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Long-Ze Lin, Hui-Ling Shieh, Cindy K. Angerhofer, John M. Pezzuto, Geoffrey A. Cordell, Liang Xue, Michael E. Johnson, and Nijsiri Ruangrungsi

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CYTOTOXIC AND ANTIMALARIAL BISBENZYLISOQUINOLINE ALKALOIDS FROM *CYCLEA BARBATA*[1]

LONG-ZE LIN, HUI-LING SHIEH, CINDY K. ANGERHOFER, JOHN M. PEZZUTO, GEOFFREY A. CORDELL,*

Program for Collaborative Research in the Pharmaceutical Sciences

LIANG XUE, MICHAEL E. JOHNSON,

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., Chicago, Illinois 60612

and NIJSIRI RUANGRUNGSI

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

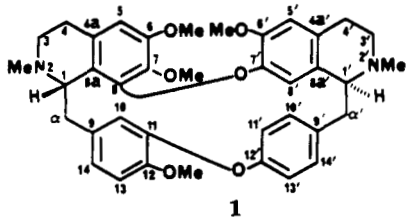
ABSTRACT.—An alkaloid extract derived from the roots of *Cyclea barbata* demonstrated cytotoxic and antimalarial activities, and five bisbenzylisoquinoline alkaloids, (+)-tetrandrine [**1**], (−)-limacine [**2**], (+)-thalrugosine [**3**], (+)-homoaromoline [**4**], and (−)-cycleapeltine [**5**], were isolated as the active principles. The complete and unambiguous assignments of the ^{1}H- and ^{13}C-nmr data of these substances were made by 1D and 2D nmr techniques (COSY, phase-sensitive ROESY, HETCOR, and FLOCK).

As judged by the long standing use of quinine and the discovery of various other agents such as artemisinin, plants are a promising source of antimalarial agents. Based on these precedents, we have investigated *Cyclea barbata* (Wall.) Miers (Menispermaceae), a plant growing in East Asia and used in Thailand for the treatment of fevers associated with malaria. This plant has previously been studied to afford a number of alkaloids (2–9). In the current investigation, an alkaloid extract of this plant showed in vitro cytotoxic and antimalarial activities, and, through bioactivity-guided fractionation, five alkaloids, (+)-tetrandrine [**1**], (−)-limacine [**2**], (+)-thalrugosine [**3**], (+)-homoaromoline [**4**], and (−)-cycleapeltine [**5**], were found to be responsible for these activities. Although these alkaloids are diversely distributed (10,11), their complete and unambiguous ^{1}H- and ^{13}C-nmr analyses have not thus far appeared due to the severe overlap of the ^{1}H and ^{13}C signals from the corresponding positions of the dimeric units. As a result, some of the previously reported ^{13}C assignments of tetrandrine [**1**], limacine [**2**], and homoaromoline [**4**] require definition and revision. High field nmr, and particularly the use of various 2D nmr techniques (COSY, ROESY, HETCOR, and FLOCK), permitted these determinations.

Tetrandrine [**1**] was previously isolated from various plants, including *C. barbata*, and nmr parameters have been partially assigned (10–13). The ^{1}H-nmr spectrum showed coupling signals for the four protons of the C′ ring, and according to convention (12), the most downfield signal (δ 7.30) should be assigned to H-14′. A decoupling study on the ^{1}H-nmr spectrum at 300 MHz showed that H-14′ coupled to H-13′ (δ 7.10) and H-10′ (δ 6.27), H-11′ (δ 6.76) coupled to H-13′ and H-10′, and H-14 (δ 6.86) coupled to H-13 (δ 6.82) and H-10 (δ 6.52). These relationships were confirmed by the COSY spectrum that also showed that H-1 (δ 3.72) coupled to two H-α protons at δ 2.48 and 2.67, and that H-1′ (δ 3.84) coupled to the two H-α′ protons at δ 2.75 and 3.22.

The ROESY spectrum (14,15) showed the presence of nOe correlation contours between H-5 and 6-OMe, H-13 and 12-OMe, H-5′ and 6′-OMe, and H-8′ and H-14′.

