Fitoterapia 98 (2014) 45–52

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Cytotoxic eburnamine-aspidospermine type bisindole alkaloids from *Bousigonia mekongensis*

Yan-hui Fu [a,b], Shun-lin Li [a], Shi-fei Li [a], Hong-ping He [a], Ying-tong Di [a], Yu Zhang [a,*], Xiao-jiang Hao [a,*]

[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, PR China
[b] Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, Hainan Normal University, Haikou 571158, PR China

ARTICLE INFO

Article history:
Received 8 June 2014
Accepted in revised form 6 July 2014
Available online 16 July 2014

Keywords:
Bousigonia mekongensis
Bisindole alkaloids
Mekongenines C–F
Cytotoxicity

ABSTRACT

Four new eburnamine-aspidospermine type bisindole alkaloids, namely, mekongenines C–F (1–4), along with 27 known indole alkaloids were isolated from the twigs and leaves of *Bousigonia mekongensis*. Their structures with the absolute configurations were elucidated by spectroscopic methods and ECD analyses. All new compounds were evaluated for their cytotoxicities against five human cancer cell lines: HL-60, SMMC-7721, A-549, MCF-7 and SW480 in vitro. Alkaloids 1–4 exhibited inhibitory effects with IC_{50} values comparable to those of cisplatin.

1. Introduction

The genus *Bousigonia* (Apocynaceae) comprises two species: *Bousigonia mekongensis* and *Bousigonia angustifolia* [1]. Phytochemical investigations on this genus only conducted in our lab have afforded about 40 monoterpenoid indole alkaloids. Among them, some alkaloids possess new skeletons, such as mekongenine A [2] and angustifonine A [3]. In continuation, mekongenines C–F (1–4), four new bisindole alkaloids constituted from the union of eburnamine and aspidospermine moieties, were isolated from the twigs and leaves of *B. mekongensis*. Their structures and relative stereochemistries were elucidated on the basis of extensive NMR and MS analyses. The absolute configurations of mekongenines C–F (1–4) were established by comparison of their ECD spectra with that of mekongenine B, whose structure and absolute configurations were determined unambiguously by a combination of spectroscopic methods, X-ray diffraction analysis, and ECD calculation [2]. In addition, 27 known alkaloids, melodinine J (5) [4], eburnamenine (6) [5], epivincanol (7) [6], *O*-methylepivincanol (8) [6], *O*-methylvincanol (9) [7], 19-hydrovincanol (10) [8], 16-epi-Δ^{14}- vincanol (11) [9], *O*-methyl-Δ^{14}-epivincanol (12) [9], 14,15-dehydrovincine (13) [10], vincamone (14) [11], 16-epivincamine (15) [12], vincine (16) [13,14], vincarodine (17) [10,15], tabersonine (18) [16,17], 11-hydroxytabersonine (19) [5], 11-methoxytabersonine (20) [18], (19*R*)-hydroxy tabersonine (21) [19,20], (19*R*)-acetoxytabersonine (22) [21,22], (19*R*)-acetoxy-11-methoxy tabersonine (23) [21,22], vincadifformine (24) [22], eburenine (25) [23], venalstonine (26) [24], vindolinine (27) [24], 10-hydroxyscandine (28) [25], epimeloscine (29) [26], 9-hydroxy epimeloscine (30) [27], and meloscandonine (31) [28], were isolated and identified by comparing the experimental and reported physical data. All new compounds were evaluated for their cytotoxicities against five human cancer cell lines in vitro. Herein, we describe the isolation, structure elucidation, and cytotoxic properties of these new alkaloids.

* Corresponding authors. Tel.: +86 871 6522 3263; fax: +86 871 6522 3070.
E-mail addresses: zhangyu@mail.kib.ac.cn (Y. Zhang), haoxj@mail.kib.ac.cn (X. Hao).

http://dx.doi.org/10.1016/j.fitote.2014.07.008
0367-326X/

2. Experimental

2.1. General experiment procedure

Optical rotations were measured on a Perkin Elmer 241 polarimeter. ECD spectra were recorded on an Applied Photophysics Chirascan spectrometer. UV spectra were recorded on a UV 210A spectrophotometer. IR spectra were taken on a Bio-Rad FTS-135 spectrophotometer (KBr). HRESIMS data were measured on a VG Auto Spec-3000 spectrometer. ESIMS data were obtained on a Finnigan MAT 90 spectrometer. NMR spectra were recorded on Bruker AM-400, DRX-500 and Avance III 600 NMR spectrometers using 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).

2.2. Plant material

The twigs and leaves of *B. mekongensis* were collected in April 2010 from Mengna County, Yunnan Province, PR China, and were identified by Mr. Jing-Yun Cui, Xishuangbanna Tropical Plant Garden. A voucher specimen (No. CUI20100419) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

