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Winchinines A and B, two unusual monoterpene indole alkaloids with a third nitrogen atom from *Winchia calophylla*†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Two novel monoterpenoid indole alkaloids (MIAs) with a third nitrogen atom, winchinines A (1) and B (2), along with three known ones (3-5) were isolated from the twigs and leaves of *Winchia calophylla*. Compound 1 is the first natural MIA possessing a 6/5/6/5/6/5 hexacyclic ring system with an oxazolidinone unit. Compound 2 is an unprecedented cyano-substituted aspidosperma-type alkaloid. Their structures with absolute configurations were elucidated by means of NMR spectroscopy, single crystal X-ray diffraction, and electronic circular dichroism data analyses.

The plant *Winchia calophylla* is a species natively abundant in Yunnan and Hainan provinces of China.[1] This plant has been traditionally used as a folk medicine to treat chronic asthma and bronchitis.[2] Previous phytochemical investigations of this plant have led to the isolation of more than 35 monoterpene indole alkaloids (MIAs) [3-6] that showed antitumor, anti-inflammatory, and antiasthma activities. [7-9] It has been proposed that MIAs arise from strictosidine, which originates from the coupling of tryptamine and secologanin by a series of biosynthetic processes.[10-11] MIAs are characterized with two nitrogen atoms and those with three nitrogen atoms are uncommon[12-15]. During our ongoing research on structurally and biologically interesting MIAs from medicinal plants[16-18], two novel MIAs with a third nitrogen atom, winchinines A (**1**) and B (**2**), and three known ones (**3**-**5**) were isolated from *W. calophylla*. Winchinine A (**1**) is the first natural MIA possessing an oxazolidinone unit with a unique 6/5/6/5/6/5 hexacyclic ring system. Winchinine B (**2**) is an unprecedented cyano-substituted aspidosperma-type alkaloid. In this paper, we describe the isolation, structural elucidation and cytotoxic activities of **1** and **2**.

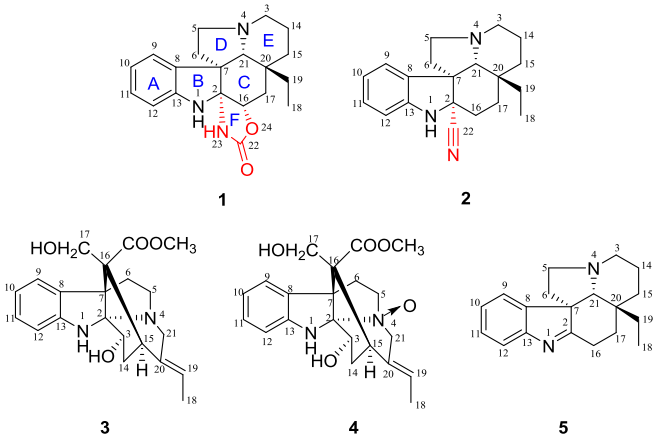

Figure 1. Structures of compounds **1**-**5**.

The powdered twigs and leaves of *W. calophylla* (16 kg) were extracted with 95% EtOH. The extract was suspended in H_2O and acidified with 5% HCl to pH 3, which was further partitioned with $CHCl_3$ to remove the neutral components. The aqueous layer was then basified with $NH_3 \cdot H_2O$ to pH 9 and re-extracted with $CHCl_3$ to obtain a total alkaloid fraction (215 g). The alkaloid fraction was separated continuously by column chromatography over silica gel, Sephadex LH-20, ODS (Octadecylsiyl) and HPLC (High Performance Liquid Chromatography) to yield compounds **1** (10.8 mg, yield: 0.067 %) and **2** (7.4 mg, yield: 0.046 %) (Fig.1).

Winchinine A (**1**) was obtained as colourless crystal. The molecular formula $C_{20}H_{25}N_3O_2$ was deduced on the basis of its HRESIMS (*m/z* 340.2027 $[M+H]^+$), which indicated the presence of ten degrees of unsaturation. The UV spectrum of **1** displayed the absorption maxima at 207 (C=C, π→π*), 245 (C=C, π→π*), and 302 (C=O, n→π*) nm. The IR spectrum showed characteristic absorptions for aromatic ring (1609, 1488 cm^{-1}), amino (3290 cm^{-1}), carbonyl (1754 cm^{-1}) and methyl (2935 cm^{-1}) groups. The ^1H NMR (Nuclear Magnetic Resonance) spectrum of **1** showed signals for an ortho-disubstituted phenyl ring [δ_H 6.99 (1H, overlapped, H-9), 6.99 (1H, overlapped, H-11), 6.71 (1H, dd, *J* = 7.5, 7.5 Hz, H-10),

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† Electronic supplementary information (ESI) available: The general experimental procedure, extraction and isolation, spectroscopic data of 1 and 2, single-crystal X-ray data of 1, NMR spectra and quantum chemical CD calculation of compounds 1 and 2. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/x0xx00000x.

