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Indole alkaloids from *Ervatamia chinensis*

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ABSTRACT

Four vobasinyl–ibogan type bisindole alkaloids, ervachinines A–D (**1**–**4**), along with 12 known terpenoid indole alkaloids, were isolated from the whole plant of *Ervatamia chinensis*. Their structures were elucidated by analysis of spectroscopic data, including 1D and 2D NMR, and the absolute configurations of **1**–**4** were determined by CD exciton chirality method. All of the compounds were evaluated for in vitro cytotoxicity against five human cancer cell lines: HL-60, SMMC-7721, A-549, MCF-7 and SW480. Bisindole alkaloids **1**–**6** exhibited inhibitory effects, with IC_{50} values comparable to those of cisplatin.

1. Introduction

The indole alkaloids found in plants of the Apocynaceae family are a group of structurally diverse natural products with potent biological activities (Dewick, 2002). Several of these compounds have attracted attention in total synthesis and pharmaceutical research (Beckers and Mahboobi, 2003; Bonjoch and Sole, 2000; Saxton, 1995). The genus *Ervatamia* (Apocynaceae) includes about 120 species, and is distributed mainly over the tropical and subtropical areas of Asia and Australia (Jiang and Li, 1977). Many of these compounds are used in folk medicine for the treatment of abdominal pain, hypertension, and sore throat (Jiang and Li, 1977). A previous chemical investigation of this genus produced a series of new monoterpenoid indole and bisindole alkaloids with antitumor activities (Hirasawa et al., 2009; Low et al., 2010; Zaima et al., 2009; Zhang et al., 2007).

E. chinensis is a common plant found mainly in the Yunnan and Guangxi provinces of China, and its chemically active constituents have not been described. As a part of our ongoing research into bioactive indole alkaloids (Tan et al., 2010; Liu et al., 2010; Zhou et al., 2010; Hu et al., 2009; Zhang et al., 2009), four new bisindole alkaloids, ervachinines A–D (**1**–**4**), together with 12 known alkaloids were isolated from whole *E. chinensis* plants. In the present paper, isolation, structure elucidation, and cytotoxic properties of these alkaloids are reported.

2. Results and discussion

2.1. Structure elucidation

The alkaloid-containing crude ethanol extract of *E. chinensis* was subjected to column chromatography on silica gel, RP-18, and Sephadex LH-20 to afford four new bisindole alkaloids (**1**–**4**) (Fig. 1). In addition, 12 known indole alkaloids were obtained and identified as 16-decarbomethoxyvoacamine (**5**) (Braga et al., 1984), tabernaecorymbosine A (**6**) (Luo et al., 2010), coronaridine (**7**) (Okuyama et al., 1992), isovoacangine (**8**) (Ladhar et al., 1981), heyneanine (**9**) (Ladhar et al., 1981), 3-(2-oxopropyl)voacangine (**10**) (Okuyama et al., 1992), 19-acetonylisovoacangine (**11**) (Agwada et al., 1975), vincadiffine (**12**) (Achenbach et al., 1994), vobasine (**13**) (Van Beek et al., 1984), difforlemeine (**14**) (Achenbach et al., 1994), (+)-voaphylline (**15**) (Torrenegra et al., 1988), and (+)-hecubine (**16**) (Torrenegra et al., 1988), respectively, on the basis of their physical and spectroscopic data and by comparison with literature data (see Fig. 1).

Ervachinine A (**1**) was obtained as a light yellow amorphous powder, and its molecular formula, $C_{44}H_{54}N_4O_6$, was established by HRESIMS (*m/z* 735.4131 $[M+H]^+$, calcd 735.4121) and ^{13}C NMR spectroscopic data (Table 1). Its UV spectrum showed absorption maxima at λ_{max} (log ε) 293 (3.82), 288 (3.83), and 227 (4.28) nm, typical of indole chromophores (Sheludko et al., 2002). IR bands at 3426 and 1725 cm^{-1} indicated the presence of NH/OH and ester functionalities, respectively. Analysis of the ^1H and ^{13}C NMR spectroscopic data (Table 1) suggested that **1** possessed 44 carbons including 6 methyls, 10 methylenes, 13 methines and 15 quater-