2.3. Extraction and isolation

The dried twigs and leaves of *B. mekongensis* (12 kg) were extracted with CH_3OH, and the pH of the crude extract was adjusted with saturated tartaric acid to 2. The acidic mixture was defatted with petroleum ether (PE) and then extracted with $CHCl_3$. The aqueous phase was basified to pH ~ 10 with saturated Na_2CO_3 and then extracted with $CHCl_3$ to obtain crude alkaloids. The crude alkaloids (60 g) were separated on a silica gel column (200–300 mesh; $CHCl_3/CH_3OH$, 1:0 → 0:1), yielding five major fractions (Fr 1–5). Fraction 1 (11.8 g) was chromatographed over a series of silica gel columns ($CHCl_3$/acetone and $CHCl_3/CH_3OH$) to afford compounds 6 (18 mg), 8 (16 mg), and 10 (24 mg). Fraction 2 (11.2 g) was further chromatographed over a reversed-phase C_{18} silica gel medium-pressure column (CH_3OH/H_2O, 1:1 → 1:0) to give four fractions (Fr 2A–2D). Fraction 2A (2.8 g) was chromatographed over a series of silica gel columns ($CHCl_3$/acetone and $CHCl_3/CH_3OH$) to afford compounds 9 (18 mg), 12 (8 mg), and 14 (24 mg). Fraction 2B (1.6 g) was then subjected to a SephadexLH-20 column, and the fractions were further separated using pre-HPLC (CH_3OH/H_2O, 80:20) added with 0.1‰ v/v diethylamine to give 1 (27 mg) and 2 (16 mg). Fraction 2C (3.2 g) was separated on a silica gel column (300–400 mesh; PE/acetone, 3:1), yielding three fractions (Fr 2C1–2C3). Fraction 2C1 (520 mg) was purified using a Sephadex LH-20 column eluted with CH_3OH, followed by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 80% CH_3OH/H_2O to afford compounds 3 (16 mg), 4 (18 mg), and 7 (72 mg). Compounds 5 (26 mg), 11 (22 mg), and 18 (16 mg) were separated from fraction 2C2 (760 mg) by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 70% CH_3CN/H_2O added with 0.1‰ v/v diethylamine. Compounds 13 (13 mg), 16 (28 mg), and 22 (36 mg) were obtained from fraction 2C3 (368 mg) by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 70% CH_3OH/H_2O added with 0.1‰ v/v diethylamine. Fraction 3 (8.8 g) was further chromatographed over a reversed-phase C_{18} silica gel medium-pressure column (CH_3OH/H_2O, 1:1 → 1:0) to give four fractions (Fr 3A–3D). Fraction 3A (482 mg) was separated by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 45% CH_3OH/H_2O to give compounds 17 (16 mg) and 20 (28 mg). Compounds 15 (19 mg) and 29 (18 mg) were obtained from fraction 3B (540 mg) by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 55% CH_3OH/H_2O. Fraction 3C (780 mg) was purified using a Sephadex LH-20 column eluted with CH_3OH, followed by semipreparative HPLC using an Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 70% CH_3OH/H_2O to afford compounds 21 (22 mg), 23 (48 mg), and 26 (38 mg). Fraction 4 (9.8 g) was further chromatographed over a reversed-phase C_{18} silica gel medium-pressure column (CH_3OH/H_2O, 1:1 → 1:0) to give four fractions (Fr 4A–4D). Fraction 4A (518 mg) was separated by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 40% CH_3OH/H_2O to give compounds 24 (9 mg), 27 (16 mg), and 30 (23 mg). Compound 25 (128 mg) was crystallized in acetone from fraction 4B (800 mg). Fraction 4C (960 mg) was purified using a Sephadex LH-20 column eluted with CH_3OH, followed by semipreparative HPLC using a Waters XBridge C_{18} (10 × 250 mm, 5 μm) column with 30% CH_3OH/H_2O to afford compounds 19 (32 mg), 28 (12 mg), and 31 (8 mg).

2.4. Mekongenine C (1)

Yellowish amorphous powder; $[\alpha]_D^{19}-16.0$ (*c* 0.11, CH_3OH); IR (KBr) ν_{max} 3429, 2926, 1724, 1679, 1618 and 1459 cm^{-1}; UV (CH_3OH) λ_{max} 228 (ε 27806), 332 nm (11043); ECD (0.00024 M, CH_3OH) λ_{max} ($\Delta\varepsilon$) 232 (+31.1), 332 (−8.9) nm; ^1H and ^{13}C NMR data (Table 1); ESIMS *m/z* 673 $[M + H]^+$; HRESIMS *m/z* 673.3756 (M + H; calcd for $C_{42}H_{49}N_4O_4$, 673.3754).

2.5. Mekongenine D (2)

Yellowish amorphous powder; $[\alpha]_D^{19}-28.0$ (*c* 0.12, CH_3OH); IR (KBr) ν_{max} 3426, 2927, 1724, 1676, 1616 and 1456 cm^{-1}; UV (CH_3OH) λ_{max} 226 (ε 24730), 331 nm (9484); ECD (0.00018 M, CH_3OH) λ_{max} ($\Delta\varepsilon$) 229 (+35.3), 332 (−15.9) nm; ^1H and ^{13}C NMR data (Table 2); ESIMS *m/z* 645$[M + H]^+$; HRESIMS *m/z* 645.3811 (M + H; calcd for $C_{41}H_{49}N_4O_3$, 645.3805).

2.6. Mekongenine E (3)

Yellowish amorphous powder; $[\alpha]_D^{19}-12.0$ (*c* 0.15, CH_3OH); IR (KBr) ν_{max} 3431, 2927, 1719, 1674, 1617 and 1455 cm^{-1}; UV (CH_3OH) λ_{max} 232 (ε 39450), 330 nm (18383); ECD (0.00013 M, CH_3OH) λ_{max} ($\Delta\varepsilon$) 231 (+44.6), 332 (−16.5) nm; ^1H and ^{13}C NMR data (Table 3); ESIMS *m/z* 659 $[M + H]^+$; HRESIMS *m/z* 659.3595 (M + H; calcd for $C_{41}H_{47}N_4O_4$, 659.3597).