[1]Part XIX in the series "Traditional Medicinal Plants of Thailand." For part XVIII, see Lin *et al.* (1).



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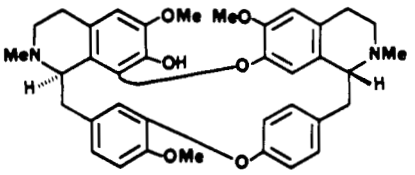

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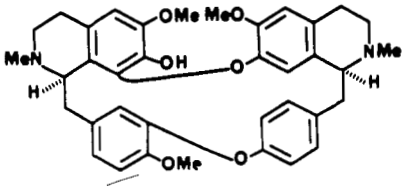

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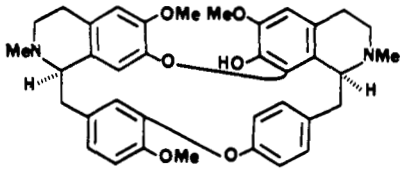

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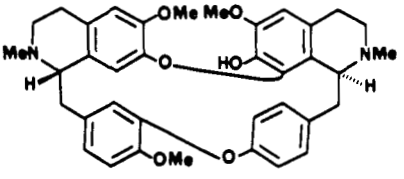

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Thus, H-14′ is spatially closer to H-8′ than H-10′, which is not apparent from the planar diagram (12). The ^{1}H-nmr data for the methyl and methine signals were consistent with the reported data (12), but there were no previous reports on the assignment of the six methylene groups, which are assigned here. The ^{13}C spectrum of **1** originally showed only 22 separated aromatic carbon signals. However, after zero-filling to 32K, two signals near δ 127.7 (C-4a and C-4a′), and two signals near δ 121.6 (C-11′ and C-13′), were disclosed, permitting the emergence of all 24 aromatic carbon signals, consistent with the structure. The HETCOR spectrum (16) displayed each of the protonated carbons, and the FLOCK spectrum (17) (Figure 1) indicated the long-range correlations between ^{1}H and ^{13}C through three bonds and two bonds.

Analysis of the corresponding cross-correlations revealed that the N-Me coupled to C-1 and C-3, the N′-Me coupled to C-1′ and C-3′, the H-1 coupled to C-8a, C-4a, C-α, C-8, and C-9, the H-1′ coupled to C-α′, the H-5 coupled to C-4, C-8a, C-7, C-6, and C-4a, the H-5′ coupled to C-7′, C-6′, C-4′, and C-8a′, the H-8′ coupled to C-6′, C-7′, C-1′, and C-4a′, the H-13 coupled to C-11, C-9, and C-10, the H-13′ coupled to C-12′, C-9′, and C-11′, the 6-OMe coupled to C-6, the 7-OMe coupled to C-7, the 12-OMe coupled to C-12, and the 6′-OMe coupled to C-6′. Therefore, this experiment permitted the complete assignment of the ^{13}C-nmr data. Retrospective analysis of the HETCOR spectrum then disclosed the assignments of the partly overlapped signals of the six methylene groups as the H-3 signals at δ 2.87 and 3.47, the H-3′ signals at δ 2.83 and 3.39, the H-4 signal at δ 2.39 and 2.89, the H-4′ signals at δ 2.69 and 2.91, the H-α signals at δ 2.48 and 2.67, and the H-α′ signals at δ 2.75 and 3.22. The unambiguous ^{1}H- and ^{13}C-nmr data of **1** are shown in Tables 1 and 2, respectively. Comparison with the reported ^{13}C data for **1** (13) indicates that the data for C-8a, C-11, and C-7′ should be revised. In addition, the parameters for the N-Me and N′-Me and for C-