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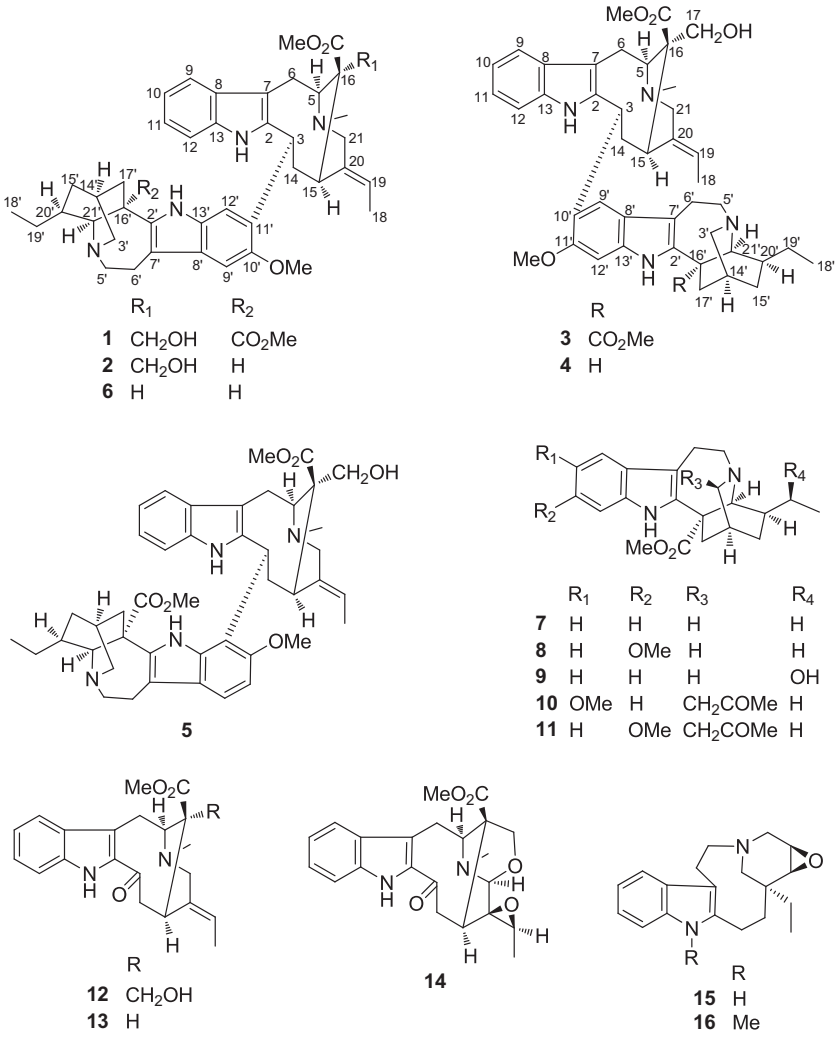

Fig. 1. Structures of alkaloids **1**–**16** isolated from *Ervatamia chinensis*.

nary carbons. Further analysis of the NMR spectra indicated that **1** was a bisindole alkaloid comprising vobasinyl and ibogan units (Kam et al., 2003). Compound **1** was highly similar to voacamine (Medeiros et al., 1999), with the exception that the C-16 methine in voacamine was replaced by a quaternary carbon due to the presence of a hydroxymethyl group at δ_C 70.3 attached to C-16 in **1**. This assignment was confirmed by ^1H–^1H COSY and HMBC spectra (Fig. 2), and was supported by the HMBC correlations from δ_H 3.46 (^1H, d, J = 11.0 Hz, H-17b) to C-5 (δ_C 60.0) and the ester carbonyl group (δ_C 173.9). As in voacamine (Medeiros et al., 1999), the pattern of the aromatic H signals and the observed correlations from δ_H 5.07 (^1H, br d, J = 13.0 Hz, H-3) to C-11′ (δ_C 129.9), C-12′ (δ_C 110.4), and C-10′ (δ_C 150.1) in the HMBC spectrum confirmed the C-3–C-11′ connectivity of the dimer. Thus, ervachinine A was assigned as **1**, shown in Fig. 2.

The relative configuration of **1** was assigned through analysis of ^1H NMR chemical shifts, ^1H–^1H coupling values, and ROESY correlations. The ROESY correlations of H-15 and CH_3-18 in unit A established the *E*-configuration of the ethylidene side-chains as shown in Fig. 3. The configuration of C-16 was proven to be *S** by the highly shielded chemical shift (δ_H 2.32) of the methoxy carbonyl group at C-16, which can be explained by the anisotropic effect of the indole ring (Nugroho et al., 2009). The β-configuration of H-3 was assigned on the basis of the key ROESY H-3/NH correlation and through comparison of the analogous H-3 coupling constants of **1** (br d, J = 13.0 Hz) and those of vobasinyl–iboga type bisindole alkaloids (br d, J = 13.0 Hz) (Kam et al., 2003). The α-configuration of H-14′, H-20′, and H-21′ in unit B was elucidated by the ROESY correlations of H-20′/H-15′a and H-21′, and H-14′/H-17′a. The conformation of **1** through the C-3–C-11′ bond was assigned by the ROESY correlations of H-12a/H-14b and H-6a as shown in a computer-generated 3D drawing (Fig. 3).

The absolute configuration of **1** was determined by applying the exciton chirality CD method (Harada and Nakanishi, 1969). The sign of the first Cotton effect [λ_{max} 242 nm ($\Delta\varepsilon$ + 35.7)] was positive, while that of the second one [λ_{max} 223 nm ($\Delta\varepsilon$ − 52.6)] was negative, indicating that the transition dipole moments of the two indole chromophores in **1** were oriented in a clockwise manner, as shown in Fig. 4. The absolute configuration of **1** was therefore assigned. To the best of our knowledge, ervachinine A (**1**) represents the first report of the determination of absolute configuration of this type bisindole alkaloids by using the exciton chirality CD method.

The molecular formula of ervachinine B (**2**) was $C_{42}H_{52}N_4O_4$, as determined by the HRESIMS signal at *m/z* 677.4058 (calcd 677.4066) and ^{13}C NMR spectroscopic data (Table 1). This molecular weight was 58 mass units lower than that of **1**. The UV spectrum with λ_{max} (logε) of 295 (3.56), 287 (3.56), and 226 (4.05) nm was typical of indole chromophores, and the IR spectrum showed NH/OH and ester carbonyl absorption bands at 3420 and

Table 1
^{1}H and ^{13}C NMR spectroscopic data of **1** and **2** (δ in ppm and J in Hz).

