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Cytotoxic Bisbenzylisoquinoline Alkaloids from *Stephania epigaea*

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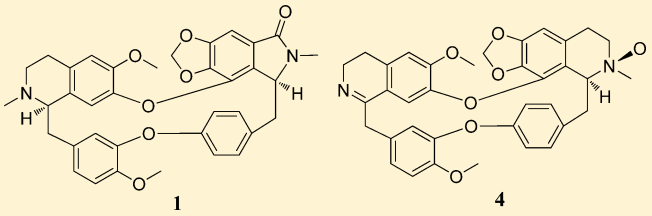
Supporting Information

ABSTRACT: Six new bisbenzylisoquinoline alkaloids (**1**–**6**) and seven known compounds (**8**–**14**) were isolated from the tubers of *Stephania epigaea*, in addition to the major alkaloid, cepharanthine (**7**). The structures of **1**–**6** were elucidated by combined spectroscopic data analysis and chemical methods, with their configurations determined from their optical rotation values and confirmed using circular dichroism. Compounds **1**–**6** belong to the oxyacanthine type of bisbenzylisoquinoline alkaloids and have a rare methylenedioxy substituent. Compound **1**, a dimer composed of benzylisoquinoline and seco-aristolactam units, represents a new type of bisbenzylisoquinoline alkaloid, while compounds **3**–**6** are bisbenzylisoquinoline *N*-oxides. These compounds were evaluated for their in vitro cytotoxicities against six human cancer cell lines (A-549, ECA109, HL-60, MCF-7, SMMC-7721, and SW480). Cepharanthine (**7**), the major component of *S. epigaea*, exhibited cytotoxicity against all of these cancer cell lines except ECA109, while its known analogue, **10**, displayed cytotoxicity against all six cancer cell lines.



1 **4**

The genus *Stephania* (Menispermaceae), containing 60 species, is distributed mainly in the warmer parts of Asia and Africa, with about two-thirds of the number of this genus growing in mainland China.[1] These species have been utilized in folk medicine for the treatment of asthma, cancer, dysentery, fever, hyperglycemia, intestinal complaints, inflammation, sleep disturbances, and tuberculosis.[2] Several chemical studies on *Stephania* spp. have been carried out over the past five decades, which have led to the identification of more than 200 hasubanan,[3] aporphine,[4] protoberberine,[5] and bisbenzylisoquinoline[6] alkaloids as the major constituents. Among these, cepharanthine (**7**) was reported as a main bisbenzylisoquinoline alkaloid having various biological activities, such as antitumor activity,[7] suppression of cytokine production,[8] and induction of apoptosis.[9]

Stephania epigaea H. S. Lo (Menispermaceae) is a herbaceous liana mainly growing in the southwest and southeast of Yunnan Province, People's Republic of China. Its tubers have been used by local people to treat fever and for sedation. Previous studies showed that it produces cepharanthine (**7**) and the other alkaloids cydeanine, delavaine, isochondodendrine, (−)-norcycleanine, and runanine.[10] In order to explore a new source and further investigate the bioactivities of cepharanthine (**7**) and its analogues, a detailed chemical investigation on the tubers of *S. epigaea* was carried out. This led to the identification of 13 minor bisbenzylisoquinoline alkaloids (**1**–**6**, **8**–**14**), in addition to the main component, cepharanthine (**7**). Compounds **1**–**6** are new cepharanthine analogues, and their structures were elucidated on the basis of detailed spectroscopic analysis and chemical methods. The isolated compounds **3**–**14** were evaluated for their cytotoxicity against six human cancer cell lines (A-549 human lung carcinoma, ECA109 human esophagus cancer, HL-60 human myeloid leukemia, MCF-7 human breast adenocarcinoma, SMMC-7721 hepatocellular carcinoma, and SW480 colon cancer), and the results obtained are discussed herein.

RESULTS AND DISCUSSION

The alkaloid portion from the tubers of *S. epigaea* was subjected to repeated column chromatography over silica gel, followed by preparative thin-layer chromatography on silica gel (GF254) and recrystallization, to afford the main component cepharanthine (**7**), together with 13 bisbenzylisoquinoline alkaloids (**1**–**6**, **8**–**14**). All showed a positive reaction to Dragendorff's reagent. The known compounds (**7**–**14**) (see Supporting Information) were identified as cepharanthine (**7**),[6] secocepharanthine (**8**),[11] cepharanoline (**9**),[12] (+)-2-norcepharanthine (**10**),[6] cepharanthine-2′*β*-*N*-oxide (**11**),[6] 3′,4′-dihydrostephasubine (**12**),[13] homaromoline (**13**),[14] and fangchinoline (**14**),[15] respectively, using authentic samples and by comparison of their spectroscopic and physical data with literature values.

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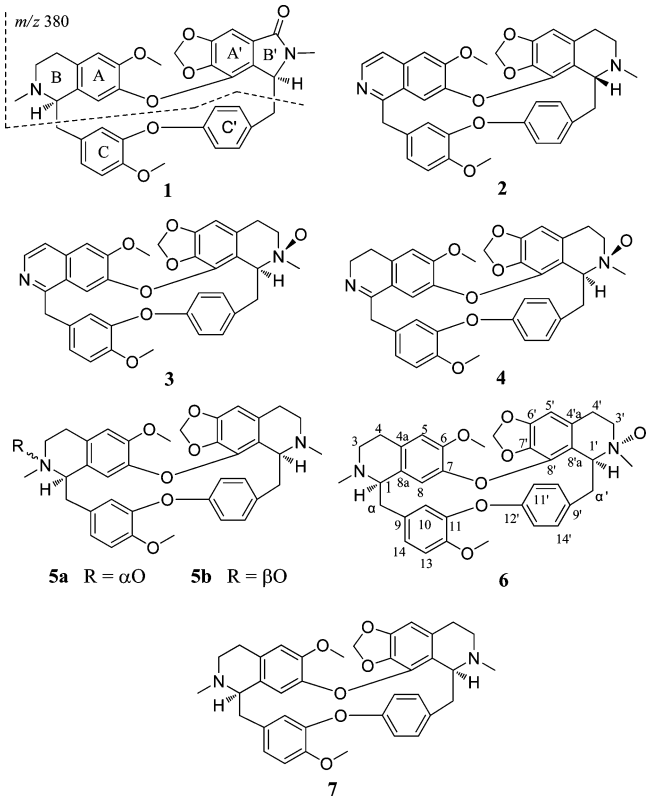

