HETEROCYCLES, Vol. 94, No. 4, 2017, pp. 743 - 749. © 2017 The Japan Institute of Heterocyclic Chemistry
Received, 24th November, 2016, Accepted, 8th March, 2017, Published online, 14th March, 2017
DOI: 10.3987/COM-16-13626

STRUCTURALLY DIVERSE INDOLE ALKALOIDS FROM *OCHROSIA ELLIPTICA*

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Abstract – A new naturally occurring indole alkaloid, 10-methoxyconolidine (**1**), together with seven known alkaloids (**2-8**), were isolated from the stems and leaves of *Ochrosia elliptica*. Among them, 10-methoxyconolidine (**1**) is a rare C5-nor stemmadenine type alkaloid, and the known compounds (**2-8**) possess diverse carbon skeletons. These structures were established on the basis of extensive spectroscopic methods. All isolated compounds were evaluated for their cytotoxicities and anti-inflammatory activities *in vitro*. New compound **1** showed no cytotoxicity while exhibited significant inhibitory activity on nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells *in vitro* with the IC_{50} value at 2.8 μM.

The genus *Ochrosia* (Apocynaceae) comprising about 39 species are trees naturally distributed in tropical or subtropical Malaysia, and west of Pacific Islands, and three species of the genus are cultivated in Guangdong, Hainan and Taiwan Provinces in China. This genus were widely used as folk medicines for antitumor.[1] Phytochemical studies on the plants of this genus led to the isolation of various skeletal types of indole alkaloids with antitumor activities, which attracted pharmacologists' attentions.[2] As a part of our ongoing research into structurally and biologically interesting alkaloids from tropical medicinal plants in China, a chemical investigation on *O. elliptica* was thus undertaken and led to the isolation and

characterization of a new C_5-nor stemmadenine type alkaloid, 10-methoxyconolidine (**1**), together with seven known alkaloids with a variety of carbon skeletons. Their structures were elucidated on the basis of extensive NMR and MS analyses. In addition, all isolated compounds were evaluated for their cytotoxicities and anti-inflammatory activities *in vitro*. Herein, we describe the isolation, structure elucidation, and biological activities of those compounds.

The EtOAc extract fraction of the stems and leaves of *O. elliptica* was subjected repeatedly to column chromatography on silica gel, sephadex LH-20, RP-18 and semi-preparative HPLC to afford a total of eight alkaloids, including a new one, as shown in Figure 1.