Position	**1**[a]		**2**[a]	
	δ_H	δ_C	δ_H	δ_C
2		138.0		138.2
3	5.07 (br d, 13.0)	37.2	5.13 (br d, 13.0)	37.3
5	3.99 (t, 8.0)	60.0	3.91 (t, 9.0)	60.1
6a	3.48 (dd, 13.8, 9.6)	17.4	3.52 (dd, 15.0, 9.0)	17.2
6b	3.26 (dd, 13.8, 8.0)		3.27 (dd, 15.0, 6.6)	
7		109.9		110.5
8		129.8		129.9
9	7.46 (br d, 7.5)	117.5	7.55 (br d, 6.0)	117.5
10	7.01 (m)	118.9	7.06 (m)	118.9
11	7.00 (m)	120.4	7.05 (m)	121.7
12	6.97 (m)	110.2	7.08 (br d, 6.6)	110.0
13		136.0		136.1
14a	2.42 (m)	36.4	2.49 (m)	36.6
14b	1.95 (m)		2.02 (ddd, 9.6, 6.6, 2.4)	
15	3.43 (m)	35.7	3.51 (t, 9.6)	36.0
16		52.0		52.0[b]
17a	3.69 (d, 11.0)	70.3	3.71 (t, 12.0)	70.7
17b	3.46 (d, 11.0)		3.71 (t, 12.0)	
18	1.58 (d, 8.0)	12.2	1.66 (d, 6.0)	12.2
19	5.27 (q, 8.0)	121.7	5.37 (q, 6.0)	119.9
20		136.1		136.7
21a	3.58 (d, 11.0)	51.8	3.57 (d, 12.0)	52.0[b]
21b	3.04 (d, 11.0)		2.94 (d, 12.0)	
NH	7.62 (br s)		7.67 (br s)	
CO_2Me	2.32 (s)	50.4	2.40 (s)	50.3
		173.9		174.0
NMe	2.59 (s)	42.0	2.55 (s)	42.0
2′		137.2		136.8
3′a	2.81 (br d, 10.0)	51.9	3.01 (br d, 9.0)	49.9
3′b	2.64 (br d, 10.0)		2.95 (br d, 9.0)	
5′a	3.30 (m)	53.0	3.35 (m)	54.2
5′b	3.07 (ddd, 18.0, 12.0, 6.0)		3.08 (dt, 13.2, 4.2)	
6′a	3.02 (br d, 9.6, 6.0)	22.1	3.30 (t, 4.2)	20.7
6′b	2.90 (br d, 9.6, 6.0)		2.59 (br d, 13.2)	
7′		110.2		108.9
8′		127.3		128.3
9′	6.85 (s)	99.1	6.92 (s)	98.6
10′		150.1		150.9
11′		129.9		128.8
12′	6.59 (s)	110.4	6.62 (s)	110.3
13′		130.2		129.2
14′	1.72 (m)	27.3	1.79 (m)	26.4
15′a	1.69 (m)	31.9	1.75 (br t, 11.4)	32.0
15′b	1.02 (m)		1.16 (m)	
16′		54.8	2.77 (m)	41.3
17′a	2.38 (br d, 16.5)	36.4	1.94 (br t, 12.0)	34.1
17′b	1.66 (br d, 16.5)		1.50 (m)[b]	
18′	0.80 (t, 8.0)	11.6	0.86 (t, 6.6)	11.9
19′a	1.56 (m)	26.7	1.50 (m)[b]	27.8
19′b	1.34 (m)		1.42 (m)	
20′	1.19 (m)	38.9	1.50 (m)[b]	41.9
21′	3.44 (br s)	57.0	2.76 (br s)	57.7
10′-OMe	3.91 (s)	56.0	3.98 (s)	55.9
CO_2Me'	3.54 (s)	52.4		
		175.2		

[a] Measured in $CDCl_3$.
[b] Overlapped.

1726 cm^{-1}. A direct comparison of the NMR spectra (Table 1) of **1** and **2** indicated that both compounds were similar and had the same basic skeleton. The only differences arose from the substituted group at C-16′, including the absence of signals associated with the C(16′)-CO_2Me group. A methine group was observed in **2** based on the NMR data at δ_C 41.3 and δ_H 2.77 (Table 1 and Supporting information). The replacement of the C(16′)-CO_2Me function with H in **2** was further supported by the HMBC correlations from H-14′, H-17′a, and H-20′ to C-16′ (δ_C 41.3). Detailed analysis of the 2D NMR spectra (HSQC, ^{1}H–^{1}H COSY, HMBC, and ROESY) confirmed that the other parts of the molecule were the same as