Compound **1** was obtained as a white, amorphous powder. Its molecular formula was established as $C_{36}H_{34}N_2O_7$, on the basis of the positive HREIMS (*m*/*z* 606.2379 $[M]^+$, calcd for $C_{36}H_{34}N_2O_7$, 606.2366), corresponding to 21 degrees of unsaturation. In the ^{13}C NMR and DEPT spectra of **1** (Table 1), 36 carbon signals were observed, assigned as four methyls, of which two are methoxy groups (δ_C 55.5, 56.2), five methylenes, including one bearing a heteroatom (δ_C 51.0) and one bearing two oxygen atoms (δ_C 102.3), two aliphatic methines bearing heteroatoms (δ_C 64.4, 64.5), a carbonyl (δ_C 168.8), and 24 aromatic carbons arising from four benzene rings. The ^1H NMR spectrum (Table 2) showed the presence of one *para*-disubstituted benzene ring [δ_H 7.41, 6.99 (each 1H, dd, *J* = 2.5, 8.3 Hz), 7.10, 6.39 (each 1H, brd, *J* = 8.4 Hz)], a 1,3,4-trisubstituted benzene ring [δ_H 6.76 (d, *J* = 8.2 Hz), 6.80 (dd, *J* = 1.8, 8.2 Hz), 5.64 (brs)], three aromatic singlet protons [δ_H 6.41, 6.67, 6.92 (each 1H, s)] due to two benzene rings, four heteroatom-bearing singlet methyls [δ_H 2.53, 3.24, 3.84, 3.69], and one methylenedioxy group [δ_H 5.69 and 5.74 (each 1H, d, *J* = 1.2 Hz)]. These NMR spectroscopic features of **1** were closely related to those of cepharanthine (**7**). However, instead of six aliphatic methylenes (δ_C 51.2, 45.9, 41.2, 38.6, 28.6, 25.9) in **7**,[6] only four methylene signals at δ_C 51.0, 38.6, 37.7, and 28.2, together with an additional carbonyl carbon at δ_C 168.8, were observed for **1**. All these characteristics suggested that compound **1** is a norcepharanthine analogue.

In the HMBC spectrum of **1** (Figure 1), three aromatic singlet protons were assigned as H-5 (δ_H 6.41), H-8 (δ_H 6.67), and H-5′ (δ_H 6.92) of the A and A′ aromatic rings, respectively, based on the correlations of δ_H 6.41 (s) with C-4/C-8a/C-6/C-7, δ_H 6.67 (s) with C-6/C-4a/C-7, and δ_H 6.92 (s) with C-6′/C-7′/C-8′a, and from the correlations of H-8 with δ_C 64.5 (CH, C-1), H-5 with δ_C 28.2 (CH_2, C-4), and the *N*-CH_3 (δ_H 2.53) group with δ_C 51.0 (CH_2, C-3). These observations allowed the B-ring of **1** to be constructed. In addition, HMBC correlations of the ABX-coupled aromatic proton at δ_H 6.80 (dd, *J* = 1.8, 8.2 Hz, H-14) with C-12 (δ_C 147.1)/C-α (δ_C 37.7, CH_2), H-1 (δ_H 3.63) with C-9, and H-α with C-1 revealed the connectivity of the 1,3,4-trisubstituted benzene C-ring to C-1 of the B-ring through a methylene group (C-α). Cross-peaks of the aromatic proton at δ_H 6.99 (H-11′) with C-9′ (δ_C 134.9), both H-10′ (δ_H 7.41) and H-14′ (δ_H 7.10) with C-12′ (δ_C 153.8), both H-α′ (δ_H 2.55, 3.56) and H-1′ (δ_H 4.75) with C-9′, and H-1′ with C-8′a (δ_C 130.9) confirmed the connection of the *para*-disubstituted benzene C′-ring via another methylene group, C-α′, to the heteroatom-bearing methine (δ_C 64.4, C-1′), which was connected to the C-8′a position of the A′-ring. The above key HMBC correlations were used to construct the cepharanthine skeleton in **1**. Also, HMBC correlations of the downfield shifted methylenedioxy protons (δ_H 5.69 and 5.74) with C-6′ and C-7′ and of the two methoxy groups (δ_H 3.69 and 3.84) with C-6 (δ_C 149.1) and C-12 (147.1), respectively, could be observed. These, together with the ROESY correlations (Figure 1) of the methoxy group signals at δ_H 3.69 with H-5 and of the other methoxy group signal at δ_H 3.84 with H-13, revealed the locations of the methylenedioxy group and two methoxy groups in **1**, which were the same as those in **7**. Furthermore, HMBC correlations of both H-5′ and 2′-*N*-CH_3 (δ_H 3.24) with the carbonyl carbon (δ_C 168.8, C-3′), and 2′-*N*-CH_3 also with C-1′, were used to determine the B′-ring in **1**. This was suppported by the IR band at 1687 cm^{-1}, produced by the characteristic absorption of a secondary amide.

In the EIMS of **1**, a fragment ion peak at *m*/*z* 380 corresponded to the upper half of the molecule, as a result of the cleavage of two benzylic bonds, C-1/C-α and C-1′/C-α′. These data confirmed that compound **1** is a head-to-head bisbenzyl-isoquinoline alkaloid.[16] It was concluded that the diphenyl ether bridge occurs between C-11/C-12′ and C-7/C-8′. The positive optical rotation value of **1** {$[\alpha]^{25}_D$ +172.8 (*c* 1.1, MeOH)} and a circular dichroism (CD) curve (Supporting Information) similar to that of **7** indicated the 1*R*, 1′*S* configurations in **1**, the same as those of cepharanthine (**7**).[15] On the basis of the above evidence, the structure of compound **1** was elucidated as 3′-nor-4′-oxocepharanthine, which is a dimer consisting of benzylisoquinoline and seco-aristolactam units.