Table 1
^{1}H and ^{13}C NMR data of mekongenine C (1) in CD_3OD.

	Unit A			Unit B	
No.	δ_H[a]	δ_C[b]	No.	δ_H[a]	δ_C[b]
2		132.8 s	2′		168.5 s
3a	3.02 (1H, d, 17.4)	44.6 t	3′a	2.92(1H, d, 15.8)	51.5 t
3b	3.13 (1H, d, 17.4)		3′b	3.80 (1H, m)	
5a	3.47 (1H, overlap)	50.6 t	5′a	2.52 (1H, m)	51.7 t
5b	3.47 (1H, overlap)		5′b	3.28 (1H, m)	
6a	2.72 (1H, dd, 16.7, 5.8)	17.8 t	6′a	1.74 (1H, dd, 11.2, 4.1)	46.0 t
6b	3.22 (1H, dd, 16.7, 8.2)		6′b	1.96 (1H, m)	
7		105.7 s	7′		55.9 s
8		123.9 s	8′		131.4 s
9	7.25 (1H, d, 8.5)	119.1 d	9′	7.00 (1H, s)	121.2 d
10	6.56 (1H, dd, 8.5, 2.0)	109.6 d	10′		122.2 s
11		156.4 s	11′		158.0 s
12	5.68 (1H, d, 2.0)	96.6 d	12′	6.90 (1H, s)	95.1 d
13		138.7 s	13′		145.6 s
14	5.64 (1H, m)	126.7 d	14′	5.68 (1H, m)	126.2 d
15	5.76 (1H, d, 9.8)	129.2 d	15′	5.56 (1H, d, 9.6)	134.0 d
16	5.19 (1H, dd, 11.7, 3.5)	50.4 d	16′		93.2 s
17a	1.94 (1H, overlap)	43.0 t	17′a	2.40 (1H, d, 15.4)	30.2 t
17b	2.31 (1H, overlap)		17′b	2.48 (1H, d, 15.4)	
18	1.03 (3H, t, 7.5)	8.6 q	18′	0.42 (3H, t, 7.4)	7.7 q
19a	1.68 (1H, q, 7.5)	34.7 t	19′a	0.72 (1H, q, 7.4)	27.8 t
19b	1.96 (1H, q, 7.5)		19′b	0.89 (1H, q, 7.4)	
20		38.6 s	20′		42.4 s
21	4.31 (1H, s)	59.2 d	21′	2.33 (1H, s)	70.3 d
OCH_3′	3.33 (3H, s)	55.1 q	$COOCH_3$′		170.0 s
			$COOCH_3$′	3.80 (3H, s)	51.6 q
			OCH_3′	3.97 (3H, s)	56.5 q

[a] Measured at 600 MHz.
[b] Measured at 150 MHz.

Table 2
^{1}H and ^{13}C NMR data of mekongenine D (2) in $CDCl_3/CD_3OD$ (1:1).

	Unit A			Unit B	
No.	δ_H[a]	δ_C[b]	No.	δ_H[a]	δ_C[b]
2		134.6 s	2′		169.6 s
3a	3.03 (1H, d, 15.8)	44.8 t	3′a	2.25 (1H, d, 15.8)	51.4 t
3b	3.12 (1H, d, 15.8)		3′b	2.97 (1H, dd, 15.8, 4.8)	
5a	3.46 (1H, m)	50.7 t	5′a	2.46 (1H, m)	52.8 t
5b	3.46 (1H, overlap)		5′b	2.86 (1H, m)	
6a	2.74 (1H, dd, 15.8, 6.4)	17.9 t	6′a	1.73 (1H, dd, 11.2, 3.6)	46.3 t
6b	3.24 (1H, dd, 15.8, 8.2)		6′b	2.03 (1H, dd, 11.2, 6.8)	
7		105.9 s	7′		56.4 s
8		129.8 s	8′		131.1 s
9	7.41 (1H, d, 7.6)	118.8 d	9′	6.96 (1H, s)	120.9 d
10	6.93 (1H, t, 7.6)	120.3 d	10′		122.3 s
11	6.69 (1H, t, 7.6)	121.5 d	11′		157.6 s
12	6.22 (1H, d, 7.6)	113.7 d	12′	6.79 (1H, s)	95.3 d
13		138.1 s	13′		145.4 s
14	5.60 (1H, m)	126.8 d	14′a	1.42 (1H, m)	22.5 t
			14′b	1.75 (1H, m)	
15	5.73 (1H, d, 10.1)	129.3 d	15′a	1.05 (1H, m)	33.8 t
			15′b	1.68 (1H, m)	
16	5.22 (1H, d, 10.1)	50.8 d	16′		93.7 s
17a	1.90 (1H, overlap)	43.2 t	17′a	2.24 (1H, overlap)	27.1 t
17b	2.34 (1H, d, 14.8)		17′b	2.63 (1H, d, 15.3)	
18	1.03 (3H, t, 7.6)	9.0 q	18′	0.50 (3H, t, 6.4)	7.9 q
19a	1.67 (1H, q, 7.6)	34.9 t	19′a	0.41 (1H, q, 6.4)	30.5 t
19b	1.96 (1H, q, 7.6)		19′b	0.91 (1H, q, 6.4)	
20		38.6 s	20′		38.7 s
21	4.28 (1H, s)	59.3 d	21′	2.16 (1H, s)	73.3 d
			$COOCH_3$′		170.4 s
			$COOCH_3$′	3.79 (3H, s)	51.9 q
			OCH_3′	3.94 (3H, s)	56.7 q

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

Table 3
^{1}H and ^{13}C NMR data of mekongenine E (3) in CD_3OD.