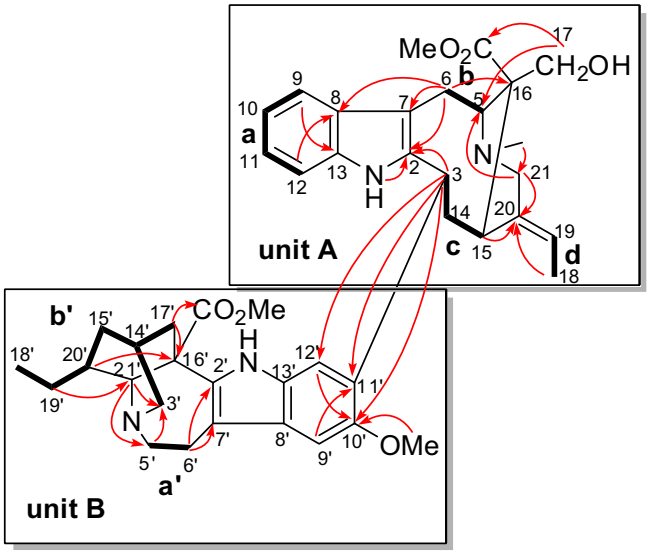

Fig. 2. ^{1}H–^{1}H COSY (bold) and key HMBC (arrow, H → C) correlations of **1**.

those of **1**. The structure of alkaloid **2** was thereby characterized as the 16′-decarbomethoxy analog of **1**.

Ervachinine C (**3**) was obtained as a light yellow amorphous powder, the ^{13}C NMR spectroscopic data (Table 2) and its positive HRESIMS signal at m/z 735.4135 ([M+H]$^+$, calcd 735.4121) established the molecular formula of $C_{44}H_{54}N_4O_6$. The UV spectrum displayed two maxima at λ_{max} (log ε) 295 (4.05) and 227 (4.64) nm. The IR spectrum indicated the presence of NH/OH (3425 cm^{-1}) and ester carbonyl groups (1723 cm^{-1}), respectively. Detailed analysis of the NMR spectroscopic data (Table 2) indicated that **3** was also a vobasinyl–iboga bisindole alkaloid, and branching of the monomeric units in **3** proceeded from C-3 of the vobasinyl unit to C-10′ of the iboga unit, as in the related compound conodiparine A (Kam et al., 2003). HMBC correlations between δ_H 5.11 (^{1}H, br d, J = 13.0 Hz, H-3) and C-10′ (δ_C 128.5), C-9′ (δ_C 118.3), and C-11′ (δ_C 154.5) confirmed the above structural features. Moreover, the NMR spectroscopic data of **3** were strikingly similar to those of conodiparine A (Kam et al., 2003), except for the presence of signals corresponding to a methylene carbon (δ_C 27.8) in place of the oxymethine carbon (δ_C 71.2) at C-19′. The hydroxyethyl side-chain at C-20′ in conodiparine A was replaced with an ethyl side-chain in

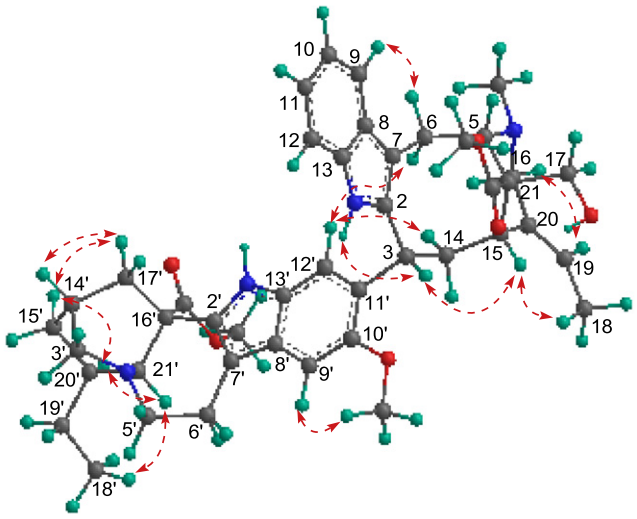

Fig. 3. Key ROESY (dashed) correlations of **1**.

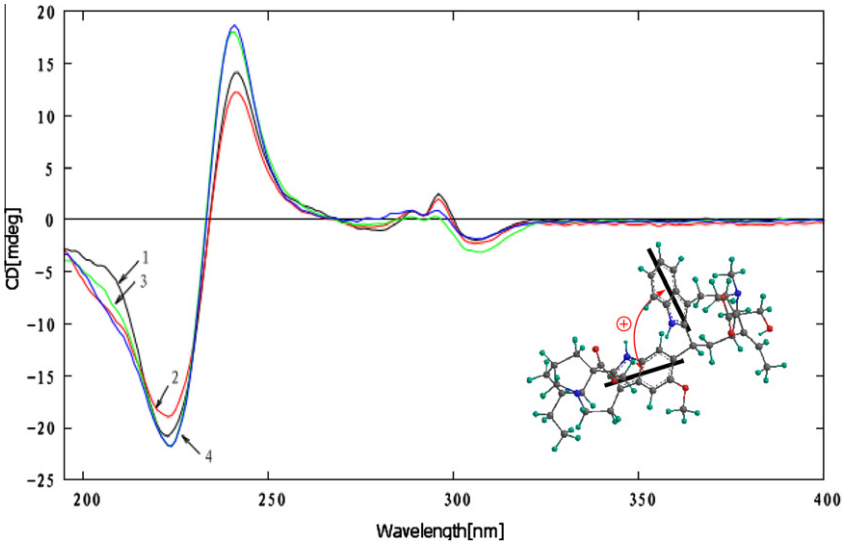

Fig. 4. CD spectra of **1**–**4**. Bold lines denote the electric transition dipole of the chromophores for **1**.