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6.52 (1H, d, *J* = 7.5 Hz, H-12)],[19] an ethyl group [δ_H 1.12 (1H, dd, *J* = 14.5, 7.4 Hz, H-19a), 0.79 (1H, dd, *J* = 14.5, 7.4 Hz, H-19b), 0.66 (3H, t, *J* = 7.4 Hz, H-18)], and an oxygenated methine proton [δ_H 4.52 (1H, dd, *J* = 11.5, 6.1 Hz, H-16). The ^{13}C NMR and DEPT spectra of **1** exhibited twenty carbon signals including a methyl, seven methylenes, six methines and six quaternary carbons. The six aromatic carbon signals [δ_C 144.7 (C-13), 134.9 (C-8), 128.2 (C-11), 122.9 (C-9), 120.0 (C-10), and 109.2 (C-12)], a spiro quaternary carbon [δ_C 54.7 (C-7)] and an isolated ethyl group [δ_C 30.2 (C-19), 7.0 (C-8)] were characteristic for aspidosperma-type alkaloid. Comparison of the NMR data of **1** with those of the known compound aspidospermidine[20] revealed that their NMR signals were similar except for the existence of signals for a carbonyl [δ_C 158.7 (C-22)], a methine [δ_C 85.0; δ_H 4.52 (1H, dd, *J* = 11.5, 6.1 Hz) (CH-16)] and an amino hydrogen [δ_H 6.56 (1H, s) (23-*N*H)], as well as the absence of signals for CH_2-16 and CH-2 in **1**. The ^{1}H–^{1}H COSY spectrum of **1** revealed the presence of five spin systems as shown in Figure 2. In the HMBC spectrum, the correlations between H-16 (δ_H 4.52) and C-22 (δ_C 158.7), between H-23 (δ_H 6.56) and C-16 (δ_C 85.0), and between H-23 (δ_H 6.56) and C-22 (δ_C 158.7) suggested the presence of a -NH-C (O)-O- fragment which was adjacent to C-2 and C-16 positions to form an unusual oxazolidinone moiety. This conclusion was further confirmed by the ten degrees of unsaturation and by the downfield chemical shifts of C-2 (δ_C 83.7) and C-16 (δ_C 85.0) in **1**. Based on the above evidence, the planar structure of **1** was established as shown in Figure 2.

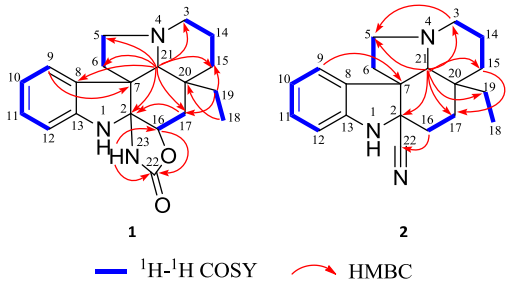


Figure 2. ^{1}H-^{1}H COSY and HMBC correlations of **1** and **2**.

In the ROESY spectrum, the correlations between H-21 (δ_H 2.29) and H-9 (δ_H 6.99)/H-3β (δ_H 1.96)/H-15β (δ_H 1.05)/H-19b(δ_H 0.79), between H-18 (δ_H 0.66) and H-16 (δ_H 4.52), as well as between H-23 (δ_H 6.56) and H-6α (δ_H 2.41) indicated the relative configuration of **1** as shown in Figure 3. Fortunately, crystals suitable for single crystal X-ray diffraction experiment were obtained from methanol solution. Thus, the structure and relative stereochemistry of **1** were unequivocally determined (Fig. 4).

