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BISAMIDES FROM *AGLAIA* SPECIES: STRUCTURE ANALYSIS AND POTENTIAL TO REVERSE DRUG RESISTANCE WITH CULTURED CELLS

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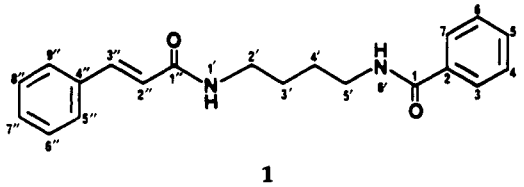
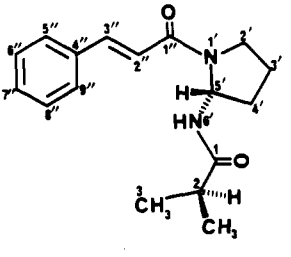
ABSTRACT.—The structure of pyramidatine [**1**], a new bisamide alkaloid from leaves of *Aglaia pyramidata*, was determined through extensive nmr studies, including homonuclear COSY, NOESY, APT, HETCOR, and selective INEPT techniques. Revision of the ^{13}C-nmr assignment of piriferine [**2**], an alkaloid previously isolated from *A. pirifera*, was achieved by examination of several 2D nmr spectra (homonuclear COSY, NOESY, and HETCOR) and confirmed by selective INEPT nmr experiments. Evaluation of the cytotoxic potential of the two alkaloids, along with two other bisamides from *Aglaia odorata*, odorine [**3**] and 5′-*epi*-odorine [**4**], was carried out in eleven human cancer cell lines. None of these bisamides showed significant cytotoxicity. Nevertheless, piriferine [**2**], odorine [**3**], and 5′-*epi*-odorine [**4**] were found to inhibit the growth of the vinblastine-resistant KB cells by enhancing the anticancer activity of vinblastine.

Cinnamic acid-derived bisamides are a unique group of alkaloids found in plants of the genus *Aglaia* (Meliaceae) (1–4). Piriferine [**2**] has been recently isolated from *Aglaia pirifera* Hance (1), whereas odorine [**3**] and 5′-*epi*-odorine [**4**] were identified from *Aglaia odorata* Lour. and *Aglaia roxburghiana* (2,3). A recent report on the antileukemic activity of (−)-odorinol [**5**] in the in vivo P-388 system (4) prompted our interest in the investigation of anticancer potential of other bisamides within this structural type, as a part of our ongoing search for anticancer agents from plants. During our phytochemical examination of *Aglaia pyramidata*, pyramidatine was isolated as a new bisamide of putrescine.



1



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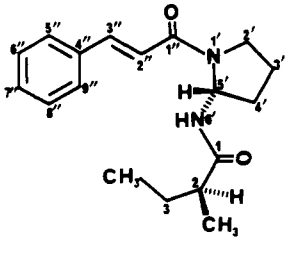
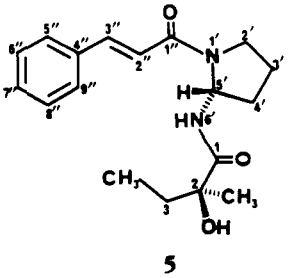


3
4 =5′-epimer



5

Pyramidatine [**1**] showed a molecular ion (hrms) at *m/z* 322.1677, indicating a molecular formula of $C_{20}H_{22}N_2O_2$ (calcd 322.1681). The presence of a cinnamoyl moiety was suggested by the ion at *m/z* 131 in the mass spectrum (1), and the trans stereochemistry was confirmed by a pair of doublets ($J = 15.8$ Hz) at δ 6.63 (H-2″) and 7.42 (H-3″) in the ^{1}H-nmr spectrum (Table 1) (1–4). Ir absorptions at 3317, 1635, and 1621 cm^{-1} revealed the presence of two amide groups, and this was further supported by proton signals at δ 8.13 and 8.48 in the ^{1}H-nmr spectrum and carbon resonances at δ 164.9 and 166.2 in the ^{13}C-nmr spectrum. Detailed analysis of the ^{1}H-nmr, ^{13}C-nmr, and APT spectra indicated the presence of four methylene protons at δ 1.43–1.64, four other downfield methylene protons appearing as two double doublets at δ 3.21 and 3.28, and ten aromatic protons in the region δ 7.35–7.84. The homonuclear COSY spectrum provided evidence for the coupling between the two-proton double doublet at δ 3.21 and the methylene protons at δ 1.43–1.58, and a similar coupling was also observed between the double doublet at δ 3.28 and the methylene multiplet at δ 1.52–1.64. On the basis of the above spectral evidence, it was proposed that pyramidatine has the structure **1**.

