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Tabernines A–C, β-Carbolines from the Leaves of *Tabernaemontana elegans*

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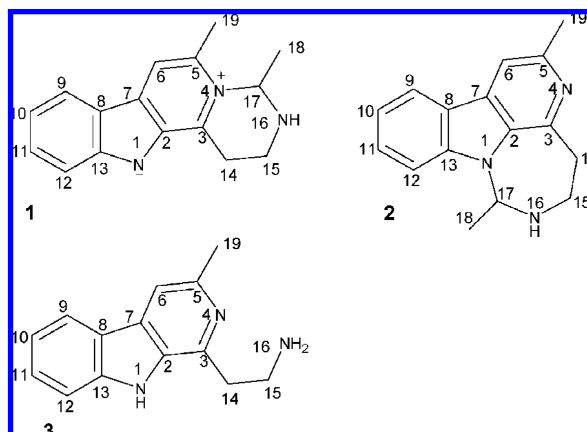
Three novel β-carboline indole alkaloids (**1**–**3**) have been isolated from a MeOH extract of the leaves of *Tabernaemontana elegans*. The structures were established by means of spectroscopic techniques including 2D NMR experiments. Compounds **1** and **2** contain a two-carbon unit, attached to a structurally related β-carboline skeleton, as part of an additional six-membered ring in **1** and a seven-membered ring in **2**. To the best of our knowledge, this is the first report of β-carboline indole alkaloids from the genus *Tabernaemontana.* Compounds **1**–**3** were evaluated for their ability to modulate multidrug resistance in mouse lymphoma cell lines. Compounds **1** and **3** exhibited a weak activity.

Indole alkaloids are pharmacologically active natural products that have been shown to possess a wide range of biological activities, including cytotoxic,[1,2] antiviral,[3] antimicrobial,[3,4] anti-inflammatory,[5] antiserotonin,[3,6] and enzyme inhibitiory.[4,7] One important subclass of indole alkaloids is β-carbolines, which possess a common tricyclic pyrido[3,4-*b*]indole ring structure.[8] The β-carboline skeleton is an important structure in drug discovery,[9] and drugs in market such as tadalafil,[10] possess this indole nucleus. β-Carbolines are derived from tryptophan. Under physiological conditions, tryptophan undergoes modifications via Pictet–Spengler-type reactions. In these reactions, the nucleophilic position 2 of the indole system that is adjacent to the nitrogen attacks the Schiff base generated from tryptamine and an aldehyde (or keto acid), resulting in the formation of tetrahydro-β-carbolines and β-carbolines.[11]

One of the major obstacles in cancer chemotherapy is intrinsic or acquired drug resistance. This broad-spectrum resistance to structurally and mechanistically diverse antitumor agents is known as multidrug resistance (MDR).[12] One of the most effective mechanisms of MDR involves P-glycoprotein (Pgp), a transmembrane protein efflux pump encoded by the human MDR1 gene that is greatly overexpressed in most cancer cells found to be resistant to therapeutic agents.[13]A promising strategy to overcome drug resistance is to find MDR modulators that can inhibit Pgp activity.[14–16] Previous phytochemical studies of *Tabernaemontana elegans* Staph. (Apocynaceae) resulted in the isolation of a variety of indole and bisindole alkaloids.[17–20] These alkaloids possess unusual and intriguing ring systems. Herein we report the isolation and structure elucidation of three unprecedented β-carboline indole alkaloids (**1**–**3**) from *Tabernaemontana elegans*, as well as the evaluation of their ability as MDR modulators.

Results and Discussion

Compound **1**, named tabernine A, was isolated as an optically active yellow oil; $[\alpha]^{22}_{D}$ +50 (*c* 0.1, MeOH). The low-resolution mass spectrum (ESIMS) contained a molecular ion at *m/z* 251, and the high-resolution mass spectrum (HREIMS) exhibited a molecular ion at *m/z* 251.1413, indicating a molecular formula of $C_{16}H_{17}N_3$, with 10 degrees of unsaturation (calculated for $C_{16}H_{17}N_3$, 251.1423). The UV spectrum showed absorptions at 296 and 238 nm. The IR spectrum displayed a band at 3367 cm^{-1}, which, together with the