	Unit A			Unit B	
No.	δ_H[a]	δ_C[b]	No.	δ_H[a]	δ_C[b]
2		134.7 s	2′		167.9 s
3a	2.93 (1H, overlap)	44.7 t	3′a	2.85 (1H, d, 15.6)	51.6 t
3b	3.06 (1H, d, 17.4)		3′b	3.23 (1H, d, 5.6, 4.8)	
5a	3.40 (1H, m)	50.5 t	5′a	2.43 (1H, m)	51.8 t
5b	3.40 (1H, m)		5′b	2.83 (1H, overlap)	
6a	2.66 (1H, dd, 15.8, 5.3)	17.8 t	6′a	1.69 (1H, dd, 11.2, 3.6)	46.4 t
6b	3.17 (1H, overlap)		6′b	1.94 (1H, overlap)	
7		105.8 s	7′		55.9 s
8		129.8 s	8′		130.8 s
9	7.34 (1H, d, 7.6)	118.6 d	9′	6.92 (1H, s)	120.0 d
10	6.85 (1H, t, 7.6)	120.8 d	10′		122.9 s
11	6.66 (1H, t, 7.6)	121.7 d	11′		158.0 s
12	6.21 (1H, d, 7.6)	112.8 d	12′	6.82 (1H, s)	95.4 d
13		138.0 s	13′		145.6 s
14	5.55 (1H, dd, 10.1, 3.6)	126.9 d	14′	5.70 (1H, d, 9.8)	127.3 d
15	5.68 (1H, d, 10.1)	129.1 d	15′	5.65 (1H, d, 9.8)	130.6 d
16	5.21 (1H, d, 8.9)	50.6 d	16′		93.5 s
17a	1.88 (1H, overlap)	43.3 t	17′a	2.32 (1H, d, 15.4)	28.5 t
17b	2.28 (1H, d, 14.8)		17′b	2.92 (1H, d, 15.4)	
18	0.98 (3H, t, 7.5)	8.6 q	18′	0.42 (3H, d, 6.4)	18.9 q
19a	1.63 (1H, q, 7.5)	34.8 t	19′a	3.21 (1H, q, 6.4)	66.3 d
19b	1.92 (1H, q, 7.5)		19′b		
20		38.6 s	20′		48.0 s
21	4.26 (1H, s)	59.2 d	21′	2.31 (1H, s)	67.8 d
			$COOCH_3$′		170.2 s
			$COOCH_3$′	3.75 (3H, s)	51.5 q
			OCH_3′	3.92 (3H, s)	56.6 q

[a] Measured at 500 MHz.
[b] Measured at 125 MHz.

2.7. Mekongenine F (4)

Yellowish amorphous powder; $[\alpha]_D^{19}-23.0$ (*c* 0.12, CH_3OH); IR (KBr) ν_{max} 3426, 2926, 1731, 1680, 1617 and 1455 cm^{-1}; UV (CH_3OH) λ_{max} 229 (ε 35811), 331 nm (17323); ECD (0.00012 M, CH_3OH) λ_{max} ($\Delta\varepsilon$) 234 (+36.9), 334 (−14.7) nm; ^{1}H and ^{13}C NMR data (Table 4); ESIMS *m/z* 701 $[M + H]^+$; HRESIMS *m/z* 701.3683 (M + H; calcd for $C_{43}H_{49}N_4O_5$, 701.3702).

2.8. 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 [29]. 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. Cisplatin (Sigma, St. Louis, MO, USA) were used as positive controls. After treatment, cell viability was measured and the cell growth curve was plotted. IC_{50} values were calculated by the Reed and Muench method [30].

3. Results and discussion

3.1. Structure elucidation

Mekongenine C (1), obtained as a yellowish amorphous powder, possessed a molecular formula of $C_{42}H_{48}N_4O_4$, as established by HRESIMS (*m/z* 673.3756 $[M + H]^+$, calcd 673.3754) and ^{13}C NMR spectroscopic data (Table 1), indicating 21° of unsaturation. The IR absorption bands implied the presence of amino or hydroxyl (3429 cm^{-1}) and ester carbonyl (1679 cm^{-1}) functions. The UV spectrum showed the absorption bands at 228 and 332 nm, which were characteristic of the aspidospermine indole alkaloid with the β-anilinoacrylate chromophore [31]. The analyses of ^{1}H, ^{13}C NMR and DEPT spectra suggested that 1 possessed 42 carbons including 5 methyls, 10 methylenes, 12 methines, and 15 quaternary carbons. Further analyses of the NMR spectra indicated that 1 was an eburnamine-aspidospermine type bisindole alkaloid. The ^{1}H and ^{13}C NMR data of 1 showed similarity with those of mekongenine B [2], except for the loss of one aromatic proton and the presence of an additional methoxyl. By comparing the ^{13}C NMR data of 1 with that of mekongenine B, the carbon signal at C-11 was significantly deshielded ($\Delta\delta_C$ +35), and the carbon signals at C-10 and C-12 were relatively upshielded ($\Delta\delta_C$ −10.5 and −13.5, respectively), suggesting that the methoxy was attached to C-11 [11,32]. The structure of 1 and the linkage of the methoxy at C-11 were confirmed by the HMBC experiment in which the methoxy proton resonanced at δ_H 3.33 correlated