3, as confirmed by the observation of a triplet at δ_H 0.81 (3H, J = 7.2, Me-18′), and two multiplets at δ_H 1.32 and 1.42 (2H, CH_2-19′) in the ^{1}H NMR of **3**. Therefore, ervachinine C (**3**) was deduced to be the 19′-dehydroxy analog of conodiparine A, which was further substantiated through 2D-experiments, including HSQC, ^{1}H–^{1}H COSY, HMBC, and ROESY spectra.

Ervachinine D (**4**) had a molecular formula of $C_{42}H_{52}N_4O_4$, as established by the HRESIMS signal at *m/z* 677.4080 ([M+H]$^+$, calcd for $C_{42}H_{53}N_4O_4$, 677.4066) and ^{13}C NMR spectroscopic data (Table 2). The UV and IR spectra were very similar to those of **3**. Comparison of the NMR data of **4** (Table 2) and **3** suggested that the alkaloids shared the same basic skeleton, except that the molecular weight of **4** was less than that of **3** by 58 mass units, namely, the methyl ester group at C-16′ in **3** was replaced with H. This finding was supported by the absence of signals associated with the ester carbonyl at C-16′, whereas the C-16′ resonance was shifted upfield from δ_C 56.2 to δ_C 41.9. A HMBC correlation between δ_H 2.70 (^{1}H, br s, H-21′) and C-16′ (δ_C 41.9) further confirmed this structural feature. Detailed analysis of the 2D NMR spectrum (HSQC, ^{1}H–^{1}H COSY, HMBC, and ROESY) confirmed that the other components of the structure were the same as those of **3**.

The absolute configurations of compounds **2**–**4** were identical to that of **1**, as determined by their similar CD curves in the CD spectra (Fig. 4).

2.2. Cytotoxic activity

Cytotoxic activities of compounds **1**–**14** were evaluated against five human cancer cell lines HL-60, SMMC-7721, A-549, MCF-7 and SW480 by using the MTT method (Mosmann, 1983), with cisplatin as a positive control. It is noteworthy that only the bisindole alkaloids **1**–**6** showed inhibitory activities (Table 3). Among them, ervachinines A (**1**) and C (**3**) showed significant inhibitory activity against the five cell lines with IC_{50} values in the range 0.84–3.66 μM, and the others exhibited more stronger inhibitory activities than that of positive control (cisplatin) in some cancer cell lines.

3. Concluding remarks

Six vobasinyl–ibogan type bisindole alkaloids including four new ones, ervachinines A–D (**1**–**4**), were isolated from whole *E. chinensis* plants together with 10 known monoterpenoid indole alkaloids. The discovery of compounds **1**–**4** is a further addition to diverse and complex array of bisindole alkaloids that are rapidly expanding. Their presence as characteristic markers may be helpful in chemotaxonomical classifications. The cytotoxicity against several human cancer cell lines of all compounds was also investigated, and found to be quite potent.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on JASCO P-1020 digital polarimeter, whereas UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. IR spectra were measured in a Bio-Rad FTS-135 spectrometer with KBr pellets, whereas CD spectra were recorded with an Applied Photophysics Chirascan spectrometer. ESIMS and HRESIMS were measured on a Finnigan MAT 90 instrument and VG Auto Spec-3000 spectrometer, respectively. 1D and 2D NMR spectra were acquired using Bruker AM-400, DRX-500 and AV-600 spectrometers with TMS as an internal standard. Silica gel (300–400 mesh, Qingdao Marine Chemical Inc., China), Silica gel H (10–40 μm, Qingdao Marine Chemical Inc., China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), and Sephadex LH-20 (40–70 μm, Amersham Biosciences, Sweden) were used for column chromatography (CC).

4.2. Plant material

Whole *E. chinensis* plants were collected in November 2008 from the Xishuangbanna area of Yunnan Province, People's Republic of China, and the plant sample was identified by Dr. Zhi Wang, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). A voucher specimen (KIB 08110613) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

4.3. Extraction and isolation

The powder of *E. chinensis* (7.5 kg) were extracted three times with EtOH–H_2O (95:5, v/v). The combined extracts were concentrated under reduced pressure, followed by partitioning between EtOAc and 3% tartaric acid. The aqueous phase was adjusted to pH 9–10 with saturated Na_2CO_3 and then extracted with $CHCl_3$

Table 2
^{1}H and ^{13}C NMR spectroscopic data of **3** and **4** (δ in ppm and J in Hz).