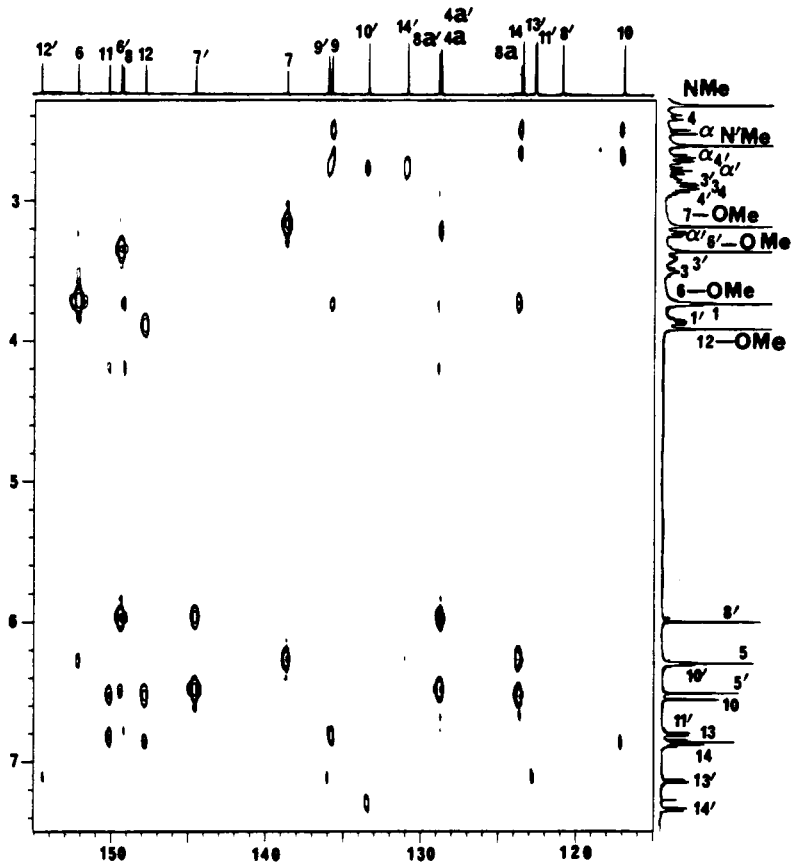

FIGURE 1. The FLOCK spectrum of tetrandrine [**1**].

α and C-α′ now have been clearly assigned, and the assignments for the four OMe signals have been defined.

Limacine [**2**] also was previously isolated from this and other plants, and partial assignment of the ^{1}H- and ^{13}C-nmr data has been reported (10–13). Like tetrandrine [**1**], a corresponding 1D and 2D nmr study led to the complete assignment of the ^{1}H- and ^{13}C-nmr data. Comparison with the reported ^{13}C data (Table 2) indicated that C-11 and C-7′ should be revised. The N-Me, N′-Me, C-3, C-3′, C-α, and C-α′ have now been clearly assigned, and assignments for the three OMe signals have been made.

Thalrugosine [**3**], a stereoisomer of limacine [**2**], was previously isolated from this and other plants, and partial assignments of the ^{1}H-nmr data have been made (10–12). The ^{1}H- and ^{13}C-nmr data have now been completely assigned by the use of COSY, ROESY, HETCOR, and FLOCK techniques. Some of the signals differ from those of **2** (Tables 1 and 2); the nmr data of both compounds were taken at the same concentration. The major difference in the ^{1}H data between **2** and **3** is that the 6′-OMe signal of **3** is 0.5 ppm farther downfield than that of **2**, the H-1 and H-5′ resonances of **3** are about 0.2 ppm farther downfield than those of **2**, and the H-10, H-14, and H-1′ signals of **3** are 0.2–0.3 ppm farther downfield than those of **2**. Furthermore, the C-5, C-8, C-4a′, and C-8a′ signals of **3** are 2–3 ppm farther up- or downfield than those of **2**.

Homoaromoline [**4**] has been isolated previously from about ten plants, but this is the first reported isolation from *C. barbata* (10,11). In our study, it was found that its ^{1}H-nmr spectra showed remarkable difference between dilute and concentrated solu-

TABLE 1. ^{1}H-Nmr Spectral Data of the Bisbenzylisoquinoline Alkaloids **1–5** from *Cyclea barbata*[a].