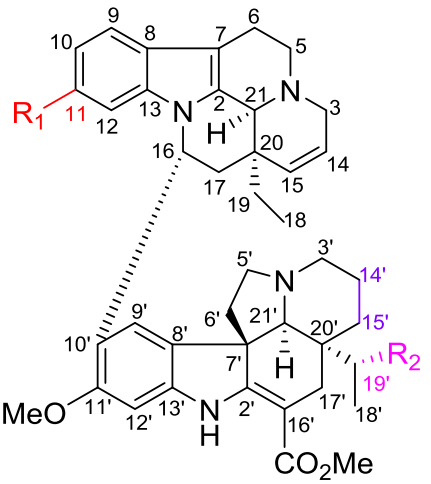
Table 4
^{1}H and ^{13}C NMR data of mekongenine F (4) in CD_3OD.

	Unit A			Unit B	
No.	δ_H[a]	δ_C[b]	No.	δ_H[a]	δ_C[b]
2		134.4 s	2′		167.8 s
3a	3.00 (1H, d, 16.8)	44.7 t	3′a	3.26 (1H, d, 15.8)	51.3 t
3b	3.13 (1H, d, 16.8)		3′b	3.74 (1H, dd, 15.8, 4.8)	
5a	3.48 (1H, m)	50.5 t	5′a	2.51 (1H, m)	51.6 t
5b	3.48 (1H, m)		5′b	2.88 (1H, overlap)	
6a	2.74 (1H, dd, 16.8, 6.4)	17.8 t	6′a	1.75 (1H, dd, 11.2, 2.8)	46.0 t
6b	3.24 (1H, dd, 16.8, 8.4)		6′b	1.97(1H, overlap)	
7		105.9 s	7′		56.1 s
8		129.8 s	8′		130.5 s
9	7.39 (1H, d, 7.6)	118.7 d	9′	6.96 (1H, s)	120.8 d
10	6.88 (1H, t, 7.6)	120.1 d	10′		122.8 s
11	6.68 (1H, t, 7.6)	121.8 d	11′		158.0 s
12	6.25 (1H, d, 7.6)	112.9 d	12′	6.91 (1H, s)	95.5 d
13		138.0 s	13′		145.6 s
14	5.63 (1H, d, 10.1)	126.7 d	14′	5.82 (1H, dd, 9.6, 3.9)	128.5 d
15	5.76 (1H, d, 10.1)	129.1 d	15′	5.68 (1H, d, 9.6)	130.0 d
16	5.24 (1H, d, 9.2)	50.6 d	16′		93.1 s
17a	1.90 (1H, dd, 14.8, 9.2)	43.1 t	17′a	2.41 (1H, d, 15.2)	28.6 t
17b	2.36 (1H, d, overlap)		17′b	2.71 (1H, d, 15.2)	
18	1.03 (3H, t, 7.4)	8.6 q	18′	0.50 (3H, d, 6.2)	15.7 q
19a	1.68 (1H, q, 7.4)	34.7 t	19′	4.51 (1H, q, 6.2)	69.8 d
19b	1.96 (1H, overlap)				
20		38.6 s	20′		47.3 s
21	4.34 (1H, s)	59.2 d	21′	2.35 (1H, s)	67.3 d
			$COOCH_3$′		169.6 s
			$COOCH_3$′	3.75 (3H, s)	51.6 q
			$OOCCH_3$′		171.8 s
			$OOCCH_3$′	1.98 (3H, s)	21.1 q
			OCH_3′	3.97 (3H, s)	56.5 q

[a] Measured at 600 MHz.
[b] Measured at 150 MHz.

to C-11 at δ_C 156.4. Thus, mekongenine C was assigned as 1, which was further verified by a combination analysis of the HSQC, ^{1}H-^{1}H COSY, HMBC and ROESY spectra (Fig. 2).

Mekongenine D (2) was obtained as a yellowish amorphous powder. Its UV spectrum showed absorption maxima attributed to an aspidospermine indole alkaloid with a β-anilinoacrylate chromophore (330 and 242 nm) [31], while the IR spectrum displayed absorption bands due to amino or hydroxyl (3426 cm^{-1}) and conjugated ester carbonyl (1676 cm^{-1}) functions. The molecular formula was established as $C_{41}H_{48}N_4O_3$ by HRESIMS (m/z 645.3811, $[M + H]^+$; calcd: 645.3805), which was larger than that of mekongenine B by 2 mass units, suggested that it had one fewer degree of unsaturation than mekongenine B. The ^{1}H and ^{13}C NMR data



	R_1	R_2	
1	OMe	H	$\Delta^{14,15}$
2	H	H	
3	H	OH	$\Delta^{14,15}$
4	H	OAc	$\Delta^{14,15}$

Fig. 1. Molecular structures of mekongenines C–F (1–4).