Compound **2**, a white, amorphous powder, gave a molecular formula of $C_{36}H_{32}N_2O_6$, as deduced by the positive HREIMS (*m*/*z* 589.2338 $[M + H]^+$, calcd for $C_{36}H_{33}N_2O_6$, 589.2338), implying 22 degrees of unsaturation. The NMR data were closely related to those of cepharanthine (**7**),[6] except for the signals arising from the B- or B′-ring. Instead of two aliphatic methylenes (δ_C 28.9, 51.2), one N-bearing methine (δ_C 64.2), and two *N*-CH_3 (δ_C 42.0, 43.9) groups in **7**,[6] signals for two aromatic methines (δ_C 120.6, 140.6), one downfield shifted aromatic quaternary carbon (δ_C 160.5), and only one *N*-CH_3 (δ_C 41.9) group were observed in **2**, indicating that the B- or B′-ring in **2** is an aromatic ring. On comparison with **7**, the ^1H NMR spectrum of **2** displayed two additional mutually coupled aromatic protons at δ_H 8.18 and 7.53 (each 1H, d, *J* = 5.9 Hz). In the HMBC spectrum, correlations of the signal at δ_H 7.53 with C-5 (δ_C 107.6) and C-8a (δ_C 123.2), of δ_H 8.18 with C-4a (137.7), and of both aromatic protons at δ_H 8.18 and 7.67 (H-8) with the downfield shifted aromatic quaternary carbon at δ_C 160.5 confirmed that the B- or B′-ring in **2** is dehydrogenated to form a pyridine ring. Thus, the aromatic proton signals at δ_H 8.18 and 7.53 were assigned at H-3 and H-4, respectively. Furthermore, the signal at δ_C 160.5 was assigned to C-1 based

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Table 1. ^{13}C NMR Spectroscopic Data for Compounds 1−6 in CD_3OD (δ in ppm)

position	1[a,c]	2[a]	3[b]	4[b]	5[a] a	5[a] b	6[b]
1	64.4, CH	160.5, C	160.6, C	169.3, C	78.2, CH	81.4, CH	64.8, CH
2-*N*-CH_3	44.1, CH_3				58.7, CH_3	60.2, CH_3	43.5, CH_3
3	51.0, CH_2	141.6, CH	141.7, CH	47.0, CH_2	60.3, CH_2	68.0, CH_2	50.7, CH_2
4	28.2, CH_2	120.6, CH	120.8, CH	26.5, CH_2	25.2, CH_2	28.1, CH_2	28.0, CH_2
4a	133.1, C	137.7, C	138.0, C	138.4, C	128.1, C	129.1, C	132.6, C
5	111.8, CH	107.6, CH	107.8, CH	112.5, CH	112.6, CH	112.6, CH	112.9, CH
6	149.1, C	156.5, C	156.1, C	155.6, C	152.0, C	152.9, C	150.6, C
7	141.5, C	146.7, C	146.1, C	143.8, C	144.1, C	145.4, C	143.5, C
8	119.2, CH	119.6, CH	119.9, CH	123.5, CH	120.0, CH	121.4, CH	119.9, CH
8a	128.1, C	123.2, C	120.7, C	121.4, C	124.2, C	127.8, C	127.8, C
α	37.7, CH_2	42.4, CH_2	42.5, CH_2	42.0, CH_2	39.0, CH_2	35.2, CH_2	38.8, CH_2
9	130.9, C	132.8, C	132.7, C	130.6, C	128.3, C	133.9, C	131.4, C
10	117.3, CH	122.5, CH	122.7, CH	124.6, CH	117.1, CH	125.4, CH	117.3, CH
11	148.7, C	150.3, C	150.2, C	150.4, C	150.2, C	149.8, C	149.7, C
12	147.1, C	150.5, C	150.8, C	151.5, C	149.9, C	152.6, C	148.6, C
13	111.4, CH	114.5, CH	114.7, CH	114.7, CH	112.9, CH	115.3, CH	112.8, CH
14	124.4, CH	125.0, CH	131.9, CH	125.3, CH	124.3, CH	126.3, CH	125.3, CH
1′	64.5, CH	61.1, CH	75.5, CH	75.4, CH	62.7, CH	60.4, CH	77.2, CH
2′-*N*-CH_3	28.2, CH_3	41.9, CH_3	56.8, CH_3	56.7, CH_3	42.0, CH_3	42.7, CH_3	58.5, CH_3
3′	168.8, C	45.3, CH_2	59.9, CH_2	60.1, CH_2	45.7, CH_2	45.4, CH_2	58.8, CH_2
4′		24.3, CH_2	27.1, CH_2	27.3, CH_2	25.9, CH_2	24.5, CH_2	25.8, CH_2
5′	97.7, CH	105.0, CH	105.0, CH	104.7, CH	103.9, CH	104.4, CH	103.2, CH
4′a	126.6, C	127.0, C	125.0, C	124.8, C	126.8, C	128.3, C	125.1, C
6′	150.5, C	149.8, C	151.3, C	151.2, C	149.2, C	149.4, C	149.5, C
7′	139.2, C	135.5, C	136.2, C	136.1, C	134.7, C	134.9, C	135.0, C
8′	137.2, C	139.8, C	138.9, C	139.4, C	139.0, C	140.7, C	139.2, C
8′a	130.9, C	122.5, C	120.8, C	121.0, C	123.6, C	121.6, C	121.0, C
α′	38.6, CH_2	42.2, CH_2	38.9, CH_2	38.8, CH_2	41.2, CH_2	41.4, CH_2	42.7, CH_2
9′	134.9, C	136.8, C	135.0, C	135.0, C	139.3, C	134.8, C	136.6, C
10′	129.2, CH	131.5, CH	132.2, CH	132.3, CH	129.7, CH	133.3, CH	133.6, CH
11′	122.5, CH	122.5, CH	122.3, CH	122.5, CH	122.9, CH	120.7, CH	122.6, CH
12′	153.8, C	158.0, C	158.8, C	159.2, C	153.3, C	160.6, C	154.4, C
13′	121.7, CH	122.4, CH	122.7, CH	122.1, CH	121.7, CH	120.3, CH	123.7, CH
14′	132.7, CH	132.1, CH	131.9, CH	131.8, CH	133.6, CH	132.0, CH	128.8, CH
OCH_2O	102.3, CH_2	102.5, CH_2	103.4, CH_2	103.1, CH_2	102.1, CH_2	102.1, CH_2	102.3, CH_2
6-OCH_3	55.5, CH_3	56.6, CH_3	56.8, CH_3	56.2, CH_3	55.6, CH_3	55.5, CH_3	55.5, CH_3
12-OCH_3	56.2, CH_3	56.8, CH_3	56.9, CH_3	56.9, CH_3	56.5, CH_3	57.1, CH_3	56.7, CH_3