Proton	Compound				
	1	**2**	**3**	**4**	**5**
H-1	3.72 (d, 9.5)	3.75 (dd, 10, 2.5)	3.98 (dd, 10.9, 2.5)	3.63 (dd, 4, 2.6)	3.45 (d, 7.7)
NMe	2.30 (s)	2.32 (s)	2.28 (s)	2.53 (s)	2.57 (s)
H-3	2.87 (m)	2.85 (m)	2.74 (m)	3.02 (m)	2.66 (m)
H-3	3.47 (m)	3.49 (m)	3.21 (m)	2.73 (m)	2.97 (m)
H-4	2.39 (m)	2.39 (m)	2.32 (dd, 15, 4.7)	2.40 (m)	2.60 (m)
H-4	2.89 (m)	2.90 (m)	2.79 (m)	2.40 (m)	2.78 (m)
H-5	6.26 (s)	6.27 (s)	6.32 (s)	6.33 (s)	6.41 (s)
6-OMe	3.70 (s)	3.70 (s)	3.72 (s)	3.60 (s)	3.34 (s)
7-OMe	3.15 (s)	—	—	—	—
H-8	—	—	—	6.66 (s)	6.44 (s)
H-α	2.48 (dd, 14, 1.8)	2.57 (d, 13.9)	2.61 (dd, 14.8, 10.9)	2.68 (m)	2.50 (d, 13.4, 7.8)
H-α	2.67 (dd, 14, 10)	2.69 (dd, 13.9, 10)	2.90 (dd, 14.8, 2.5)	2.94 (dd, 14.1, 4)	3.05 (d, 13.4)
H-10	6.52 (d, 2.2)	6.57 (d, 2.2)	6.26 (d, 2.2)	5.54 (br s)	6.60 (d, 2.2)
H-11	—	—	—	—	—
12-OMe	3.88 (s)	3.91 (s)	3.88 (s)	3.88 (s)	3.94 (s)
H-13	6.82 (d, 8.2)	6.83 (d, 8.2)	6.76 (d, 8.3)	6.74 (d, 8.4)	6.94 (d, 8.4)
H-14	6.86 (dd, 8.2, 2.2)	6.85 (dd, 8.2, 2.2)	6.60 (dd, 8.3, 2.2)	6.70 (dd, 8.4, 2.3)	6.88 (dd, 8.4, 2.2)
H-1′	3.84 (dd, 11, 5.9)	3.87 (dd, 10.9, 5.6)	3.58 (dd, 11.5, 5.0)	4.13 (d, 6.5)	4.19 (br.d, 9.8)
N′Me	2.58 (s)	2.59 (s)	2.45 (s)	2.43 (s)	2.48 (s)
H-3′	2.83 (m)	2.83 (m)	2.74 (m)	2.55 (m)	2.84 (m)
H-3′	3.39 (m)	3.49 (m)	3.31 (m)	2.86 (m)	3.37 (m)
H-4′	2.69 (m)	2.72 (m)	2.88 (m)	2.73 (m)	2.63 (m)
H-4′	2.91 (m)	2.94 (m)	2.90 (m)	3.08 (m)	2.93 (m)
H-5′	6.48 (s)	6.51 (s)	6.70 (s)	6.20 (s)	6.38 (s)
6′-OMe	3.33 (s)	3.33 (s)	3.86 (s)	3.76 (s)	3.75 (s)
H-8′	5.96 (s)	6.05 (s)	6.04 (s)	—	—
H-α′	2.75 (dd, 11.0, 12.3)	2.75 (dd, 12.5, 10.9)	2.76 (m)	2.61 (m)	2.77 (dd, 12.7, 9.8)
H-α′	3.22 (dd, 12.3, 5.9)	3.22 (dd, 12.5, 5.6)	3.18 (m)	3.22 (d, 13.8)	3.13 (dd, 12.7, 1.6)
H-10′	6.27 (dd, 8.2, 2.3)	6.30 (dd, 8.2, 2.3)	6.41 (dd, 8.3, 2.2)	6.95 (dd, 8.4, 2.2)	6.84 (dd, 8.4, 2.2)
H-11′	6.76 (dd, 8.2, 2.3)	6.79 (dd, 8.2, 2.3)	6.80 (dd, 8.3, 2.2)	6.40 (dd, 8.4, 2.2)	6.84 (dd, 8.4, 2.2)
H-13′	7.10 (dd, 8.2, 2.3)	7.12 (dd, 8.2, 2.3)	7.02 (dd, 8.3, 2.2)	6.91 (dd, 8.4, 2.2)	7.06 (br.d, 2.2)
H-14′	7.30 (dd, 8.2, 2.3)	7.32 (dd, 8.2, 2.3)	7.28 (dd, 8.3, 2.2)	7.33 (dd, 8.4, 2.2)	7.30 (br.d, 2.2)