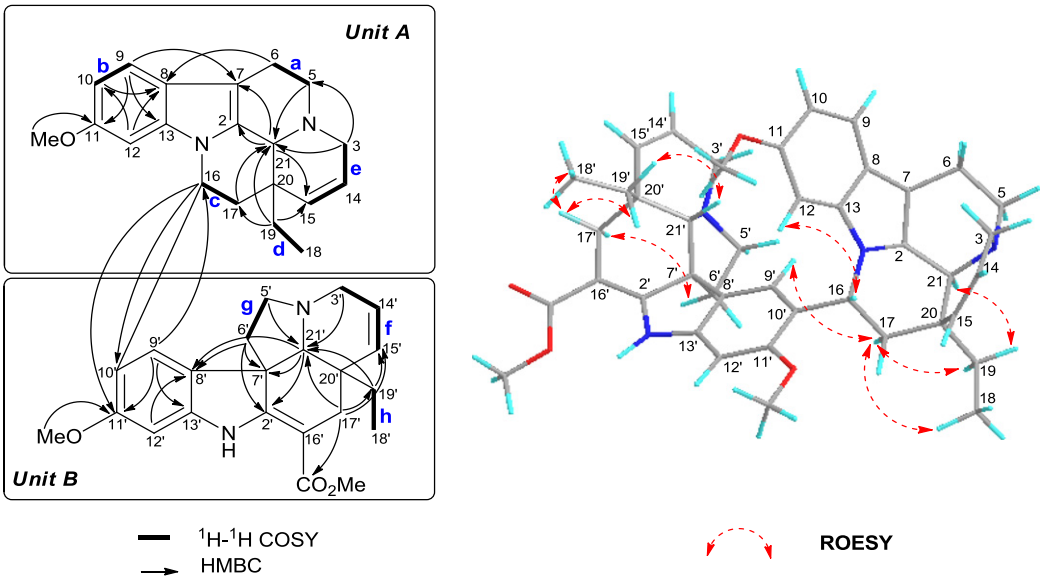


Fig. 2. Selected 2D NMR correlations for mekongenine C (1).

(Table 2) revealed that 2 possessed 41 carbon signals including 4 methyls, 12 methylenes, 11 methines, and 14 quaternary carbons, which were analogous to those of mekongenine B. The only significant differences were the presence of two methylene carbon signals instead of a double bond ($C_{14'}$–$C_{15'}$) carbon signals in 2. Detailed analysis of spectroscopic data revealed that the double bond ($C_{14'}$–$C_{15'}$) in mekongenine B was hydrogenated in 2, especially supported by the HMBC correlations of H_2-19′ and H-21′ to C-15′ (δ_C 33.8), H_2-3′ to C-14′ (δ_C 22.5) and C-15′, as well as the key ^{1}H-^{1}H COSY correlations of H_2-3′/H_2-14′/H_2-15′. The relative configurations of 2 were assigned to be the same as in mekongenine B by nearly identical ROESY correlations. Thus, the relative stereostructure of 2 was assigned as the same as mekongenine B, as shown in Fig. 1.

The molecular formula of mekongenine E (3) was established as $C_{41}H_{46}N_4O_4$ by HRESIMS (*m/z* 659.3595, $[M + H]^+$; calcd: 659.3597), which was larger than that of mekongenine B by 16 mass units. The ^{1}H and ^{13}C NMR data (Table 3) suggested that 3 possessed 41 carbon signals which resembled those of mekongenine B except for the chemical shifts of an ethyl group ($C_{18'}$–$C_{19'}$). Further analysis of spectroscopic data suggested a hydroxyl group was located at C-19′ in 2, especially supported by the HMBC correlations of H_3-18′ to C-19′ (δ_C 66.3). The similar ROESY correlations between 3 and mekongenine B indicated that 3 had the same relative configurations at the chiral centers (C-16, C-20, C-21, C-7′, C-20′, C-21′) with mekongenine B except for C-19′ (see detail ROESY data in Supplementary material). Moreover, the absolute configuration of C-19′ could be affirmed

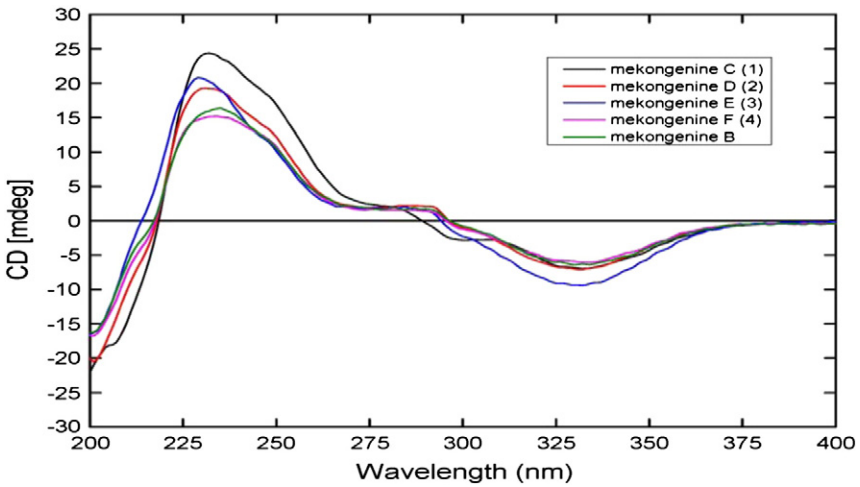


Fig. 3. ECD spectra of mekongenine B and mekongenines C–F (1–4).

Table 5
Cytotoxicities of compounds 1–4[a] (IC_{50}[b], μM).