[a]Data were recorded at 150 MHz. [b]Data were recorded at 100 MHz. [c]Data were detected in CD_3OD + $CDCl_3$ (1:1).

on the HMBC correlations of H-α (δ_H 4.52 and 4.11) with δ_C 160.5, C-9 (δ_C 132.8), and C-10 (δ_C 122.5). Other HMBC, $^1H-^1H$ COSY, and ROESY correlations (Figure 1) were used to confirm the planar structure of **2**. In the EIMS of **2**, an ion peak at m/z 482 $[M - 106]^+$, together with a corresponding base peak at m/z 481 $[M - 107]^+$ due to the loss of a C′-ring, indicated the characteristics of a bisbenzylisoquinoline with C-7/C-8′ and C-11/C-12′ diphenyl ether bridges.[16] A weak ion peak at m/z 362 (3%) corresponded to the upper half of compound **2**. By comparing with 1,2-dehydro-2-norlimacusine $\{[\alpha]^{25}_D$ −94 (c 0.2, MeOH)$\}$,[17] with the same C-7/C-8′ and C-11/C-12′ diphenyl ether bridge linkages, the negative optical rotation value of **2** $\{[\alpha]^{25}_D$ −13.5 (c 1.1, MeOH)$\}$ was used to confirm the 1′*R* configuration. Therefore, compound **2** was elucidated as (−)-1,3,4-dehydrocepharanthine.

Compound **3** was obtained as a white, amorphous powder. Its molecular formula was established as $C_{36}H_{32}N_2O_7$ according to the positive HREIMS (604.2198 $[M]^+$, calcd for $C_{36}H_{32}N_2O_7$, 604.2210), 16 Da more than that of **2**. The ^1H NMR and ^{13}C NMR spectroscopic data were very similar to those of **2**, except for the significantly downfield chemical shifts of C-1′, 2′-*N*-CH_3, C-3′, and C-4′ with $\Delta\delta$ of 14.4, 14.9, 14.6, and 2.8 ppm, respectively, suggesting that *N*-2′ in **3** is oxygenated. This was confirmed by the EIMS, in which a weak molecular ion peak at m/z 604 $[M]^+$ (25%) and a major fragment ion peak at m/z 588 $[M - 16]^+$ (100%) were observed, accompanied by the base peak at m/z 587, due to the loss of oxygen. A somewhat weak ion peak at m/z 379 corresponded to the upper half of **3**. On comparing with compound **7**, the proton signals of H-1′ (δ_H 4.96) and 2′-*N*-CH_3 (δ_H 3.31) were shifted downfield by 0.35 and 0.70 ppm, respectively, suggesting a *trans* relationship between the *N*-oxygen and H-1′ in **3**.[16] The ROESY correlation of 2′-*N*-CH_3 with H-1′ (Figure 1) also supported the opposite orientation of H-1′ with the *N*-oxygen in **3**. The 1′*S* configuration of **3** was determined by its same positive $[\alpha]^{25}_D$ value (+40.7) to that of (+)-coclobine $\{[\alpha]^{20}_D$ +130 (c 0.5, $CHCl_3$)$\}$[18] and confirmed by the different CD spectrum of **3** with that of (−)-1,3,4-dehydrocepharanthine (**2**) (Supporting Information). There-

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Table 2. ^{1}H NMR Spectroscopic Data for Compounds 1−6 in CD_3OD (δ in ppm)

position	1[a,c]	2[a]	3[b]	4[b]	5a[a]	5b[a]	6[b]
1	3.63 m				4.54 brs	4.25 m	3.86 m
2-*N*-CH_3	2.53 s				3.45 s	2.98 s	2.63 s
3	2.43 brd, 2.77 m	8.18 d (5.9)	8.20 d (6.0)	3.61 m	3.00 m, 3.08 m	3.69 m, 3.75 m	2.56 m, 2.78 m
4	2.45 m	7.53 d (5.9)	7.54 d (6.0)	2.67 m	2.36 m, 3.23 m	3.16 m	2.39 m, 2.57 m
5	6.41 s	7.23 s	7.23 s	6.76 s	6.64 s	6.72 s	6.56 s
8	6.67 s	7.67 s	7.69 s	7.21 s	6.79 s	6.93 s	6.75 s
α	2.87 dd (3.8, 14.7)	4.11 d (12.7)	4.14 d (13.8)		3.23 m	2.70 m	2.95 m
	3.12 brd	4.52 d (12.7)	4.51 d (13.8)		3.46 m	4.14 brd (13.6)	3.18 dd (15.0, 3.5)
10	5.64 brs	7.15 d (1.6)	7.20 brs	7.10 brs	5.28 brs	7.06 brs	5.40 brs
13	6.76 d (8.2)	6.94 d (8.6)	6.88 d (8.6)	6.94 brd (8.5)	6.72 m	7.16 d (8.7)	6.87 m
14	6.80 dd (1.8, 8.2)	7.03 dd (1.6, 8.6)	7.01 dd (1.6, 8.6)	7.03 dd (2.0, 8.5)	6.89 m	7.31 brd (8.7)	6.87 m
1′	4.75 brd (11.0)	4.61 m	4.96 m	4.87 m	4.26 m	4.38 dd (2.8, 11.1)	4.84 brd (6.8)
2′-*N*-CH_3	3.24 s	2.61 s	3.31 s	3.23 s	2.49 s	2.49 s	3.69 s
3′		3.03 m, 3.51 m	3.60 m, 3.93 m	3.52 m, 3.87 m	2.90 m, 3.22 m	2.90 m, 3.21 m	3.47 m
4′		2.89 m, 3.04 m	3.26 m, 3.37 m	3.20 m, 3.33 m	2.73 m, 3.03 m	2.87 m, 2.96 m	2.95 m, 3.46 m
5′	6.92 s	7.23 s	6.56 s	6.53 s	6.39 s	6.40 s	6.47 s
α'	2.55[d] m	2.95 m	2.77 dd (11.4, 12.6)	2.71[d] m	2.80 m	2.84[d] m	3.04 dd (6.8, 15.2)
	3.56 dd (1.2, 14.2)	3.36 m	4.43 brd (12.6)	4.40 brd (12.5)	3.35 brd (12.0)	3.19[d] m	3.60 brd (15.2)
10′	7.41 dd (2.5, 8.3)	7.05 dd (8.3, 1.8)	7.52 dd (8.6, 2.4)	7.51 brd (8.5)	7.39 brd (8.3)	7.28 brd (8.8)	7.10 dd (2.0, 8.5)
11′	6.99 dd (2.5, 8.3)	7.19 dd (8.3, 2.5)	6.60 dd (8.6, 2.4)	6.65 dd (2.5, 8.5)	6.79 m	6.55 brd (8.8)	6.44 dd (2.0, 8.5)
13′	6.39 brd (8.4)	6.63 dd (8.5, 2.5)	7.19 m	7.14 dd (2.5, 8.0)	6.49 dd (2.3, 8.2)	6.97 dd (2.4, 8.3)	6.92 dd (2.0, 8.5)
14′	7.10 brd (8.4)	7.40 dd (8.5, 1.8)	7.03 dd (8.5, 2.4)	6.92 dd (2.5, 8.0)	7.10 brd (8.2)	6.57 brd (8.3)	7.57 dd (2.0, 8.5)
OCH_2O	5.69 d (1.2)	5.49 brs	5.51 brs	5.56 brs	5.55 brs	5.43 brs	5.60 brs
	5.74 d (1.2)	5.66 brs	5.69 brs	5.72 brs	5.56 brs	5.60 brs	5.61 brs
6-OCH_3	3.69 s	3.77 s	3.75 s	3.63 s	3.74 s	3.52 s	3.71 s
12-OCH_3	3.84 s	3.85 s	3.81 s	3.86 s	3.84 s	3.93 s	3.84 s