[a]Recorded in $CDCl_3$. Chemical shift values are reported as δ values (ppm) from TMS at 500 MHz; signal multiplicity and coupling constants (Hz) are shown in parentheses. In order to follow the convention (12), the most downfield signal is assigned for H-14′, one of the four protons of ring C′.

tions in $CDCl_3$; one of the N-Me signals moved upfield by more than 0.1 ppm. In this study, a sample (40 mg) of **4** in about 0.4 ml of $CDCl_3$ was used, and its complete ^{1}H- and ^{13}C-nmr data were assigned by a combination of COSY, ROESY, HETCOR, and FLOCK experiments (Tables 1 and 2). Comparison with the reported ^{1}H-nmr data (12)

TABLE 2. ^{13}C-Nmr Spectral Data of Bisbenzylisoquinoline Alkaloids **1–5** from *Cyclea barbata*.[a]

Carbon	Compound				
	1	**2**	**3**	**4**	**5**
C-1	61.19	61.36	60.09	64.26	65.31
NMe	42.05	42.25	42.13	43.72	42.36
C-3	43.91	44.14	43.72	51.10	46.78
C-4	21.81	21.76	22.34	28.45	26.59
C-4a	127.72	123.21	122.14	130.56	127.92
C-5	105.55	104.80	107.46	111.10	112.38
C-6	151.18	145.75	146.84	148.50	149.12
C-7	137.63	134.57	136.32	143.96	144.15
C-8	148.19	141.86	144.20	116.93	120.73
C-8a	122.64	123.41	124.22	128.02	131.31
C-α	41.70	41.85	39.06	38.32	40.38
C-9	134.68	134.96	133.17	130.95	133.90
C-10	115.99	116.14	114.77	117.00	120.46
C-11	149.12	149.29	150.10	148.70	148.58
C-12	146.83	146.93	146.51	146.64	148.50
C-13	111.33	111.45	111.35	110.71	112.77
C-14	122.56	122.67	121.78	123.65	123.45
C-1′	63.64	63.65	64.92	60.46	60.22
N′Me	42.32	42.52	42.89	41.50	41.54
C-3′	45.00	45.21	45.80	44.96	44.26
C-4′	24.92	25.35	25.39	24.96	22.70
C-4a′	127.72	128.03	130.57	122.99	122.95
C-5′	112.50	112.99	112.18	104.50	105.82
C-6′	148.39	148.71	148.96	147.61	146.42
C-7′	143.57	143.50	143.17	133.39	134.91
C-8′	119.96	120.57	121.15	142.37	143.06
C-8a′	127.84	128.64	130.77	122.91	122.95
C-α′	37.93	37.86	37.93	38.20	43.99
C-9′	134.91	135.10	135.17	138.17	136.46
C-10′	132.42	132.49	131.85	131.49	131.69
C-11′	121.63	121.86	122.79	121.12	120.38
C-12′	153.58	153.67	154.36	152.74	155.42
C-13′	121.63	121.82	122.49	121.90	121.55
C-14′	129.90	130.07	129.93	128.34	129.78
6-OMe	55.55	56.00	55.84	55.21	55.21
7-OMe	59.99	—	—	—	—
12-OMe	55.87	56.04	56.07	55.79	56.22
6′-OMe	55.56	56.19	55.89	55.68	55.82

[a]Recorded in $CDCl_3$. Chemical shift values are reported as δ values (ppm) at 125.8 MHz.

indicated that the assignments for N-Me and N′-Me should be revised. Comparison with the reported ^{13}C-nmr data (11,18) indicated that the assignments of C-9, C-9′, C-3, C-3′, C-11, C-12, C-4a′, C-8a′, 6-OMe, and 6′-OMe also should be revised, and that C-4a, C-9′, C-5, C-13, N-Me, and N′-Me have been definitively assigned.