Position	**3**[a]		**4**[a]	
	δ_H	δ_C	δ_H	δ_C
2		139.7		139.9
3	5.11 (br d, 12.8)	39.1	5.13 (br d, 13.0)	39.1
5	3.74 (t, 10.4)	62.1	3.78 (t, 11.2)	62.1
6a	3.51 (dd, 16.2, 7.2)	18.7	3.56 (m)	18.7
6b	3.23 (dd, 16.2, 8.4)		3.26 (dd, 14.8, 8.0)	
7		111.3		111.2
8		131.3		131.4
9	7.51 (dd, 7.0, 2.5)	118.4	7.53 (br d, 7.0)	118.2
10	6.98 (m)[b]	119.2	7.00 (m)[b]	119.2
11	6.98 (m)[b]	122.0	7.00 (m)[b]	122.0
12	7.10 (dd, 7.2, 2.8)	111.0	7.10 (dd, 7.2, 2.4)	111.0
13		138.0		138.1
14a	2.48 (m)	38.6	2.51 (m)	38.7
14b	2.00 (m)		2.01 (m)	
15	3.62 (dd, 12.0, 5.6)	36.4	3.63 (dd, 11.6, 6.8)	36.4
16		54.0		54.1
17a	3.78 (d, 10.8)	70.3	3.81 (d, 12.0)	70.3
17b	3.56 (d, 10.8)		3.60 (d, 12.0)	
18	1.65 (d, 6.0)	12.4	1.69 (d, 6.4)[b]	12.3
19	5.29 (q, 6.0)	120.3	5.36 (q, 6.4)	120.2
20		138.7		138.9
21a	3.48 (d, 10.0)	52.9	3.57 (d, 13.2)	53.1
21b	2.75 (d, 10.0)		2.87 (d, 13.2)	
CO_2Me	2.28 (s)	50.3	2.31 (s)	50.4
		174.6		174.5
NMe	2.39 (s)	42.5	2.49 (s)	42.6
2′		136.7		141.5
3′a	3.10 (dd, 13.2, 7.2)	54.5	2.90 (br d, 8.4)	50.6
3′b	2.81 (dd, 13.2, 6.4)		2.75 (br d, 8.4)	
5′a	2.66 (dd, 9.6, 7.2)	53.7	3.04 (br d, 13.2)	55.4
5′b	2.56 (m)		2.82 (br d, 13.2)	
6′a	2.71 (t, 7.2)2.59 (t, 7.2)	22.7	3.00 (m)2.20 (m)	21.3
6′b				
7′		110.4		108.6
8′		123.5		124.7
9′	6.70 (s)	118.3	6.67 (s)	118.1
10′		128.5		128.2
11′		154.5		154.0
12′	6.83 (s)	93.9	6.83 (s)	93.7
13′		136.6		135.6
14′	1.55 (m)	28.6	1.69 (m)[b]	27.7
15′a	1.59 (m)	33.0	1.79 (m)	33.0
15′b	0.96 (m)		1.12 (m)	
16′		56.1	2.89 (br d, 8.4)	41.9
17′a	2.55 (br d, 14.0)	36.9	2.01 (br d, 12.4)	35.3
17′b	1.71 (br d, 14.0)		1.48 (br d, 12.4)	
18′	0.81 (t, 7.2)	12.1	0.88 (t, 6.8)	12.3
19′a	1.42 (m)	27.8	1.48 (q, 6.8)	28.5
19′b	1.32 (m)		1.42 (q, 6.8)	
20′	1.24 (m)	39.9	1.53 (m)	43.3
21′	3.40 (br s)	58.0	2.70 (br s)	59.4
11′-OMe	3.92 (s)	56.2	3.95 (s)	56.3
CO_2Me′	3.62 (s)	52.8		
		176.4		

[a] Measured in CD_3OD.
[b] Overlapped.

to give crude alkaloids (40.0 g). The crude alkaloid extract was then subjected to a silica gel CC (300–400 mesh) using a petroleum ether–Me_2CO gradient (1:0–0:1) to obtain four major fractions A–D. Fraction A (3.5 g) was subjected to silica gel CC eluting with petroleum ether–Me_2CO–Et_2NH (20:1:0.1–8:1:0.1, v/v) to give **7** (20 .0 mg), **10** (25.0 mg), **11** (12.0 mg) and **16** (35.0 mg). Fraction B (3.8 g) was further purified by silica gel CC (300–400 mesh) with petroleum ether–Me_2CO–Et_2NH (10:1:0.2–4:1:0.2, v/v), then followed by Sephadex LH-20 (CH_3OH) to afford **1** (20.0 mg), **3** (25.0 mg), **6** (60.0 mg), and **13** (70.0 mg). Compounds **14** (220.0 mg) and **15** (63.0 mg) was isolated from fraction C (800 mg) by successive reversed phase chromatography using a C_{18} column (MeOH–H_2O, 40:60–100:0) and Sephadex LH-20 (MeOH). Fraction D (4.4 g) was further purified by reversed phase chromatography on a C_{18} column (MeOH–H_2O, 20:80–100:0) to give three subfractions (D_1–D_3). Subfraction D_2 (1.2 g) was subjected to silica gel CC eluted with petroleum ether–EtOAc (10:1–3:1, v/v), then followed by Sephadex LH-20 CC (CH_3OH) to afford **2** (17.0 mg), **4** (17.5 mg), and **8** (22.0 mg). Subfraction D_3 (1.0 g) was further purified by silica gel eluting with petroleum ether–Me_2CO (8:1–4:1, v/v), and then Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to yield **5** (30.0 mg), **9** (17.0 mg), and **12** (16.8 mg).

Table 3
Cytotoxicity of compounds **1**–**6**[a] (IC_{50}[b], μM).