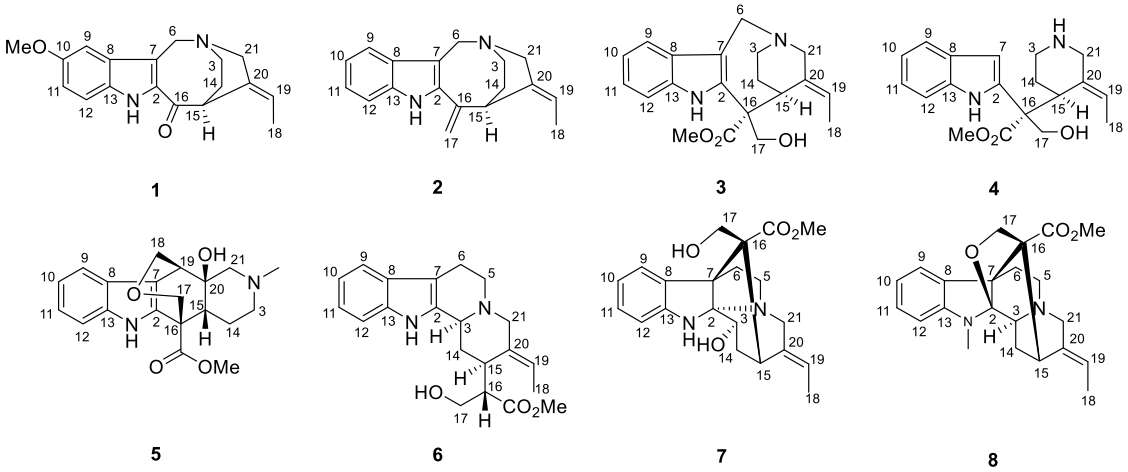

Figure 1. Structures of compounds **1**-**8** isolated from *O. elliptica*

10-Methoxyconolidine (**1**) was obtained as a white amorphous powder with a specific rotation of +38.8 (*c* 0.13, MeOH). Its molecular formula, $C_{18}H_{20}N_2O_2$, was established by HRESI-MS (*m/z* 297.1608 $[M + H]^+$, calcd 297.1603). Its IR spectrum showed the presence of an amino group (3338 cm^{-1}), a conjugated ketone carbonyl group (1634 cm^{-1}) and a phenyl group (1618, 1588 and 1458 cm^{-1}). The UV spectrum showed the absorption bands at 242 and 312 nm, which were characteristic of a 2-acylindole chromophore. The ^{13}C NMR and DEPT data revealed the presence of 18 carbon atoms, including 11 sp^2 carbon atoms, one sp^3 methines, four sp^3 methylenes and two methyl groups. In addition, the 11 sp^2 carbon atoms were attributable to one trisubstituted double bond, one indole ring group and one ketone carbonyl group. The above data revealed that the structure of **1** was similar to that of conolidine, except for the pattern of substitution of benzene ring group.[5-8] In the ^1H NMR spectrum of **1**, three downfield proton signals at δ_H 7.28 (1H, d, *J* = 8.6 Hz, H-12), 6.97 (1H, d, *J* = 2.0 Hz, H-9), and 6.92 (1H, dd, *J* = 8.6, 2.0 Hz, H-11), revealed the presence of an ABX coupling system. In the HMBC spectrum of **1,** the HMBC correlations of the methoxy group resonating at δ_H 3.75 (3H, s), H-9, H-11 and H-12 to C-10 implied that the methoxy group was located at C-10 (δ_C 153.4) (Figure 2), which was further supported by the ROESY correlations of H-9 to H-6*a*, 10-OCH_3 to H-9 and H-11, as well as H-12 to N_1-H. Detailed

analysis of 2D NMR (HSQC, HMBC, ^{1}H-^{1}H COSY and ROESY) spectra confirmed the planar structure of **1** as shown in Figure 2. The observation of strong cross-peak in the NOESY spectrum between H-15 and H-18 permitted the assignment of the geometry of the olefinic bond between C-19 and C-20 as *E*. The relative configuration of **1** was assigned on the basis of the NOESY correlations and the coupling constant of proton signals, which were almost identical to those of conolidine (Figure 2).[3] Thus, the structure of **1** was determined to be 10-methoxyconolidine as shown in Figure 1.

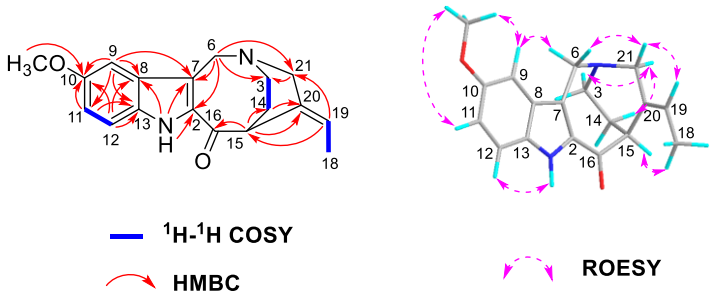

Figure 2. Selected 2D NMR correlations for 10-methoxyconolidine (**1**)

Table 1. ^{1}H and ^{13}C NMR data of 10-methoxyconolidine (**1**) in DMSO-d_6

Position	δ_H[a]	δ_C[b]
2		130.4 s
3α	2.93 (1H, m)	44.0 t
3β	3.23 (1H, overlapped)	
6α	4.14 (1H, d, J = 18.6 Hz)	53.1 t
6β	4.59 (1H, d, J = 18.6 Hz)	
7		119.1 s
8		127.2 s
9	6.97 (1H, d, J = 2.0 Hz)	100.6 d
10		153.4 s
11	6.92 (1H, dd, J = 8.6, 2.0 Hz)	117.2 d
12	7.28 (1H, d, J = 8.6 Hz)	113.3 d
13		132.0 s
14α	1.91 (1H, m)	22.4 t
14β	2.06 (1H, m)	
15	3.82 (1H, d, J = 6.2 Hz)	48.0 d
16		192.6 s
18	1.43 (1H, d, J = 6.8 Hz)	12.3 q
19	5.40 (1H, q, J = 6.8 Hz)	120.9 d
20		134.7 s
21α	3.24 (1H, overlapped)	54.3 t
21β	3.72 (1H, d, J = 15.2 Hz)	
10-OMe	3.75 (3H, s)	55.3 q
N_1-H	11.17 (1H, s)	

[a] Measured at 400 MHz. [b] Measured at 100 MHz.