Compounds	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	3.24 ± 0.12	5.73 ± 0.09	17.05 ± 0.16	11.08 ± 0.04	13.91 ± 0.06
2	4.91 ± 0.16	12.30 ± 0.16	10.08 ± 0.24	15.20 ± 0.08	9.73 ± 0.12
3	10.88 ± 0.08	38.88 ± 0.82	20.94 ± 0.40	21.66 ± 0.34	14.38 ± 0.18
4	5.16 ± 0.16	10.98 ± 0.18	14.04 ± 0.06	8.04 ± 0.12	15.06 ± 0.10
Cisplatin[c]	1.56 ± 0.02	10.82 ± 0.22	14.26 ± 0.18	19.28 ± 0.26	25.69 ± 0.88

[a] All results are expressed as mean + SD; n = 3 for all groups.
[b] IC_{50}: 50% inhibitory concentration.
[c] Positive control.

as 19′*R* by comparison of the NMR data of C-18′ (δ_C 18.9) and C-19′ (δ_C 66.3) with those of (19*R*)-hydroxytabersonine [17,19,20]. Therefore, mekongenine E (3) was deduced to be the (19′*R*)-hydroxyl analog of mekongenine B, which was further substantiated through 2D NMR (HSQC, ^1H-^1H COSY, HMBC, and ROESY).

The molecular formula of mekongenine F (4) was $C_{43}H_{48}N_4O_5$, as determined by HRESIMS (*m/z* 701.3683, $[M + H]^+$; calcd: 701.3702) and ^{13}C NMR spectroscopic data (Table 4). Comparison of the NMR data of 4 (Table) with those of 3 suggested that both alkaloids shared the same basic skeleton, but the molecular weight of 4 is larger than that of 3 by 42 units, namely, the hydroxyl group at C-19′ in 3 was replaced by an acetoxy group (δ_H 1.98 (3H, s) and δ_C 21.1 and 171.8). The above elucidation was supported by the HMBC correlations of H_3-18′ to C-19′ (δ_C 69.8) and H-19′ to the ester carbonyl carbon (δ_C 171.8). Moreover, the absolute configuration of C-19′ could be also affirmed as 19′*R* according to the NMR data of C-18 and C-19 of (19*R*)-acetoxytabersonine [21,22]. Thus, the structure of mekongenine F (4) was thus established as the (19′*R*)-acetoxyl analog of mekongenine B as shown in Fig. 1.

From a structural and biogenetic point of view, mekongenine B [2], whose structure and absolute configuration had been determined by X-ray diffraction and ECD calculation, has the same chromophores with those of alkaloids 1–4 and could be considered as a model compound for assignment of the absolute configuration by comparison of the ECD curves [33,34]. Hence, the absolute configurations of alkaloids 1–4 were identical to that of mekongenine B, as determined by their similar patterns of Cotton effects in the ECD spectra corresponding to the UV absorption maxima (Fig. 3).

3.2. Cytotoxic activity

Selected compounds were evaluated for cytotoxicity against five human cancer cell lines, HL-60, SMMC-7721, A-549, MCF-7 and SW480 using the MTT method, with cisplatin as a positive control [27]. Compounds 1–4 showed significant cytotoxicity, while the other compounds were inactive (IC_{50} values of >40 μmol) (Table 5). It is noteworthy that bisindole alkaloids 1, 2 and 4 exhibited significant inhibitory effects against five human cancer cell lines with IC_{50} values similar to those of cisplatin, even stronger inhibitory activities than that of cisplatin in some cancer cell lines.

4. Concluding remarks

Four new eburnamine-aspidospermine type bisindole alkaloids, mekongenines C–F (1–4), as well as 27 known monoterpenoid indole alkaloids, were isolated from the twigs and leaves of *B. mekongensis*. The discoveries of compounds 1–4 as well as mekongenines A and B reported in our previous research are not only a further addition to the diverse and complex array of bisindole alkaloids that are rapidly expanding, but also, 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.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The work was financially supported by the National Natural Science Foundation of China (No. 31100259 and 21302181). The authors thank Mr. Jing-Yun Cui, Xishuangbanna Tropical Plant Garden, Chinese Academy of Sciences (CAS), for the collection and identification of the plant material.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.07.008.

References

[1] Zhen M, Min TL. Flora of China, vol. 63. Beijing: Science Press; 1980 30–5.
[2] Fu YH, He HP, Di YT, Li SL, Zhang Y, Hao XJ. Mekongenines A and B, two new alkaloids from *Bousigonia mekongensis*. Tetrahedron Lett 2012;53:3642–6.
[3] Fu YH, Di YT, He HP, Li SL, Zhang Y, Hao XJ. Angustifonines A and B, cytotoxic bisindole alkaloids from *Bousigonia angustifolia*. J Nat Prod 2014;77:56–62.
[4] Feng T, Li Y, Wang YY, Cai XH, Liu YP, Luo XD. Cytotoxic indole alkaloids from *Melodinus tenuicaudatus*. J Nat Prod 2010;73:1075–9.
[5] Rahman A, Zaman K, Perveen S, Habib R, Muzaffar A, Choudhary MI, et al. Alkaloids from *Rhazya stricta*. Phytochemistry 1991;30:1285–93.
[6] Pfaffli P, Hauth H. Addition reactions on the alicyclic double bond of eburnamenin and apovincamine. Helv Chim Acta 1978;61:1682–95.
[7] Kam TS, Tan PS, Wei C. Absolute configuration of C-16 of eburnane alkaloids from *Kopsia larutensis*. Phytochemistry 1993;33:921–4.
[8] Baassou S, Mehri H, Plat M. 19(*R*)-hydroxyeburnamine and 19(*R*)-hydroxyisoeburnamine from *Kopsia dasyrachis*. Nat Prod Lett 1998;12:293–8.

