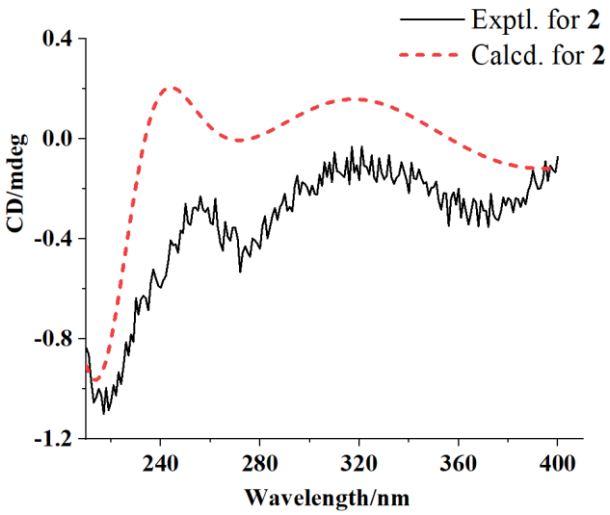

Figure 4. Experimental and calculated ECD spectra of **2** at the M062X/def2svp level in methanol.

The xanthine oxidase inhibitory effects of ophiorrhines L (**1**), ophiorrhines M (**2**) and allopurinol (positive control) have been evaluated by measuring the production of uric acid. Both in the assays of

uric acid production, compounds **1** and **2** exhibited moderate inhibitory activities on XOD with IC_{50} values 42.5 and 22.6 μM, respectively, compared to the positive control allopurinol with an IC_{50} value of 11.1 μM.

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Supporting Information

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

ORCID

Yu He: 0009-0006-1011-7255
Fan Xu: 0009-0001-5972-9416
Chun Tian: 0009-0006-6091-3102
Hong-Lian Ai: 0000-0003-2670-2054
Bao-Bao Shi: 0000-0001-9138-5628
Ji-Kai Liu: 0000-0001-6279-7893

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