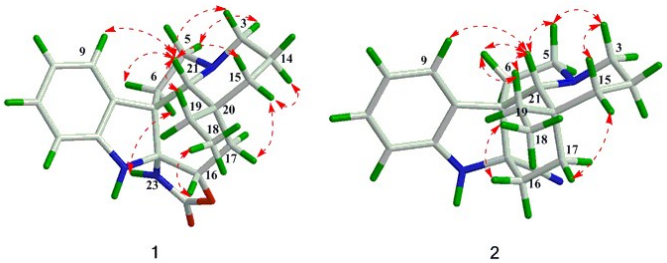


Figure 3. Key ROESY correlations of **1** and **2**.

To determine the absolute configuration of winchinine A (**1**), ECD curves for the two possible enantiomers (2*R*, 7*S*, 16*S*, 20*R*, 21*R*-**1** and 2*S*, 7*R*, 16*R*, 20*S*, 21*S*-**1**) were calculated using the TD-DFT theory method (see the Supporting Information). As shown in Figure 5, the experimental ECD spectrum of **1** showed a negative Cotton effect at 300 nm and positive Cotton effects at 245 and 207 nm, which were similar to the calculated one for the isomer with 2*R*, 7*S*, 16*S*, 20*R*, and 21*R* configurations. Therefore, the absolute stereochemistry of **1** was assigned as 2*R*, 7S, 16*S*, 20*R*, and 21*R*.

Table 1. ^{1}H and ^{13}C NMR data of **1** and **2**

	1[a]		**2**[a]	
No.	δ_H[b]	δ_C	δ_H[b]	δ_C
1-*N*H	4.77 s		3.93 s	
2	–	83.7	–	64.8
3	α 3.07 br d (9.3) β 1.96 td (12.1, 3.9)	53.6	α 3.03 br d (9.3) β 1.97	53.5
5	α 3.17 td (9.1, 4.3) β 2.33 d (9.1)	53.2	α 3.25 td (9.2, 4.3) β 2.24 d (9.2)	51.8
6	α 2.41 m β 1.56 m	36.1	α 2.66 m β 1.50	36.7
7	–	54.7	–	57.9
8	–	134.9	–	133.3
9	6.99	122.9	7.05	123.3
10	6.71 dd (7.5, 7.5)	120.0	6.79 dd (7.6, 7.6)	120.8
11	6.99	128.2	7.05	128.2
12	6.52 d (7.5)	109.2	6.63 d (7.6)	111.1
13	–	144.7	–	146.5
14	α 1.69 m β 1.47 dd (13.3, 3.9)	21.5	α 1.50 β 1.07 dd (13.4, 3.7)	21.6
15	α 1.62 β 1.05 m	34.1	α 1.62 d (13.7) β 1.12 dd (13.7, 3.7)	34.3
16	4.52 dd (11.5, 6.1)	85.0	α 1.97 β 1.78	32.7
17	α 2.24 d (11.5) β 1.62	30.0	α 2.28 dd (11.0, 6.0) β 1.78	22.1
18	0.66 t (7.4)	7.0	0.63 t (7.5)	7.1
19	a 1.12 dd (14.5, 7.4) b 0.79 dd (14.5, 7.4)	32.0	a 1.38 dd (14.5, 7.5) b 0.91 dd (14.5, 7.5)	29.9
20	–	36.0	–	36.0
21	2.29 s	73.3	2.35 s	69.7
22	–	158.7	–	122.2
23-*N*H	6.56 s		–	

[a] Data were recorded in $CDCl_3$ at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. [b] Overlapped signals are reported without designating multiplicity.

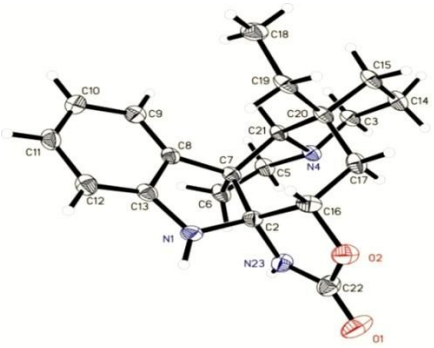


Figure 4. X-ray structure of **1** drawn by ORTEP.

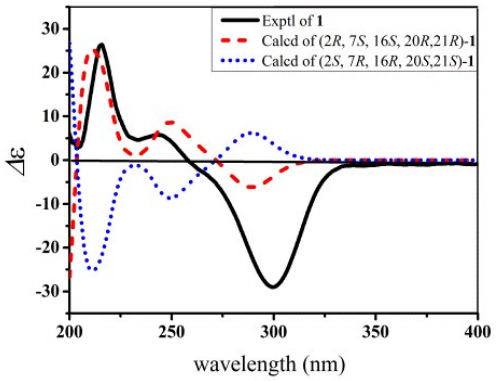


Figure 5. Calculated and experimental CD spectra of **1**.