Cycleapeltine [**5**], a stereoisomer of homoaromoline [**4**], was isolated previously from *Cyclea peltata* (19), and this is the first isolation from *C. barbata* (10,11). A sample (13 mg) of **5** in about 0.4 ml of $CDCl_3$ was used for the nmr studies, and the ^{1}H- and ^{13}C-nmr data of **5** were assigned by the use of the COSY, ROESY, HETCOR, and FLOCK techniques (Tables 1 and 2).

Alkaloids **1–3** belong to a group of bisbenzylisoquinoline alkaloids with two ether linkages between carbons 8–7′ and 11–12′, and their H-8′ signal is farther upfield than

TABLE 3. Biological Activities of the Alkaloid Extract and Compounds **1–5** from *Cyclea barbata*.

Compound	Cytotoxic Activity, ED_{50} (μg/ml)			Antimalarial activity, ED_{50} (ng/ml)	
	P-388	KB-3	KB-V1	Strain D-6	Strain W-2
Alkaloid Extract	1.5	3.6	12	163	224
Tetrandrine [**1**]	0.40	2.1	3.7	179	160
Limacine [**2**]	0.25	9.8	11	52.7	164
Thalrugosine [**3**]	0.36	3.4	11	65.1	78.0
Homoaromoline [**4**] . . .	0.31	3.6	15	232	451
Cycleapeltine [**5**]	0.57	2.2	4.4	29.0	40.6
Quinine	>5	>20	>20	7.6	30.8

that of H-5′. In contrast, in alkaloids **4** and **5**, which have ether linkages between carbons 7–8′ and 11–12′, the H-8 signal is farther downfield than that of H-5. Furthermore, in contrast to the alkaloids **1**, **2**, and **3**, and also in contrast to the normal bisbenzylisoquinolines with two or three ether linkages (with certain exceptions, such as candicusine, 12-*O*-desmethyllaberine, and osornine) (10–12), alkaloids **4** and **5** showed the ^1H resonance of the N′-Me farther upfield than the signal for the corresponding N-Me. A study to re-examine the spectra of other alkaloids with these structural characteristics is currently being undertaken.

As summarized in Table 3, compounds **1**, **2**, and **5** were capable of inhibiting the growth of cultured *Plasmodium falciparum* strains D-6 and W-2. This is consistent with a recent patent in which antimalarial activity was reported for tetrandrine [**1**] and its structural relatives (20). Heretofore, activity has not been reported with bisbenzylisoquinoline alkaloids bearing 7–8′ and 11–12′ ether linkages, such as **4** and **5**.