TABLE 1. ^{1}H- and ^{13}C-nmr Assignments of Pyramidatine [**1**][a].

Position	^{1}H	^{13}C
1		166.2
2		134.7
3,7	7.84 (dd, 8.3, 1.6)	127.2
4,6	7.44 (dd, 8.3, 8.3)	128.3
5	7.48 (m)	131.0
1′-NH	8.13 (dd, 6.6, 6.6)	
2′	3.21 (dd, 12.5, 6.6)	38.5
3′	1.43–1.58 (m)	26.8
4′	1.50–1.64 (m)	26.8
5′	3.28 (dd, 12.5, 6.6)	38.9
6′-NH	8.48 (dd, 6.6, 6.6)	
1″		164.9
2″	6.63 (d, 15.8)	122.3
3″	7.42 (d, 15.8)	138.5
4″		134.9
5″,9″	7.54 (dd, 8.0, 1.6)	127.5
6″,8″	7.39 (dd, 8.0, 8.0)	128.9
7″	7.35 (m)	129.4

[a]Recorded in DMSO-d_6; chemical shift values are reported as ppm from internal TMS at 300 MHz for ^{1}H and 75.6 MHz for ^{13}C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

In order to confirm the structure, and also to obtain the complete ^{1}H- and ^{13}C-nmr spectral assignments of pyramidatine [**1**], a NOESY experiment was performed. From the NOESY spectrum, the resonance at δ 7.54 showed an nOe with H-2″ and was therefore assigned to H-5″(9″). Protons at C-6″(8″) and C-7″ were assigned to resonances at δ 7.39 and 7.35, based on the homonuclear decoupling spectra. H-3(7), deshielded from the anisotropic effect caused by the carbonyl group (C-1), appeared at δ 7.84. The nOe observed between H-3(7) and 6′-N*H* (which was assigned through the selective INEPT technique; see below) in the NOESY spectrum substantiated this assignment. Protons at C-4(6) and C-5 were assigned by examination of the homonuclear decoupling spectra.

Selective INEPT, APT, and HETCOR experiments (5,6) were carried out to further confirm the structure of pyramidatine [**1**]. These studies also led to the unequivocal assignments for all of the quaternary carbon resonances, as well as a distinction between the two amide protons. Polarization transfer following irradiation of H-3(7) at δ 7.84 resulted in enhancements of the C-1 and C-5 resonances at δ 166.2 and 131.0, respectively. The amide proton at δ 8.48 was assigned to 6′-N*H* because it displayed long-range coupling with C-1 (δ 166.2) on selective INEPT irradiation emphasizing two-bond coupling. Analogous enhancement of C-1″ (δ 164.9) was observed when the 1′-N*H* (δ 8.13) was selectively irradiated. Magnetization transfer via irradiation of H-2″ at δ 6.63 enhanced the C-1″ (δ 164.9) and C-4″ (δ 134.9) resonances. Enhancements of the C-3″ and C-7″ signals were observed through polarization transfer by irradiation of H-5″(9″). Successive selective INEPT irradiation of H-2′ and H-5′ led to the enhancement of C-1′ and C-4′, and of C-1 and C-3′, respectively, as expected.

The assignments of C-2″ and C-3″ merit some discussion. The HETCOR spectrum of pyramidatine [**1**] indicated that C-2″ (δ 122.3) resonated at a higher field than C-3″ (δ 138.5), suggesting that possibly the chemical shifts of C-2″ and C-3″ in the cinnamoyl moiety of the bisamides in previous papers (1–4) had been incorrectly reported and that the assignments for these two carbons should be reversed.

Conclusive evidence of the need to reassign these two carbon resonances came from our nmr studies on piriferine [**2**]. Except for the two magnetically equivalent methyl groups on C-2, all other protons could be readily assigned by examination of the homonuclear COSY and NOESY spectra. As a result, unequivocal assignment of all protonated carbons was easily achieved by the application of the APT and HETCOR pulse sequences. The HETCOR spectrum of piriferine [**2**] clearly showed the C-2 resonance at 118.0 ppm and the C-3 signal at 142.7 ppm (Table 2). These reassignments

TABLE 2. ^{1}H- and ^{13}C-nmr Assignments of Piriferine [**2**].