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	3.58	3.35	2.86	3.20	2.39
2	11.77	11.40	15.98	15.17	15.33
3	0.84	3.46	3.25	3.25	3.66
4	3.41	4.63	3.10	11.76	3.63
5	3.46	5.44	8.26	17.21	15.70
6	4.00	10.86	10.18	3.71	2.77
Cisplatin[c]	1.00	17.05	26.75	16.97	18.32

[a] Other compounds were not active (IC_{50} greater than 40 μM).
[b] IC_{50}: 50% inhibitory concentration.
[c] Positive control.

4.4. Ervachinine A (*1*)

Light yellow amorphous powder; $[\alpha]_D^{25}$ −13.8 (*c* 0.39, MeOH); UV (MeOH) λ_{max} (log ε) 293 (3.82), 288 (3.83), 227 (4.28); CD (0.00012 M, MeOH) λ_{max} ($\Delta\varepsilon$) 223 (−52.6), 242 (+35.7), 296 (+6.3), 306 (−5.0) nm; IR (KBr) υ_{max} 3426, 2927, 1724, 1630, 1463, and 1287 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESIMS 735 $[M+H]^+$; HRESIMS *m/z* 735.4131 $[M+H]^+$ (calcd for $C_{44}H_{55}N_4O_6$, 735.4121).

4.5. Ervachinine B (*2*)

Light yellow amorphous powder; $[\alpha]_D^{25}$ −29.9 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 295 (3.56), 287 (3.56), 226 (4.05); CD (0.00010 M, MeOH) λ_{max} ($\Delta\varepsilon$) 223 (−57.4), 241 (+37.2), 296 (+6.0), 306 (−6.9) nm; IR (KBr) υ_{max} 3420, 2925, 1726, 1628, 1464, and 1287 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESIMS 677 $[M+H]^+$; HRESIMS *m/z* 677.4058 $[M+H]^+$ (calcd for $C_{42}H_{53}N_4O_4$, 677.4066).

4.6. Ervachinine C (*3*)

Light yellow amorphous powder; $[\alpha]_D^{25}$ −42.7 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 295 (4.05), 227 (4.64); CD (0.000077 M, MeOH) λ_{max} ($\Delta\varepsilon$) 224 (−85.7), 241 (+71.0), 295 (+1.3), 308 (−12.2) nm; IR (KBr) υ_{max} 3426, 2927, 1723, 1629, 1464, and 1287 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 2; ESIMS 735 $[M+H]^+$; HRESIMS *m/z* 735.4135 $[M+H]^+$ (calcd for $C_{44}H_{55}N_4O_6$, 735.4121).

4.7. Ervachinine D (*4*)

Light yellow amorphous powder; $[\alpha]_D^{25}$ −43.4 (*c* 0.34, MeOH); UV (MeOH) λ_{max} (log ε) 295 (4.12), 226 (4.72); CD (0.000068 M, MeOH) λ_{max} ($\Delta\varepsilon$) 224 (−97.0), 241 (+83.4), 296 (+4.1), 306 (−8.2) nm; IR (KBr) υ_{max} 3415, 2927, 1722, 1629, 1464, and 1288 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 2; ESIMS 677 $[M+H]^+$; HRESIMS *m/z* 677.4080 $[M+H]^+$ (calcd for $C_{42}H_{53}N_4O_4$, 677.4066).

4.8. Cytotoxicity bioassays

Five human cancer cell lines, human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7, and colon cancer SW480 cells, were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method in 96-well micro-plates (Mosmann, 1983). Briefly, 100 μL of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while 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 test compound dissolved in DMSO at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μmol in triplicates for 48 h with cisplatin (Sigma, USA) as the positive controls. After compound treatment, cell viability was measured and a cell growth curve was plotted. IC_{50} values were calculated by Reed and Muench's method (Reed and Muench, 1938).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.11.002.