[9] Batchily F, Baassou S, Mehri H, Plat M, Sevenet T, Pusset J. Plants from New Caledonia. Part 90. Alkaloids from *Melodinus insulae-pinorum* Boiteau. Ann Pharm Fr 1985;43:359–64.
[10] Neuss N, Boaz HE, Potier P, Ran C, Plat MM, Plat M. The structure of vincarodine. Helv Chim Acta 1973;56:2660–6.
[11] Novak L, Rohaly J, Szantay C. Synthesis of *vinca* alkaloids and related compounds. VI. Syntheses of eburnamonine and isoeburnamonine. Heterocycles 1977;6:1149–56.
[12] Bombardelli E, Bonati A, Gabetta B, Martinelli EM, Mustich G, Danieli B. Structure of cuanzine. Tetrahedron 1974;30:4141–6.
[13] Szabo L, Kalaus G, Szantay C. Synthesis of *Vinca* alkaloids and related compounds. Part LXXX. Synthesis of (+)-vincine and (+)-apovincine. Nat Prod Lett 1996;8:237–40.
[14] Strouf O, Trojanek J. Alkaloids. X. Structure of vincine. Collect Czechoslov Chem Commun 1964;29:447–56.
[15] Cordall GA, Weiss SG, Farnsworth NR. Structure elucidation and chemistry of *Catharanthus* alkaloids. XXX. Isolation and structure elucidation of vincarodine. J Org Chem 1974;39:431–4.
[16] Levy J, Laronze JY, Laronze J. A synthesis of tabersonine. Tetrahedron Lett 1978;18:1579–80.
[17] Wenkert E, Cochran DW, Hagaman EW, Schell FM, Neuss N, Katner AS, et al. Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. XIX. *Aspidosperma* alkaloids. J Am Chem Soc 1973;9:4990–5.
[18] Baassou S, Mehri H, Plat M. Plants of New Caledonia. Part 49. Alkaloids of *Melodinus aeneus*. Phytochemistry 1978;17:1449–50.
[19] Lounasmaa M, Kan SK. A 400 MHz proton NMR study of five aspidospermane-type alkaloids. Acta Chem Scand 1980;B34:379–81.
[20] Langlois N, Andriamialisoa RZ. Studies on vindolinine. 6. Partial synthesis of aspidospermane-type alkaloids. J Org Chem 1979;44: 2468–71.
[21] Kutney JP, Choi LSL, Kolodziejczyk P, Sleigh SK, Stuart KL, et al. Alkaloid production in *Catharanthus roseus* cell cultures: isolation and characterization of alkaloids from one cell line. Phytochemistry 1980;19: 2589–95.
[22] Chazelet I, Batchily F, Mehri H, Plat M, Rabaron A. Plants of New Caledonia. CIV. Alkaloids from the fruit of *Melodinus scandens* Forster. Ann Pharm Fr 1986;44:355–62.
[23] Hugel G, Levy J. Thermal rearrangements of some indole alkaloid derivatives. Tetrahedron 1980;40:1067–73.
[24] Ahond A, Janot MM, Langlois N, Lukacs G, Potier P, et al. Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. XXIII. Structure of vindolinine. J Am Chem Soc 1974;96: 633–4.
[25] Zhou YL, Ye JH, Li ZM, Huang ZH. Study on the alkaloids of *Melodinus tenuicaudatus*. Planta Med 1988;54:315–7.
[26] Bernauer K, Englert G, Vetter W, Weiss E. Constitution of the Melodinus alkaloids (+)-meloscine, (+)-epimeloscine, and (+)-scandine. Helv Chim Acta 1969;52:1886–904.
[27] Mehri H, Diallo AO, Plat M. An alkaloid from leaves of *Melodinus scandens*. Phytochemistry 1995;40:1005–6.
[28] Plat M, Hachem-Mehri M, Koch ML, Scheidegger U, Potier P. Structure and stereochemistry of meloscandonine, an alkaloid of *Melodinus scandens*. Tetrahedron Lett 1970;11:3395–8.
[29] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[30] Reed LJ, Muench H. A simple method of estimating fifty percent end points. Am J Hyg 1938;27:493–7.
[31] Weissberger I, Taylor EC. Indoles: The Monoterpenoid Indole Alkaloids. New York: Wiley-Interscience; 1983. p. 357.
[32] Zeches M, Lounkokobi J, Richard B, Plat M, Le Men-Olivier L, Sevenet T, et al. Alkaloids of *melodinus guillauminii*. Phytochemistry 1984;23: 171–4.
[33] Fang X, Zhang Q, Tan CJ, Mu SZ, Lu Y, Lu YB, et al. Cipadonoids B–G, six new limonoids from *Cipadessa cinerascens*. Tetrahedron 2009;65: 7408–14.
[34] Chen HD, Yang SP, He XF, Ai J, Liu ZK, Liu HB, et al. Trigochinins A–C: three new daphnane-type diterpenes from *Trigonostemon chinensis*. Org Lett 2010;12:1168–71.