From structural and biogenetic point of view, conolidine, whose structure and absolute configuration had been determined by a combination of spectroscopic and chemical methods, hold the same chiral center at C-15 with that of **1**, and could be considered as a model compound for assignment of the absolute configuration of **1** by comparison of their rotation values. Hence, the absolute configuration of **1** at C-15 could be assigned as *S*-configuration, identical to that of conolidine, in consideration of its similar specific rotation with that of conolidine ($[\alpha]_D$ +32).[3]

In addition to new alkaloid **1**, seven known alkaloids were isolated and identified as apparicine (**2**),[4] vallesamine (**3**),[5] yunnanensine (**4**),[6] angustilodine (**5**),[7] 16-*epi*-isositsirikine (**6**),[8] (-)-echitainine (**7**),[9] and pseudoakuammigine (**8**),[9] by comparing their experimental and reported physical data in the literature.

All isolated compounds were evaluated for their cytotoxicities against five human cancer cell lines, HL-60, SMMC-7721, A-549, MCF-7, and SW480 using the MTT method with doxorubicin as a positive control. However, all alkaloids showed no inhibitory effect in this assay (IC_{50} > 40.0 μM).

In addition, all isolated compounds were evaluated for their anti-inflammatory activities *via* examining the inhibitory activity on nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells *in vitro*. As a result, compounds **1** and **2** showed significant inhibitory activities with the IC_{50} values at 2.4 ± 0.08 μM and 5.8 ± 0.13 μM, respectively. Compounds **4** and **6** showed moderatet inhibitory activities with the IC_{50} values at 9.8 ± 0.22 μM and 12.6 ± 0.10 μM, respectively. However, other alkaloids showed no inhibitory effect in this assay (IC_{50} > 40.0 μM). While the positive control, hydrocortisone, showed a inhibitory activity with the IC_{50} value at 3.4 ± 0.18 μM. No cytotoxicity was observed in all compounds treated cells (cell viability > 90%).

A new rare C_5-nor stemmadenine type alkaloid, 10-methoxyconolidine (**1**), together with seven known alkaloids (**2-8**), were isolated from the stems and leaves of *O. elliptica*. All of them possessed diverse carbon skeletons. The discovery of **1** is not only a further addition to diverse and complex array of C_5-nor stemmadenine type alkaloids, usually possessing potent non-opioid analgesic properties,[6] but also, its presence as characteristic marker may be helpful in chemotaxonomical classifications. In addition, the anti-inflammatory activities of **1** and **2** were also investigated, and found be quite potent. To the best of our knowledge, this is the first time to evaluate the anti-inflammatory activity of any natural product in this class.

EXPERIMENTAL

General experimental procedures. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were obtained on a Nicolet 6700 spectrophotometer. NMR spectra were run on Bruker 400 MHz spectrometers using TMS as an internal standard. HRESIMS spectra were measured on a Q-TOF Ultima Global

GAA076 LC mass spectrometer. Semi-preparative HPLC was performed on an Agilent 1260 LC series with a DAD detector using an Agilent Eclipse XDB-C_{18} column (250 × 9.4 mm, 5 μm). Silica gel (Qing Dao Hai Yang Chemical Group Co.; 200-300 mesh) and RP-18 silica gel (YMC; 50 μm) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin layer chromatography (TLC).

Plant Material. The stems and leaves of *O. elliptica* were collected from Guangzhou, Guangdong Province, China, in July 2014, and identified by Prof. Dong-Ming Zhang, South China Botanical Garden, Chinese Academy of Sciences. A voucher specimen (No. 20140728) has been deposited at the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, Hainan Normal University.