[a]Data were recorded at 600 MHz. [b]Data were recorded at 500 MHz. [c]Data were detected in CD_3OD + $CDCl_3$ (1:1). [d]Overlapped with singlet 2-*N*-CH_3 signal.

fore, compound **3** was determined to be (+)-1,3,4-dehydrocepharanthine-2′β-*N*-oxide.

The molecular formula of compound **4** was assigned as $C_{36}H_{34}N_2O_7$, according to the HREIMS (m/z 606.2366 $[M]^+$, calcd for $C_{36}H_{32}N_2O_7$, 606.2366), with 21 degrees of unsaturation. The ^{13}C NMR spectrum of **4** was very close to that of 1,3,4-dehydrocepharanthine-2′β-*N*-oxide (**3**), except that the aromatic methines of C-3 and C-4 in **3** were replaced by two aliphatic methylenes at δ_C 47.0 and 26.5 in **4**. Their corresponding mutually coupled proton signals were at δ_H 3.61 and 2.67, respectively. In addition, the chemical shift of C-1 in **4** was shifted downfield to δ_C 169.3 (δ_C 160.6 for **3**). The EIMS of **4** exhibited a fragment ion peak at m/z 588 $[M - 16]^+$ (100%), suggesting the presence of a bisbenzylisoquinoline *N*-oxide functionality. The two aliphatic methylenes at δ_C 47.0 and 26.5 were assigned at C-3 and C-4, respectively, due to the HMBC correlation of H-5 (δ_H 6.76, s) with δ_C 26.5 (C-4), while the downfield shifted carbon signal at δ_C 169.3 was assigned at C-1, based on its HMBC correlations with H-8 (δ_H 7.21) and H-3 (δ_H 3.61). Other ^{1}H−^{1}H COSY and HMBC correlations (Figure 1) were used to confirm the structure of **4**. The *trans* relationship between the 2′-*N*-oxygen and H-1′ was revealed by the ROESY correlation of 2′-*N*-CH_3 with H-1′. The similar positive $[\alpha]^{25}_D$ (+40.7) and CD Cotton effects (Supporting Information) to those of **3** supported the 1′*S* configuration in **4**. Consequently, compound **4** was deduced as (+)-1-dehydrocepharanthine-2′β-*N*-oxide.

Compound **5** was obtained as a white, amorphous powder and gave a molecular formula of $C_{37}H_{38}N_2O_7$, as deduced from the positive HREIMS (m/z 623.2757 $[M + 1]^+$, calcd for $C_{36}H_{33}N_2O_7$, 623.2757), 16 Da more than that of cepharanthine (**7**). The ^{1}H and ^{13}C NMR spectra of **5** displayed two sets of signals with an integral ratio of 6.5:3.5, implying the occurrence of a pair of compounds, **5a** (major) and **5b** (minor). The protons and their corresponding carbons of **5a** and **5b** were fully separated and assigned on the basis of detailed analysis of the 1D- and 2D-NMR spectra. The ^{1}H and ^{13}C NMR features of **5a** and **5b** were closely related to those of cepharanthine (**7**), except for the chemical shifts arising from the B-ring. On comparing to those of **7** (δ_C 64.5, 44.1, and 51.0), C-1, 2-*N*-CH_3, and C-3 of **5a** and **5b** were downfield shifted to δ_C 78.2/81.4, 58.7/60.2, and 60.3/68.0, respectively, suggesting that both **5a** and **5b** are *N*-oxides of **7**. This was supported by the reduction of **5** with zinc power and HCl at room temperature, which yielded only cepharanthine (**7**) as the product. The *N*-oxide positions for **5a** and **5b** were both determined to be at the 2-*N* position from the HMBC correlations of H-8 (δ_H 6.79/6.93) and 2-*N*-CH_3 (δ_H 3.45/2.98) with δ_C 78.2/81.4 (C-1), H-5 (δ_H 6.64/6.72) with δ_C 25.2/28.1 (C-4), H-1 (δ_H 4.54/4.25) with C-α (δ_C 39.0/35.2), and H-α (δ_H 3.23/2.70, 3.46/4.14) with C-9 (δ_C 128.3/133.9) and C-10 (δ_C 117.1/125.4). Other ^{1}H−^{1}H COSY and HMBC correlations (Figure 1) helped confirm the same planar structures of **5a** and **5b**. The only difference between **5a** and **5b** was the oxygen orientation at the 2-*N* position. In the ^{1}H NMR spectrum, the chemical shifts of 2-*N*-CH_3 for **5a** and **5b** were downfield shifted by 0.89 and 0.42 ppm, respectively, compared with that of **7**, suggesting that **5a** is cepharanthine-2α-*N*-oxide and **5b** is cepharanthine-2β-*N*-oxide.[19] This was confirmed by the weak ROESY correlation of H-1 with 2-*N*-CH_3 in **5b**, but no correlation between H-1 and 2-*N*-CH_3 was observed in **5a** (Figure 1). The large positive optical rotation