Position	$^{1}H^{a}$	$^{1}H^{b}$	$^{13}C^{a}$	$^{13}C^{b}$
1			176.2	176.35
2	2.44 (dddddd, 6.9)	2.50 (m)	36.4	21.73
3	1.10 (d, 6.9)	1.10 (d, 8)	19.3[c]	19.40
	1.18 (d, 6.9)	1.18 (d, 8)	19.4[c]	19.51
1′				
2′α	3.42 (m)	3.46 (m)	46.1	46.22
β	3.59 (m)			
3′α	1.89 (m)		21.5	35.54
β	1.92 (m)			
4′α	1.98 (m)		34.4	34.57
β	2.20 (m)			
5′	6.14 (m)	6.13 (m)	62.7	63.01
1″			165.8	165.85
2″	6.92 (d, 15.6)	6.83 (d, 14)	118.0	142.71
3″	7.69 (d, 15.6)	7.76 (d, 14)	142.7	118.49
4″			134.7	135.07
5″(9″)	7.55 (m)		128.2	128.89
6″(8″)	7.37 (m)		128.8	128.24
7″	7.35 (m)		129.9	129.87

[a]Recorded in $CDCl_3$; chemical shift values are reported as ppm from internal TMS at 300 MHz for ^{1}H and 75.6 MHz for ^{13}C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

[b]Data are from Saifah *et al.* (1).

[c]Assignments are exchangeable.

TABLE 3. Evaluation of Cytotoxic Potential of the Bisamides.

Compound	Cell line tested[a] (ED_{50}, μg/ml)										
	BCA-1	HT-1080	LUC-1	MEL-2	COL-1	KB	KB-V1	KB-V1*	A-431	LNCaP	ZR-75-1
Pyramidatine [**1**]	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
Piriferine [**2**]	>20	>20	>20	>20	>20	>20	10	8.5	>20	>20	>20
Odorine [**3**]	>20	>20	>20	>20	>20	>20	>20	6.4	>20	>20	>20
5′-*epi*-Odorine [**4**]	>20	>20	>20	>20	>20	>20	>20	4.2	>20	>20	>20
Vinblastine	—	—	—	—	—	0.002	2.6	—	—	—	—

[a]BCA-1 = human breast cancer; HT-1080 = human fibrosarcoma; LUC-1 = human lung cancer; MEL-2 = human melanoma; COL-1 = human colon cancer; KB = human oral epidermoid carcinoma; KB-V1 = vinblastine-resistant KB; KB-V1* = KB-V1 with 1 μg/ml vinblastine; A-431 = human epidermoid carcinoma; LNCaP = hormone-dependent human prostate cancer; ZR-75-1 = hormone-dependent breast cancer.

were also supported by selective INEPT experiments (5,6). Selective irradiation of H-3″ (δ 7.69) enhanced the C-1″ and C-5″ signals at δ 165.8 and 128.2, respectively. In addition, the enhancements of C-3″ (δ 142.7) and C-9″ (δ 128.2) resonances were observed when H-5″ was irradiated. The assignments of C-2, C-3′, C-5″(9″), and C-6″(8″) were also revised according to the HETCOR spectrum, as shown in Table 2.