Extraction and Isolation. The powdered air-dried stems and leaves of *O. elliptica* (10.0 kg) were extracted with 90% EtOH (30.0 L) at room temperature for four times, each for 7 days. The solvent were combined and condensed *in vacuum* to yield a crude extract (1200.0 g). After suspended in water (5.0 L), the crude extract was extracted successively with petroleum ether (5.0 L × 3) and EtOAc (5.0 L × 3), to obtain the petroleum ether extract and the EtOAc extract. The EtOAc extract (240.8 g) was subjected to silica gel column chromatography eluted with $CHCl_3$/MeOH (100:1 to 50:500, *v*/*v*) yielding six fractions (Fr.1-Fr.6). Fraction 4 (6.8 g) was subjected to column chromatography on silica gel using $CHCl_3$/acetone (100:1 to 50:50, *v*/*v*) to afford seven subfractions 4A-4G. Fraction 4B (0.8 g) was purified using Sephadex LH-20 column chromatography eluted with MeOH, then separated by silica gel column chromatography eluted with petroleum ether/acetone 60:40 to yield compounds **2** (82.6 mg) and **5** (16.5 mg). Fraction 4D (1.2 g) was purified using Sephadex LH-20 column chromatography eluted with MeOH, then separated by semi-preparative HPLC (Agilent Eclipse XDB-C_{18} column, i.d. 250 × 9.4 mm, 5 μm, 75% MeOH, 2.5 mL/min, t_R 32.3, 35.8 and 38.7 min) to afford compounds **1** (28.3 mg), **6** (15.8 mg) and **8** (33.2 mg). Fraction 4E (1.5 g) was purified using Sephadex LH-20 column chromatography eluted with MeOH, then separated by semi-preparative HPLC (Agilent Eclipse XDB-C_{18} column, i.d. 250 × 9.4 mm, 5 μm, 60% MeOH, 2.5 mL/min, t_R 23.6, 26.7 and 30.2 min) to afford compounds **3** (8.9 mg), **4** (16.9 mg) and **7** (23.6 mg).

10-Methoxyconolidine (1): White amorphous powder; $[\alpha]_D^{19}$ + 38.8 (*c* 0.13, MeOH); IR (KBr) ν_{max} 3338, 2928, 1634, 1628, 1588, 1458, and 1380 cm^{-1}; UV (MeOH) λ_{max} (log ε) 242 (4.08), 312 nm (4.32); ESI-MS *m/z* 297 $[M+H]^+$; HRESI-MS *m/z* 297.1608 (M+H; calcd for $C_{18}H_{21}N_2O_2$, 297.1603);

Cytotoxicity Bioassays. The following human tumor cell lines were used: HL-60, SMMC-7721, A-549, MCF-7, and SW480. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) in 5% CO_2 at 37 °C. The cytotoxicity assay was performed using the MTT method in 96-well microplates.[10] Briefly, adherent cells (100 μL) were

seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, and suspended cells were seeded just before drug addition with an initial density of 1×10^5 cells/mL. Each tumor cell line was exposed to the tested compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μM in triplicate for 48 h. Doxorubicin (Sigma, St. Louis, MO, USA) was used as the positive control. After treatment, cell viability was measured and the cell growth curve was plotted. IC_{50} values were calculated by the Reed and Muench method.[11]

Anti-inflammatory Bioassays. The RAW 264.7 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell concentration was adjusted to 5×10^5 cells/mL, and 200 μL of cell suspension was seeded in each well of a 96-well plate. After 1 h incubation, cells were treated with LPS (1 μg/mL) and test samples were dissolved in DMSO (final DMSO concentration 0.2%, *v*/*v*) for 24 h at 37 °C. A 100 μL sample of the culture supernatant was determined by the Griess reaction,[12] The Griess reagent (50 μL of 1% sulfanilamine in 5% H_3PO_4, and 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride) was added to each well. After 10 min, the reaction products were colorimetrically quantitated at 540 nm using a microplate reader. The experiments were performed in triplicate. Hydrocortisone was used as a positive control. The cytotoxicity assay was performed using the MTT method in 96-well microplates.[10] An MTT solution (200 μg/mL) was added after the 24 h treatment and then incubated for another 4 h at 37 °C. The reduced MTT-formazan was solubilized with 150 μL of DMSO, and the absorbance of the MTT-formazan solution at 570 nm was measured by an immunoreader. The percentage of suppression was calculated by comparing the absorbance of sample treated cells with that of nontreated cells.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 21302181, 21662011 and 31660097), the Applied Technology Research and Development and Demonstration Project of Hainan Province (No. ZDXM2015063), the Key Research and Development Project of Hainan Province (No. ZDYF2016221), the Program for Science and Technology plans to youth academic innovation of Hainan Association (No. HAST201636), the Program for Innovative Research Team in University (IRT-16R19) and the Natural Science Foundation of Hainan Province (Nos. 20162021 and 20162024).

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