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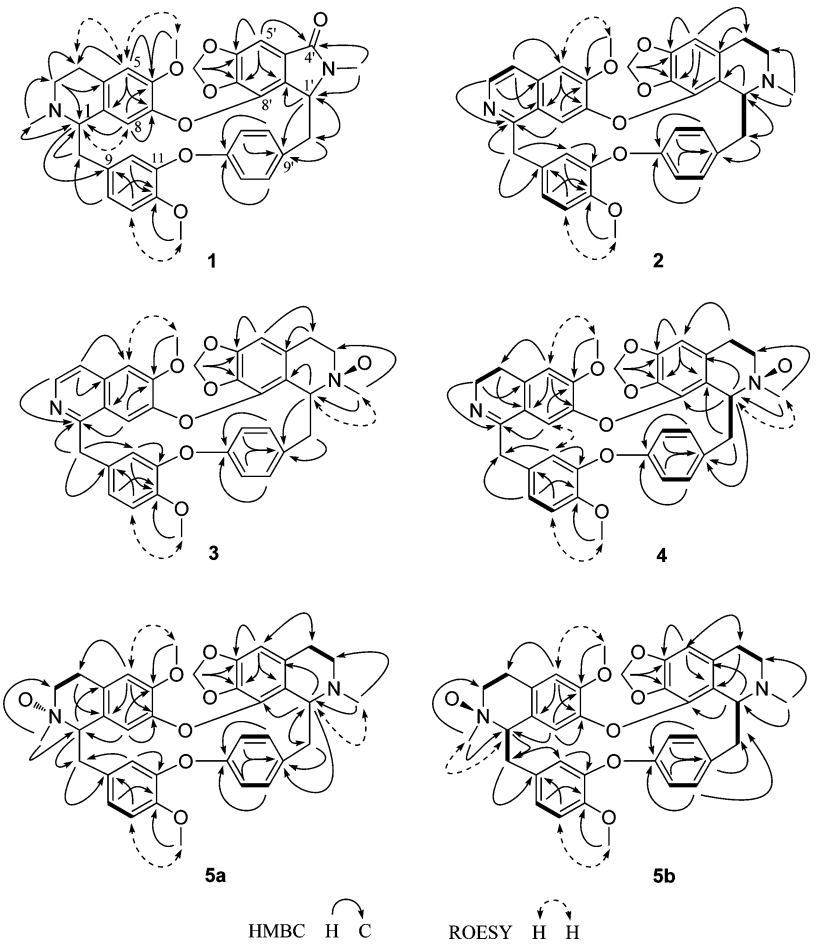

Figure 1. Key HMBC and ROESY correlations of compounds **1**−**5**.

value of $[\alpha]^{25}_{D}$ +204.2 (*c* 1.0, MeOH) together with the chemical reduction of **5** with zinc power yielding only **7** implied the same 1*R*, 1′*S* configurations in both compounds **5a** and **5b** as those in **7**. Therefore, **5** was determined to be a mixture of cepharanthine-2*α*-*N*-oxide (**5a**) and -2*β*-*N*-oxide (**5b**).

Compound **6** was obtained as a white, amorphous powder. Its molecular formula was determined as $C_{37}H_{38}N_2O_7$ due to the positive HREIMS (*m*/*z* 622.2387 $[M]^+$, calcd for $C_{36}H_{32}N_2O_7$, 622.2679), which was also 16 Da more than that of cepharanthine (**7**). Comparison the ^1H and ^{13}C NMR spectroscopic data (Table 2) with those of cepharanthine-2′*β*-*N*-oxide revealed that compound **6** has a similar structure. However, the downfield shifted H-1′ [δ_H 4.84 (**6**), δ_H 4.63 (**11**)] and 2′-*N*-CH_3 [δ_H 3.69 (**6**), δ_H 3.31 (**11**)] signals suggested that compound **6** is a 2′*α*-*N*-oxide of cepharanthine (**7**). The proton signals at δ_H 4.84 and 3.69 were assigned to H-1′ and 2′-*N*-CH_3, respectively, on the basis of their HMBC correlations with C-4′a (δ_C 125.1)/C-8′ (δ_C 139.2)/C-9′ (δ_C 136.6) and C-3′ (δ_C 58.8)/C-1′ (δ_C 77.2). Since no NOE effects between H-1′ and 2′-*N*-CH_3 were observed, this proved indirectly that H-1′ is oriented on the same side of the molecule as the oxygen of *N*-oxide. The large positive optical rotation value of **6** ($[\alpha]^{25}_{D}$ +229.0 (*c* 1.0, MeOH)) and its similar CD spectrum to that of cepharanthine (**7**) implied that compound **6** has the same 1*R*, 1′*S* configurations as **7**. The reduction of **6** with zinc power and HCl at room temperature yielded cepharanthine (**7**). Consequently, compound **6** was determined to be cepharanthine-2′*α*-*N*-oxide.

The isolated compounds **3**−**14** were evaluated for their cytotoxicity against human lung carcinoma (A-549), human esophagus cancer (ECA109), human myeloid leukemia (HL-60), human breast adenocarcinoma (MCF-7), hepatocellular carcinoma (SMMC-7721), and colon cancer (SW480) cell lines. Tanespimycin (17-AAG) and cisplatin were used as positive control substances. Among these, compounds **4**, **7**, **10**, **13**, and **14** showed cytotoxic potency against the above six human cancer cell lines (Table 3), and the other compounds tested were inactive (IC_{50} > 10 μM). It is noted that cepharanthine (**7**) as the major component of *S. epigaea* exhibited inhibitory activity against all cancer cell lines except ECA109. The known analogue (+)-2-norcepharanthine (**10**) also showed cytotoxicity against all six cancer cell lines, with

Table 3. Cytotoxicities of Compounds 4, 7, 10, 13, and 14

	IC_{50} (μM)					
compound	A-549	ECA109	HL-60	MCF-7	SMMC-7721	SW480
4	>10	10.0	>10	>10	>10	>10
7	5.0	>10	9.2	2.9	9.9	4.7
10	3.7	3.2	2.8	2.3	4.8	3.7
13	>10	>10	>10	9.5	>10	>10
14	>10	>10	>10	5.0	>10	>10
cisplatin	7.3	ND[a]	1.2	>10	6.7	>10
tanespimycin	ND	1.1	ND	ND	ND	ND

[a]Not determined.