presence of 11 signals in the aromatic region of the ^{13}C NMR spectrum (six quaternary carbons and five methines), suggested an amino-heteroaromatic ring system. The ^1H NMR spectroscopic data (Table 1), with signals at δ 8.04 (dd, *J* = 8.0, 1.5 Hz, H-9), 7.86 (s, H-6), 7.48 (dd, *J* = 8.0, 1.5 Hz, H-12), 7.37 (td, *J* = 8.0, 1.5 Hz, H-11), and 7.15 (td, *J* = 8.0, 1.5 Hz, H-10), suggested that the heteroaromatic rings were part of a 3,4-disubstituted-β-carboline moiety,[21] satisfying the requirement for 9 of the degrees of unsaturation. The remaining unsaturation was due to the presence of an additional six-membered ring attached at C-3 and N-4. The other signals observed in the ^1H NMR spectrum were assigned as follows: a singlet at δ 2.50 was ascribed to Me-19 on the basis of its $^2J_{H-C}$ and $^3J_{H-C}$ correlations observed in the HMBC spectrum to C-5 and C-6, at δ 140.5 and 116.0, respectively. Moreover, the ^1H NMR exhibited signals at δ 3.12 (m, H-14_a) and 3.18 (m, H-14_b), 3.41 (m, H-15_a) and 3.61 (m, H-15_b), 4.67 (q, *J* = 6.6 Hz, H-17), and 1.79 (d, *J* = 6.6 Hz, Me-18). Apart from the aromatic signals, the combined analysis of the ^{13}C and DEPT spectra (Table 1) showed the presence of five sp^3 carbons in the aliphatic region, one methine, two methylene, and two methyl groups. These features, coupled with the HMQC and COSY data, allowed the establishment of the $-CH_2-CH_2-NH-CH(CH_3)$ fragment A in **1** (Figure 1). The connection of this fragment to the β-carboline moiety of compound **1** was deduced from the long-range correlations observed in the HMBC spectrum between H-14 of the fragment and the sp^2 carbons C-2 (δ_C 128.3) and C-3 (δ_C 143.9) (Table 1). Moreover, the $^3J_{H-C}$ cross-peaks between the methine H-17 (δ_H 4.67) and the aromatic carbon C-5 (δ_C 140.5) indicated that the aliphatic six-membered ring must be linked at N-4.

Tabernine B (**2**) was isolated as an optically active, yellow oil, $[\alpha]^{22}_{D}$ +52 (*c* 0.1, MeOH). The low-resolution ESIMS of **2**

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Table 1. NMR Spectroscopic Data (400 MHz, CD_3OD) of Tabernines A−C (**1**−**3**)

	1		**2**		**3**	
position	δ_H (*J* in Hz)[a]	δ_C,[b] mult[c]	δ_H (*J* in Hz)[a]	δ_C,[b] mult[c]	δ_H (*J* in Hz)[a]	δ_C,[b] mult[c]
2		128.3, C		130.4[d], C		133.9, C
3		143.9, C		145.3, C		144.2, C
5		140.5, C		139.9, C		145.0, C
6	7.86 (s)	116.0, CH	7.76 (s)	115.7, CH	8.61 (s)	123.3, CH
7		122.9, C		122.4, C		123.1, C
8		124.4, C		124.8[d], C		124.6, C
9	8.04 (dd, 8.0, 1.5)	121.0, CH	7.97 (dd, 8.0, 1.5)	120.8, CH	8.15 (dd, 8.0, 1.5)	121.8, CH
10	7.15 (td, 8.0, 1.5)	119.9, CH	7.10 (td, 8.0, 1.5)	119.6, CH	7.28 (td, 8.0, 1.5)	121.5, CH
11	7.37 (td, 8.0, 1.5)	126.8, CH	7.31 (td, 8.0, 1.5)	126.3, CH	7.47 (td, 8.0, 1.5)	128.0, CH
12	7.48 (dd, 8.0, 1.5)	111.8, CH	7.42 (dd, 8.0, 1.5)	111.8, CH	7.55 (dd, 8.0, 1.5)	112.7, CH
13		142.1, C		142.1, C		142.2, C
14a	3.12 (m)	25.4, CH_2	2.97 (m)	27.5, CH_2	3.18 (t, 7.5)	23.9, CH_2
14b	3.18 (m)		2.91 (m)			
15a	3.41 (m)	41.0, CH_2	3.39 (m)	42.3, CH_2	3.81 (t, 7.5)	43.0, CH_2
15b	3.61 (m)		3.13 (m)			
17	4.67 (q, 6.6)	53.5, CH	4.34 (q, 8.0)	53.5, CH		
18	1.79 (d, 6.6)	20.9, CH_3	1.62 (d, 8.0)	22.6, CH_3		
19	2.50 (s)	13.1, CH_3	2.44 (s)	13.1, CH_3	2.5 (s)	12.9, CH_3

[a] Multiplicities and coupling constants are in parentheses (400 MHz). [b] Measured at 100 MHz. [c] Multiplicities were calculated by DEPT (100 MHz). [d] Assignments were made by HMBC experiment.