Pyramidatine [**1**], piriferine [**2**], odorine [**3**], and 5′-*epi*-odorine [**4**] were evaluated for their cytotoxic potential using a battery of cell lines (Table 3). Pyramidatine [**1**] was found to be totally inactive in all cell lines (ED_{50}>20 μg/ml). The other three bisamides, which contain a pyrrolidine ring in their structure, were also not cytotoxic, with the exception of **2**, which displayed a relatively weak response with KB-V1 cells assayed in the presence or absence of vinblastine (1 μg/ml). On the other hand, neither compound **3** nor **4** was active with cultured KB-V1 cells (ED_{50} >20 μg/ml), but appreciable activity was observed when vinblastine was added to the culture medium at a concentration of 1 μg/ml (Table 3). Since KB-V1 cells are normally cultured in the presence of vinblastine (1 μg/ml) without adversely affecting growth, these data indicate a synergistic response resulting from administering the test compounds with vinblastine. The most likely explanation is that these bisamides reverse resistance to vinblastine. Similar observations have been made with some lignans (7).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Kofler hot plate and are uncorrected. Uv spectra were obtained on a Beckman DU-7 spectrometer, and the ir spectrum on a Nicolet MX-1 FT-IR spectrometer. ^{1}H-nmr, homonuclear COSY, NOESY, ^{13}C-nmr, APT, and HETCOR spectra were recorded in $CDCl_3$, with TMS as internal standard, employing a Varian XL-300 instrument. Standard Varian pulse sequences were used. Selective INEPT experiments were performed at 90.8 MHz using a Nicolet NMC-360 nmr spectrometer. Data sets of 16K covering a spectral width of 10 MHz were acquired. Proton pulse widths were calibrated using a sample of HOAc in 10% C_6D_6 (^{1r}J = 6.7 Hz) in a 5-mm nmr tube. The radio frequency field strength for the soft proton pulse was on the order of 25 Hz for these experiments. For aromatic protons 10 Hz was used as $^3J_{CH}$, and for aliphatic and amide protons, 6 Hz. Low and high resolution mass spectra were obtained with a Varian MAT 90 instrument operating at 70 eV.

PLANT MATERIAL.—The leaves of *A. pyramidata* were collected from Khao Kitchagoot National Park, Chantaburi Province, Thailand in December 1989. Authentication was achieved by comparison with herbarium specimens (BKF 17974) in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. A voucher specimen (ES 11289) is deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

EXTRACTION AND ISOLATION.—The air-dried and powdered leaves (6.2 kg) of *A. pyramidata* were exhaustively extracted with MeOH (10, 6, and 4 liters) at room temperature. The extracts were pooled and the solvent removed in vacuo. The residue (1.3 kg) was mixed with Kieselguhr, packed in a column and eluted with hexane, $CHCl_3$, and MeOH, successively. The $CHCl_3$ extract, after removal of the organic sol-

vent, was suspended in 10% HOAc, filtered, and made alkaline with NH_3. Partitioning the aqueous solution with $CHCl_3$ yielded a fraction which subsequently was chromatographed over Si gel using 4% MeOH in $CHCl_3$ as the eluent. Fractions 15–27 were combined and evaporated under reduced pressure to afford, after recrystallization from Me_2CO, pyramidatine (81 mg, 0.0013%).

Pyramidatine [**1**].—Mp 173–174° (Me_2CO); uv λ max (MeOH) 204 (log ε 4.12), 217 (4.14), 223 (4.13), 272 (4.15), 298 (3.73) nm; ir ν max (KBr) 3317, 3057, 2947, 1635, 1621, 1534, 1490 cm^{-1}; ^1H and ^{13}C nmr see Table 1; ms *m/z* (rel. int.) $[M]^+$ 322 (43), 201 (100), 175 (72), 174 (70), 131 (70); hrms 322.1677 (calcd for $C_{20}H_{22}N_2O_2$, 322.1681).

Piriferine [**2**] was isolated from *A. pirifera* by one of us (E.S.) (1). A sample of odorine [**3**] and 5′-*epi*-odorine [**4**] mixture was kindly provided by Prof. J.D. Connolly, University of Glasgow, Glasgow, Scotland. The separation of these isomers was achieved by repetitive tlc on a Si gel plate, using $CHCl_3$ and MeOH as solvents. Identification of the compounds was carried out by comparison of their ^1H-nmr spectra with those reported earlier (3). A $CDCl_3$ solution of each isomer was left standing at room temperature for 7 days and checked every 24 h for any chemical changes by examining its ^1H-nmr spectra. No isomerization or other chemical changes were observed, in contrast to the results in a previous study (3).

EVALUATION OF CYTOTOXIC POTENTIAL.—Compounds **1–4** were evaluated for their cytotoxic potential with human cell lines specified in Table 3, as described previously (8). Under normal circumstances, KB-V1 cells are cultured in the presence of vinblastine (1 μg/ml) to assure retention of the drug-resistant phenotype. For testing procedures, cells are cultured with test compounds in the presence or absence of vinblastine. As indicated, at the highest concentration tested (20 μg/ml), >50% of the cells survived the treatment, with the exception of KB-V1 cells treated with **2** in the absence of vinblastine, or KB-V1 cells treated with **2**, **3**, or **4** in the presence of vinblastine (1 μg/ml). In these cases, dose-response studies were performed in order to assess ability to reverse resistance to vinblastine, as described previously (9).

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