Also shown in Table 3 is the cytotoxic activity of these compounds. Similar to the *Plasmodium* strains, activity was demonstrated with both drug-sensitive and drug-resistant cultures of KB cells. Cytotoxic effects were also obtained with P-388 cells in culture. These respective activities yield ratios (i.e., ED_{50} in cultured mammalian cells/ ED_{50} in cultured *P. falciparum* strains) in the range of 2–100, whereas agents such as quinine or artemisinin typically yield ratios >1000. As described in greater detail in the following paper (21), a "selectivity index" of >1000 obtained with drug-sensitive KB cells and drug-resistant *P. falciparum* appears to indicate that an agent merits further investigation. While this does not as yet apply to these isolated bisbenzylisoquinoline alkaloids, we are continuing to explore this class of compounds for their potential to afford more highly selective agents which might be subjected to in vivo testing.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Kofler hot-stage apparatus and are uncorrected. The optical rotations were determined on a Perkin-Elmer 241 polarimeter. Uv spectra were taken in MeOH on a Beckman Du-7 spectrophotometer. Ir spectra were recorded in a KBr pellet on a Nicolet MX-1 interferometer. Nmr spectra were recorded with General Electric GEΩ 500 and Varian XL-300 spectrometers, in $CDCl_3$ solution. ^{13}C-Nmr spectra were recorded at 125.8 MHz in 8K data points, processing was carried out by zero filling to 32K data points, and resolution enhancement was applied when necessary. ^1H-Nmr spectra with integrals were recorded at 500 MHz in 8K data points; processing was carried out by zero filling to 16K data points and multiplying with Gauss-Lorentz windows to achieve resolution enhancement as necessary. APT spectra were recorded at 75.4 MHz; HETCOR spectra were obtained at 300/75.4 MHz or 500/125.8 MHz using standard programs from the Varian library or GE library; COSY and ROESY spectra were obtained at 500 MHz using standard programs from the GE library. FLOCK spectra were taken at 500/125.8 MHz on a GEΩ 500 instrument with $^nJ_{CH} = 6.3$ Hz. Eims (70 eV) were recorded with a Varian MAT-112S mass spectrometer, and high resolution mass spectra were recorded with a Finnigan MAT-90 instrument.

PLANT MATERIAL.—The roots of *C. barbata* were collected in 1989 in Thailand and were identified by comparison with materials in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

EXTRACTION AND SEPARATION.—The powdered roots of *C. barbata* (3.75 kg) were extracted with MeOH at room temperature three times, and the MeOH solution was evaporated in vacuo. The residue was treated with 1% HCl, and the acidic solution was basified with NH_4OH to pH 9, then extracted with $CHCl_3$. After evaporation of the $CHCl_3$ solution, the crude alkaloid extract (45 g, yield 1.2%) was dissolved in Me_2CO and gave crystalline tetrandrine [**1**] (12 g). Chromatography of the remaining extract (30 g) on Si gel, eluting with increasingly polar mixtures of $CHCl_3$/MeOH and followed by preparative tlc, afforded alkaloids **1** (6 g, total 18 g, 0.48%), **2** (0.5 g, 0.013%), **3** (0.7 g, 0.019%), **4** (0.4 g, 0.011%), and **5** (15 mg, 0.0004%) which were identified as tetrandrine [**1**], limacine [**2**], thalrugosine [**3**], homoaromoline [**4**], and cycleapeltine [**5**] by comparison of mp, $[\alpha]_D$, uv, ir, ms, and nmr data with reported data (10–12). Compounds **3** and **4** were identified by direct comparison with authentic samples from *Stephania erecta* Craib in this laboratory (21).

ANTIMALARIAL ASSAY PROTOCOL.—Cultures of *Plasmodium falciparum* (chloroquine-sensitive strain D-6 derived from CDC Sierra Leone, and chloroquine-resistant strain W-2 derived from CDC Indochina III) were maintained in human erythrocytes in vitro according to established methods (22). Parasites were inoculated into type A+ human erythrocytes at a hematocrit of 6% in RPMI-1640 culture medium (GIBCO Laboratories, Grand Island, NY) supplemented with 32 mM $NaHCO_3$ (GIBCO), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma Chemical Co., St. Louis, MO), and 10% heat-inactivated human plasma type A+. Parasitemia was maintained below 4% under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 in 25-cm^2 culture flasks at 37°. The antimalarial activities of test compounds were assessed with an in vitro radioisotope-incorporation method (23) as described in the following paper (21). Each test compound was assayed in duplicate over a concentration range of 125,000–3 ng/ml, and quinine was tested over a range of 250–0.3 ng/ml. Concentrations of both test compounds and positive controls that inhibited parasite-specific incorporation of [^{3}H]hypoxanthine by 50% (ED_{50}) were determined by non-linear regression analysis. Zero-drug controls defined 100% incorporation.

EVALUATION OF CYTOTOXIC ACTIVITY.—Cytotoxity was determined utilizing cultured P-388, KB-3, and KB-V1 cells as described in the following paper (21).

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