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IC_{50} values ranging from 2.3 to 4.8 μM. In turn, the new compound (+)-1-dehydrocepharanthine-2′β-*N*-oxide (**4**) displayed selective cytotoxicity for the ECA109 cell line (Table 3). Both **4** and **10** are cepharanthine analogues bearing only one *N*-CH_3 in their respective structures.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were performed on a P-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra was measured on a Bruker Tensor 27 spectrometer with KBr pellets. 1D- and 2D-NMR spectra were run on Bruker AM-400, DRX-500, and AVANCE III-600 NMR spectrometers operating at 400, 500, and 600 MHz for ^{1}H and 100, 125, and 150 MHz for ^{13}C, respectively. Coupling constants are expressed in Hz, and chemical shifts are given on a ppm scale with tetramethylsilane as internal standard. The MS data were recorded on a VG Auto Spec-3000 spectrometer (VG, Manchester, U.K.) with glycerol as the matrix. HREIMS was recorded on an API Qstar Pulsa LC/TOF spectrometer. Silica gel (200−300 mesh, Qingdao Haiyang Group Co., Ltd., Qingdao, People's Republic of China) was used for column chromatography. TLC and preparative TLC were carried out on precoated silica gel GF254 plates, which were visualized by spraying with Dragendorff's reagent or immersing in I_2 vapor.

Plant Material. The tubers of *S. epigaea* was collected in October 2009 in Dali City, Yunnan Province, People's Republic of China, and identified by one of the authors (C.R.Y.). A voucher specimen (KUN 0432112) has been deposited in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried tubers of *S. epigaea* (350 kg) were extracted with 1% hydrochloric acid solution (700 L × 3) at room temperature. The extract was adjusted to pH 10 with 5% NaOH to give a precipitate (72.8 kg). The precipitate was refluxed with ethanol to obtain a total alkaloid portion (1.8 kg), which was subjected to passage over a silica gel column, eluting with $CHCl_3$−CH_3OH (20:1), to afford four major fractions. Fraction 1 (9.0 g) was chromatographed on a silica gel column (EtOAc−CH_3OH, 15:1−7:1), followed by preparative TLC, to afford **1** (3 mg, petroleum ether−acetone−diethylamine (2:1:0.02) and **8** (19 mg, EtOAc−CH_3OH (10:1)). Fraction 2 (1.4 kg) was recrystallized four times in methanol to give **7** (895 g). Fraction 3 (12 g) was applied to repeated column chromatography over silica gel ($CHCl_3$−CH_3OH, 10:1) to give **2** (2 mg, EtOAc−CH_3OH, 5:1−3:1), **9** (506 mg, EtOAc−CH_3OH−NH_3·H_2O, 15:1:0.15), **10** (210 mg, $CHCl_3$−CH_3OH−NH_3·H_2O, 20:1:0.07), **12** (30 mg, EtOAc−petroleum ether−diethylamine, 10:1:0.1), **13** (250 mg, EtOAc−CH_3OH−$NH_3.H_2O$, 30:1:0.3), and **14** (33 mg, EtOAc−CH_3OH−NH_3·H_2O, 30:1:0.3). Fraction 4 (40 g) was chromatographed on a silica gel column (EtOAc−CH_3OH−NH_3·H_2O, 4:1:0.1), followed by preparative TLC, to give **3** (70 mg, $CHCl_3$−CH_3OH−NH_3·H_2O, 10:1:0.1), **4** (17 mg, EtOAc−CH_3OH−diethylamine, 4:1:0.01), a mixture of **5a** and **5b** (700 mg, EtOAc−CH_3OH−$NH_3.H_2O$, 4:1:0.1), **6** (70 mg, EtOAc−CH_3OH−NH_3·H_2O, 4:1:0.02), and **11** (1.0 g, $CHCl_3$−CH_3OH−NH_3·H_2O, 10:1:0.07).

3-Nor-4-oxocepharanthine (1): white, amorphous powder; $[\alpha]^{25}_D$ +172.8 (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.52), 281 (3.76) nm; IR (KBr) ν_{max} 3427, 2924, 1688, 1629, 1511, 1270, 1128, 1067 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 606 $[M]^+$, 432, 380, 225, 195; HREIMS *m*/*z* 606.2379 $[M]^+$ (calcd for $C_{36}H_{34}N_2O_7$, 606.2366).

(−)-1,3,4-Dehydrocepharanthine (2): white, amorphous powder; $[\alpha]^{25}_D$ −13.5 (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.58), 235 (4.49) nm; IR (KBr) ν_{max} 3427, 2926, 1629, 1505, 1274, 1126, 1065 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 588 $[M]^+$, 481, 294; HREIMS *m*/*z* 589.2333 $[M + 1]^+$ (calcd for $C_{36}H_{33}N_2O_6$, 589.2338 $[M + 1]$).

(+)-1,3,4-Dehydrocepharanthine-2′β-*N*-oxide (3): white, amorphous powder; $[\alpha]^{25}_D$ +40.7 (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.76), 240 (4.58), 281 (3.89) nm; IR (KBr) ν_{max} 3424, 2925, 1629, 1509, 1274, 1126, 1068 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 604 $[M]^+$, 588, 574, 481, 379, 295; HREIMS *m*/*z* 604.2198 $[M]^+$ (calcd for $C_{36}H_{32}N_2O_7$, 604.2210).

(+)-1-Dehydrocepharanthine-2′β-*N*-oxide (4): white, amorphous powder; $[\alpha]^{25}_D$ +40.7 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.73), 280 (3.99) nm; IR (KBr) ν_{max} 3431, 2925, 1626, 1509, 1270, 1126, 1069 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 605 $[M - 1]^+$, 590, 547, 483, 295; HREIMS *m*/*z* 606.2380 $[M]^+$ (calcd for $C_{36}H_{32}N_2O_7$, 606.2366).

Cepharanthine-2α-*N*-oxide (5a) and Cepharanthine-2β-*N*-oxide (5b): white, amorphous powder; $[\alpha]^{25}_D$ +204.2 (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.82), 280 (3.83) nm; IR (KBr) ν_{max} 3426, 2930, 1625, 1512, 1272, 1127, 1065 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 622 $[M - 1]^+$, 606, 379; HREIMS *m*/*z* 623.2748 $[M + 1]^+$ (calcd for $C_{36}H_{33}N_2O_7$, 623.2757).