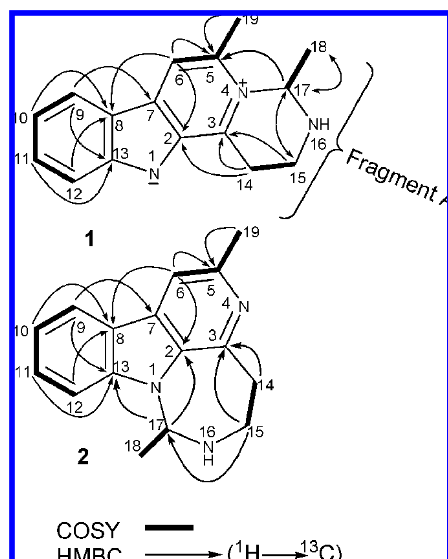

Figure 1. Key COSY and HMBC correlations of compounds **1** and **2**.

exhibited the molecular ion at *m/z* 251. The molecular formula of **2** was determined as $C_{16}H_{17}N_3$ by HREIMS, with the molecular ion at *m/z* 251.1428 (calcd for $C_{16}H_{17}N_3$, 251.1423), indicating the presence of 10 degrees of unsaturation, as in compound **1**. The NMR data of **2** for the β-carboline moiety was similar to that of compound **1**, suggesting that it was a structural isomer of **1**. When comparing the aromatic region of the NMR spectra of **2** to those of the **1**, the main differences were found at carbons C-2 (δ130.4) and C-3 (δ145.3), which showed paramagnetic effects of 2.1 and 1.4 ppm in compound **2**, respectively. However, significant differences were observed in the NMR chemical shifts of the carbons and protons of the fourth ring. In the ^{13}C NMR spectra (Table 1), excepting the C-17-methine, the remaining three aliphatic carbons were shifted downfield in compound **2**, 2.1, 1.3, and 1.7 ppm at C-14, C-15, and C-18, respectively. Significant differences were also found in the corresponding proton chemical shifts. The chemical shifts of H_2-14, H_2-15, H-17, Me-18, and Me-19 of **2** were significantly upfield shifted as compared to those of **1**. The H-14_a and H-14_b signals in **1** appeared at δ 3.12 (m) and 3.18 (m), respectively. In compound **2** these two protons resonated at δ 2.97 (m) and 2.91 (m). The protons H-15_a and H-15_b in **1** appeared at δ 3.41 (m) and 3.61 (m), respectively, while in **2** these displayed upfield shifts at δ 3.39 (m) and 3.13 (m). Similarly, the resonances corresponding to H-17, Me-18, and Me-19 of **1** were shifted upfield in compound **2**, by 0.33, 0.17, and 0.06 ppm, respectively. These differences in the NMR spectroscopic data indicated that the ring attached to the β-carboline moiety of **2** differed from that of **1**. COSY correlations between H-14/H-15 and H-17/H-18 were clear, allowing, together with the HMQC spectrum, the establishment of a spin system by these protons. The HMBC correlations were crucial to define the ring system in **2** (Figure 1). Key $^3J_{H-C}$ correlations between H-17 and the aromatic carbons C-2 and C-13 indicated that C-17 was attached to N-1 instead of N-4 as in the case of **1**.

Compound **3**, named tabernine C, was isolated as a yellow oil. Its low-resolution SIMS showed a pseudomolecular ion peak $[M + Na]^+$ at *m/z* 248. The molecular formula was determined as $C_{14}H_{15}N_3$ on the basis of a pseudomolecular ion at *m/z* 248.1151 $[M + Na]^+$ in the HRSIMS (calculated for $C_{14}H_{15}N_3Na$, 248.1164), representing 9 degrees of unsaturation. The ^1H and ^{13}C NMR spectra of **3** (Table 1) provided evidence for the presence of a β-carboline moiety similar to that of **1** and **2**, but the aliphatic region was rather simple. In the ^1H NMR of **3**, two methylene groups appeared as triplets at δ 3.18 (t, $J = 7.5$ Hz, 2H, H-14) and 3.81 (t, $J = 7.5$ Hz, 2H, H-15) coupled to each other, as confirmed by the COSY spectrum. HMBC correlations for H_2-14/C-3, H_2-14/C-2, and H_2-15/C-3 unambiguously indicated that this two-carbon nitrogen-containing side chain was attached at C-3. The H-17 methine and its *vicinal* Me-18, present in both **1** and **2**, were missing in compound **3**. A key HMBC correlation was also observed from Me-19 to C-5, placing this methyl at C-5 as in compounds **1** and **2**. A structurally similar β-carboline alkaloid, plakortamine A, was isolated from the marine sponge *Plakortis nigra*, containing a bromine atom at C-11 and a dimethyl group at N-16.[21]