Winchinine B (**2**), a white amorphous powder, possessed the molecular formula $C_{20}H_{25}N_3$ according to its HRESIMS at *m/z* 308.2128 $[M+H]^+$ (calcd for $C_{20}H_{26}N_3^+$ 308.2121). The UV spectrum showed absorption maxima at 206 (C=C, π→π*) and 296 (C=C, n→π*) nm. The IR spectrum of **2** suggested the presence of NH (3323 cm^{-1}), CN (2362 cm^{-1}), methyl (2937 cm^{-1}) and benzene ring (1680, 1540, 1457 cm^{-1}). The ^1H and ^{13}C NMR data of **2** were similar to those of **1**, except for the signals for CH-16 (δ_H 4.52, δ_C 85.0) and C-22 (δ_C 158.7) in **1** were replaced by a methylene (δ_H 1.97, 1.78; δ_C 32.7) and a cyano group (δ_C 122.2) in **2**, respectively. Based on the ten degrees of unsaturation and the ^{13}C NMR data of **2**, a cyano group was proposed to connect at C-2 position, which was further confirmed by the HMBC correlation between H-16 (δ_H 1.78) and C-22 (δ_C 122.2) (Fig. 2) and by the fragment ion peak at *m/z* 281.2033 in the HRESIMS that originated by the loss of cyano unit (see the Supporting Information).

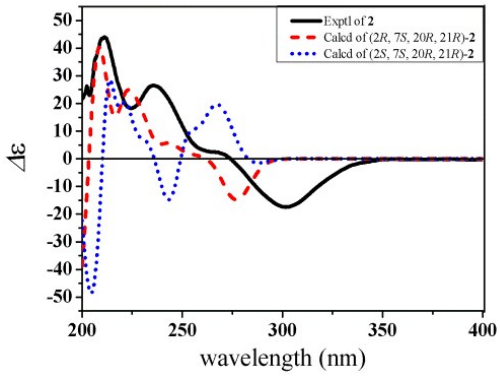


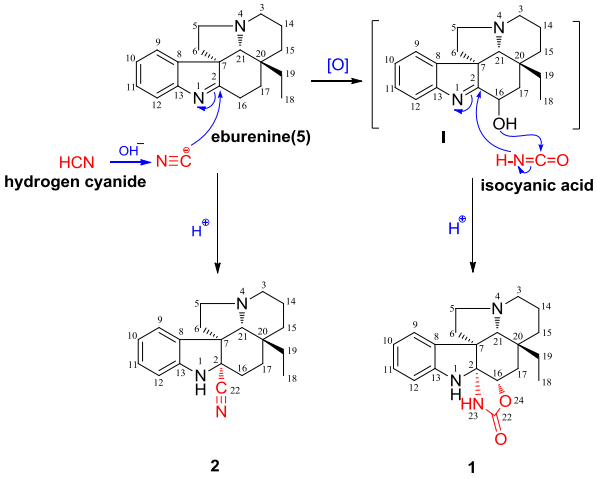
Figure 6. Calculated and experimental CD spectra of **2**.

The relative configurations of C-7, C-20 and C-21 of **2** were deduced to be the same as **1** according to the ROESY experiment (Fig. 3). However, the stereochemistry of the cyano group cannot be determined by ROESY spectrum. To determine the absolute configuration of **2**, a comparison between the experimental and calculated CD spectra using the time-dependent DFT method was performed (see the Supporting Information).[21] ECD curves for the two possible isomers (2*R*,7*S*,20*R*,21*R*-**2** and 2*S*,7*S*,20*R*,21*R*-**2**) were calculated. The measured CD spectrum of **2** (Fig. 6) exhibited a negative Cotton effect at λ_{max} 302 nm and positive Cotton effects at λ_{max} 205 and 248 nm, which were similar with the calculated curves of 2*R*,7*S*,20*R*,21*R*-**2**. Thus, the structure of **2** was determined.

The known compounds N_b-demethylechitamine (**3**)[22], N_b-demethylechitamine-N_b-oxide (**4**)[23], and eburenine (**5**)[24] were identified by comparison of their physical and spectroscopic data with those reported in the literature.