Cepharanthine-2′α-*N*-oxide (6): white, amorphous powder; $[\alpha]^{25}_D$ +229.0 (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.77), 283 (3.84) nm; IR (KBr) ν_{max} 3423, 2926, 1628, 1511, 1272, 1127, 1071 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Tables 1 and 2; EIMS *m*/*z* 622 $[M]^+$, 606, 379, 365, 190, 174, 145; HREIMS *m*/*z* 622.2687 $[M]^+$ (calcd for $C_{36}H_{32}N_2O_7$, 622.2679).

Reduction of Compounds 5 and 6. Compounds **5** (i.e., **5a** and **5b**) (20 mg) and **6** (10 mg) were separately dissolved in 10% HCl (30 mL), and then zinc powder (100 mg) was added. After being stirred at room temperature for 2 h, the reaction mixture was extracted with $CHCl_3$ three times. The organic layer was subjected to preparative TLC ($CHCl_3$−CH_3OH−NH_3·H_2O, 20:1:0.07) to afford cepharanthine (**7**) (4 and 7 mg from **5** and **6**, respectively).

Cytotoxicity Assay. The six cancer cell lines (A-549 lung cancer, ECA109 human esophagus cancer, HL-60 human myeloid leukemia, MCF-7 breast cancer, SMMC-7721 hepatocellular carcinoma, and SW480 colon cancer) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin in a humidified incubator in a 5% CO_2 atmosphere at 37 °C. Cells (5 × 10^3/well) were plated in 96-well plates in 100 μL of medium, in which the test samples were added at various concentrations. After 48 h incubation, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] solution (5 mg/mL in phosphate-buffered saline) was added (20 μL/well), while MTT [[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added instead of MTS for the ECA109 cancer cell line. The incubation was continued for another 4 h to give a formazan product. In each well, 100 μL of 20% SDS was added after 100 μL of medium was removed and then incubated overnight for the formazan product to dissolve completely. The absorbance of the solution was measured at 570 nm using a Bio-Rad 680 instrument. Compound concentrations inhibiting 50% of cell growth (IC_{50} values) were calculated by the Reed and Muench method.[20] Tanespimycin (17-AAG) was used as the positive control for the ECA109 cell line, and cisplatin was used as the positive control for the other cancer cell lines.

ASSOCIATED CONTENT

Supporting Information

Structures of the known compounds **8**−**14** from *S. epigaea* and the ^{1}H and ^{13}C NMR, HSQC, HMBC, ^{1}H−^{1}H COSY, ROESY, EIMS, and CD spectra for compounds **1**−**6** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ma, Y. *J. Northwest For. Coll.* **2004**, *19*, 125−130.
(2) Semwal, D. K.; Badoni, R.; Semwal, R.; Kothiyal, S. K.; Singh, G. J. P.; Rawat, U. *J. Ethnopharmacol.* **2010**, *132*, 369−383.
(3) Vanwyk, A. J. *J. South African Chem. Inst.* **1975**, *28*, 284−286.
(4) Blanchfield, J. T.; Sands, D. P. A.; Kennard, C. H. L.; Byriel, K. A.; Kitching, W. *Phytochemistry* **2003**, *63*, 711−720.
(5) Semwal, D. K.; Rawat, U.; Semwal, R.; Singh, R.; Singh, G. J. P. *J. Asian. Nat. Prod. Res.* **2010**, *12*, 99−105.
(6) Patra, A.; Freyer, A. J.; Guinaudeau, H.; Shamma, M.; Tantisewie, B.; Pharadai, K. *J. Nat. Prod.* **1986**, *49*, 424−427.
(7) Harada, K.; Bando, T.; Yoshida, H.; Sato, M. *Oral Oncol.* **2001**, *37*, 643−651.
(8) Okamoto, M.; Ono, M.; Baba, M. *Biochem. Pharmacol.* **2001**, *62*, 747−753.
(9) Wu, J. M.; Chen, Y.; Chen, J. C.; Lin, T. Y.; Tseng, S. H. *Cancer Lett.* **2010**, *287*, 187−195.
(10) Jin, H.-Z.; Wang, H.-B.; Wang, Y.-B.; Lin, L.-P.; Ding, J.; Qin, G.-W. *Chin. J. Nat. Med.* **2007**, *5*, 112−114.
(11) Kunitomo, J.; Murakami, Y.; Oshikata, M.; Akasu, M.; Kodama, K.; Takeda, N.; Harada, K.; Suzuki, M.; Tatematsu, A. *Chem. Pharm. Bull.* **1985**, *33*, 135−143.
(12) Tomita, M.; Sawada, T.; Kozuka, M.; Takeuchi, M.; Akasu, M. *Yakugaku Zasshi* **1969**, *89*, 1678−1681.
(13) Patra, A.; Mandal, T. K.; Mukhopadhyay, P. K.; Ranu, B. C. *Phytochemistry* **1988**, *27*, 653−655.
(14) Lin, L.-Z.; Shieh, H.-L.; Angerhofer, C. K.; Pezzuto, J. M.; Cordell, G. A.; Xue, L.; Johnson, M. E.; Ruangrungsi, N. *J. Nat. Prod.* **1993**, *56*, 22−29.
(15) Guha, K. P.; Mukherjee, B.; Mukherjee, R. *J. Nat. Prod.* **1979**, *42*, 1−84.
(16) Guinaudeau, H.; Freyer, A. J.; Shamma, M. *Nat. Prod. Rep.* **1986**, *3*, 477−488.
(17) Lavault, M.; Fournet, A.; Guinaudeau, H.; Bruneton, J. *Chem. Pharm. Bull.* **1986**, *34*, 1148−1152.
(18) Berthou, S.; Lebœuf, M.; Cavé, A.; Guinaudeau, H. *J. Nat. Prod.* **1989**, *52*, 95−98.
(19) Wino, T.; Sato, T.; Sasaki, H.; Chin, M.; Mitsuhashi, H. *Heterocycles* **1988**, *27*, 1149−1153.
(20) Reed, L. J.; Muench, H. *Am. J. Hyg.* **1938**, *27*, 493−497.