References

Achenbach, H., Waibel, R., Zwanzger, M., 1994. Indole alkaloids from *Tabernaemontana glandulosa*. Phytochemistry 37, 1737–1743.
Agwada, V.C., Morita, Y., Renner, U., Hesse, M., Schmid, H., 1975. Alkaloids. 155. Alkaloids of *Gabunia eglandulosa*. Helv. Chim. Acta 58, 1001–1016.
Beckers, T., Mahboobi, S., 2003. Natural, semisynthetic and synthetic microtuble inhibitors for cancer therapy. Drugs Future 28, 767–785.
Bonjoch, J., Sole, D., 2000. Synthesis of strychnine. Chem. Rev. 100, 3455–3482.
Braga, R.M., Filho, H.F.L., Reis, F., De, A.M., 1984. ^{13}C analysis of alkaloids from *Peschiera fuchsiaefolia*. Phytochemistry 23, 175–178.
Dewick, P.M., 2002. Alkaloids, in: medicinal natural products: a biosynthetic approach. John Wiley & Sons Ltd., Chichester, pp. 350–359.
Harada, N., Nakanishi, K., 1969. Determining the chiralities of optically active glycols. J. Am. Chem. Soc. 91, 3989–3991.
Hirasawa, Y., Miyama, S., Hosoya, T., Koyama, K., Rahman, A., Kusumawati, I., Zaini, N.C., Morita, H., 2009. Alasmontamine A, a first tetrakis monoterpene indole alkaloid from *Tabernaemontana elegans*. Org. Lett. 11, 5718–5721.
Hu, X.J., Di, Y.T., Wang, Y.H., Kong, L.Y., Gao, S., Li, C.S., Liu, H.Y., He, H.P., Ding, J., Xie, H., Hao, X.J., 2009. Carboline alkaloids from *Trigonostemon lii*. Planta Med. 75, 1157–1161.
Jiang, Y., Li, B.T. Flora of China Zhongguo Zhiwu Zhi, vol. 63, Science Press, Beijing, 1977. p. 98.
Kam, T.S., Sim, K.M., Pang, H.S., 2003. New bisindole alkaloids from *Tabernaemontana corymbosa*. J. Nat. Prod. 66, 11–16.
Ladhar, F., Damak, M., Ahond, A., Poupat, C., Potier, P., Moretti, C., 1981. Study of American *Tanbernaemontana*. III. Alkaloids from *Anartia cf. meyeri*. J. Nat. Prod. 44, 459–465.
Liu, L.L., Di, Y.T., Zhang, Q., Fang, X., Zhu, F., Chen, D.L., Hao, X.J., He, H.P., 2010. Aminocadamines A and B, two novel indole alkaloids from *Neolamarckia cadamba*. Tetrahedron Lett. 51, 5670–5673.
Low, Y.Y., Lim, K.H., Choo, Y.M., Pang, H.S., Etoch, T., Hayashi, M., Komiyama, K., Kam, T.S., 2010. Structure, biological activity, and a biomimetic partial synthesis of the lirofolines, novel pentacylic indole alkaloids from *Tabernaemontana*. Tetrahedron Lett. 51, 269–272.
Luo, X.D., Cai, X.H., Li, Y., 2010. Bisindolyl alkaloid compound, its medical composition, preparation and application in preparing anticancer drugs, Faming Zhuanli Shenqing Gongkai Shuomingshu.
Medeiros, W.L., Vieira, I.J.C., Mathias, L., Braz-Fiho, R., Leal, K.Z., Rodrigues-Filho, E., Schripsema, J., 1999. Two known bis-indole alkaloids isolated from *Tabernaemontana laeta*: complete ^{1}H and ^{13}C chemical shift assignments. Magn. Reson. Chem. 37, 676–681.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Nugroho, A.E., Hirasawa, Y., Kawahara, N., Goda, Y., Awang, K., Hadi, H.A., Morita, H., 2009. Bisnicalaterine A, a vobasine–vobasine bisindole alkaloid from *Hunteria zeylanica*. J. Nat. Prod. 72, 1502–1506.
Okuyama, E., Gao, L.H., Yamazaki, M., 1992. Analgesic components bornean medicinal plants, *Tabernaemontana pauciflora* Blume and *Tabernaemontana pandacaqui* Poir. Chem. Pharm. Bull. 40, 2075–2079.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. 27, 493–497.
Saxton, J.E., 1995. Recent progress in the chemistry of the monoterpenoid indole alkaloids. Nat. Prod. Rep. 12, 385–411.
Sheludko, Y., Gerasimenko, I., Kolshorn, H., Stockigt, J., 2002. New alkaloids of the sarpagine group from *Rauvolfia serpentina* hairy root. J. Nat. Prod. 65, 1006–1010.
Tan, C.J., Di, Y.T., Wang, Y.H., Zhang, Y., Si, Y.K., Zhang, Q., Gao, S., Hu, X.J., Fang, X., Li, S.F., Hao, X.J., 2010. Three new indole alkaloids from *Trigonostemon lii*. Org. Lett. 12, 2370–2373.
Torrenegra, R., Pedrozo, Julio.A.P., Achenbach, H., Bauereiss, P., 1988. Alkaloids of *Stemmadenia grandiflora*. Phytochemistry 27, 1843–1848.
Van Beek, T.A., Kuijlaars, F.L.C., Thomassen, P.H.A.M., Verpoorte, R., Svendsen, A.B., 1984. Antimicrobially active alkaloids from *Tabernaemontana pachysiphon*. Phytochemistry 23, 17711778.
Zaima, K., Hirata, T., Hosoya, T., Hirasawa, Y., Koyama, K., Rahman, A., Kusumawati, I., Zaini, N.C., Shiro, M., Morita, H., 2009. Biscarpamontamines A and B, an aspidosperma–iboga bisindole alkaloid and an aspidosperma–aspidosperma bisindole alkaloids, from *Tabernaemontana sphaerocarpa*. J. Nat. Prod. 72, 1686–1690.
Zhang, H., Wang, X.N., Lin, L.P., Ding, J., Yue, J.M., 2007. Indole alkaloids form three species of the *Ervatamia* genus: *E. Officinalis*, *E. divaricata*, and *E. divaricata* Gouyahua. J. Nat. Prod. 70, 54–59.
Zhang, Z., Di, Y.T., Wang, Y.H., Zhang, Z., Mu, S.Z., Fang, X., Zhang, Y., Tan, C.J., Zhang, Q., Yan, X.H., Guo, J., Li, C.S., Hao, X.J., 2009. Gelegamines AE: five new oxindole alkaloids from *Gelsemium elegans*. Tetrahedron 65, 4551–4556.
Zhou, H., He, H.P., Wang, Y.H., Hao, X.J., 2010. A novel alkaloid from *Melodinus henryi*. Helv. Chim. Acta 93, 2030–2032.