In comparison with the known MIAs, the structures of **1** and **2** incorporated a third nitrogen atom to form unusual oxazolidinone unit and cyano group. Compounds **1** and **2** represent the first natural MIA with an oxazolidinone unit and unusual cyano-substituted aspidosperma-type alkaloid, respectively.

On the basis of the literature and our research results, a plausible biosynthetic pathway to compounds **1**–**2** is proposed (Scheme 1). The alkaloid eburenine (**5**) is a major component of *W. calophylla*.[4] The nucleophilic attack from the cyanide ion to the imine group of **5** could afford **2**. In addition, oxidation of eburenine (**5**) could afford an intermediate I, which could incorporate with an isocyanic acid moiety by nucleophilic addition to give compound **1**.



Scheme 1. A putative biosynthesis pathway of **1** and **2**.

The inhibitory effects of compounds **1**-**5** on the viability of HepG2, MDA-MB-231, PC3 and A549 cells were determined using the MTT method as reported previously.[16] Doxorubicin (Dox, Sigma, USA) was used as the positive control. Compound **2** showed moderate cytotoxicity against PC3, MDA-MB-231, and A549 cells with IC_{50} values of 31.51±8.06, 43.43±4.26 and 40.77±2.93 μM, respectively. The other compounds were inactive (IC_{50} values >50 μM) (Table 2).

Table 2. Cytotoxic Activities of **1**–**5** on HepG2, MDA-MB-231, A549 and PC-3 Cancer Cells lines

Compounds	[a]IC_{50} (x ± SD) μM			
	HepG2	MDA-MB-231	A549	PC-3
1	>50	>50	>50	>50
2	>50	43.43±4.26	40.77±2.93	31.51±8.06
3	>50	>50	>50	>50
4	>50	>50	>50	>50
5	>50	>50	>50	>50
Dox[b]	0.25±0.03	0.32±0.04	0.18±0.06	0.40±0.08

[a]IC_{50}: Concentration of the tested compound inhibits 50% of cell growth. [b]Doxorubicin is the positive control in the test

Acknowledgements

This work was financially supported by the Program for National Natural Science Foundation of China (No. U1401225, 81273391, 81573307), the Ministry of Science and Technology of China (Nos. 2013DFM30080, 2013BAI11B05, 2012ZX09103201-056), the Guangdong Natural Science Foundation for Distinguished Young Scholar (No. 2015A030306022).

Notes and references

§ Crystal data for winchinine A (**1**): $C_{20}H_{25}N_3O_2$, triclinic, *P*1, *a* = 7.8960(10) Å, *b* = 8.4207(7) Å, *c* = 14.3158(8) Å, *β* = 81.536(10)˚, *γ* = 89.868(8)˚, *V* = 929.27(17) Å^3, *T* = 173(2) K, *Z* = 2, *D*c = 1.328 mg/mm^3, *F*(000) = 400. The final refinement gave *R* = 0.0556, *R*w = 0.1540, *S* = 1.116 and Flack parameter = -0.4(3). Crystal data of **1** were deposited in the Cambridge Crystallographic Data Centre (CCDC 1451513).

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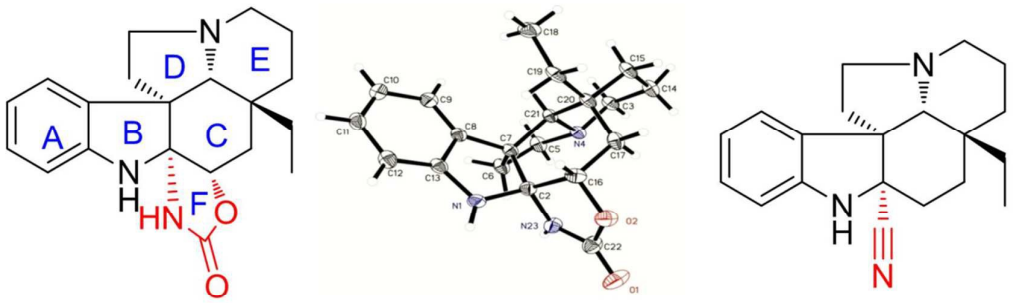
Graphical abstract

Two novel monoterpenoid indole alkaloids with a **third nitrogen atom** were isolated from the twigs and leaves of *Winchia calophylla.*



1 **2**