The new feature shared by β-carboline indole alkaloids **1**−**3** is the presence of a methyl group at C-5. Furthermore, compounds **1** and **2** contained an additional two-carbon unit (C-17 and C-18) at N-16, which is connected to N-4 in compund **1** and N-1 in compound **2**, to form additional six- and seven-membered rings, respectively. Therefore, the β-carbolines **1** and **2** can be considered as compounds with new skeletal features. To the best of our knowledge, this is the first report on the isolation of β-carboline indole alkaloids from the genus *Tabernaemontana.*

Compounds **1**−**3** were evaluated for their P-gp modulating properties on human MDR1 gene-transfected and parental L5178 mouse lymphoma cell lines, by flow cytometry, using the rhodamine-123 exclusion test. Verapamil, a well-known MDR modifier, was used as a positive control. The results are summarized in Table 2.

Table 2. Effects of **1**−**3** on Reversal of Multidrug Resistance (MDR) on Human *MDR1* Gene-Transfected Mouse Lymphoma Cells

compound	conc. (μM)	FSC[a]	SSC[b]	FL-1[c]	FAR[d]
PAR[e]		443	185	972	
PAR		443	175	891	
MDR[f]		452	251	10.7	
verapamil	22.2	439	251	98.9	9.25
1	20	454	239	18.5	1.73
	2	450	244	7.7	0.72
2	20	458	243	10.7	0.99
	2	454	243	8.9	0.78
3	20	446	242	15.3	1.43
	2	460	232	8.4	0.79
DMSO		466	242	10.3	0.97

[a] FSC: forward scatter count of cells in the samples.[27] [b] SSC: side scatter count of cells in the samples.[27] [c] FL-1: mean fluorescence intensity of the cells (see Experimental Section). [d] FAR: fluorescence activity ratio; values were calculated by using the equation given in the Experimental Section. [e] PAR control: a parental cell without MDR gene. [f] MDR: a parental cell line transfected with human MDR1 gene.

Table 3. Antiproliferative Effects of Compounds **1**−**3** on Parental (PAR) and Multidrug Resistance (MDR) Mouse Lymphoma Cells (L5178)

compound	PAR-L5178 ID_{50} (μM)	MDR-L5178 ID_{50} (μM)
1	45.9 ± 6.4	37.5 ± 2.1
2	46.6 ± 9.2	39.7 ± 0.7
3	70.6 ± 2.1	51.5 ± 0.7

Their antiproliferative effects on these cell lines are presented in Table 3. According to several authors, a highly effective Pgp modulator candidate is supposed to be a lipophilic molecule, possessing at least two aromatic rings, to be an H-bond acceptor, and to contain at least one basic nitrogen atom.[13,22] Although tabernines A−C showed high calculated log *P* values (log $P \geq 3.86$)[23] and fulfilled the remaining above-mentioned criteria required to be effective Pgp modulators, they were inactive at the lowest concentration. Compounds **1** and **3** displayed weak MDR reversal activity when tested at the highest concentration (FAR = 1.73 and 1.43, at 20 μM, respectively). According to several studies, steric properties are also an important factor for Pgp modulation. Small molecules are not Pgp modulators and the range of appropriate molecular weights varies between 250 and 2000.[22] Therefore, the low molecular weight of the compounds **1**−**3** (228 for **3** and 251 for compounds **1** and **2**) may contribute to their lack of activity.

Experimental Section

General Experimental Procedures. Optical rotations were obtained using a Perkin-Elmer 241 polarimeter. UV spectra were taken on a Shimadzu UV1240 spectrometer. IR spectra were determined on a FTIR Nicolet Impact 400, and NMR spectra recorded on a Bruker ARX-400 NMR spectrometer (^{1}H 400 MHz; ^{13}C 100.61 MHz), using CD_3OD as solvent. ESIMS was taken on a Micromass Quattro micro API spectrometer. SIMS, HRSIMS, and HREIMS were recorded on a Micromass Autospec Finnigan Mat 711 spectrometer. Column chromatography (CC) was carried out on SiO_2 (Merck 9385). TLC was performed on precoated SiO_2 F254 plates (Merck 5554 and 5744) and visualized under UV light and by spraying with Dragendorff's reagent or sulfuric acid−MeOH (1:1) followed by heating.

Plant Material. The leaves of *Tabernaemontana elegans* were collected in Maputo, Mozambique, in March 2006, and identified by one of the authors (S.M.) and Dr. Teresa Vasconcelos of Instituto Superior de Agronomia, Universidade de Lisboa. A voucher specimen (No. 23 SM) has been deposited at the herbarium (LMA) of Instituto de Investigação Agronomica of Mozambique.

Extraction and Isolation. The air-dried powdered leaves (2.1 kg) of *T. elegans* were extracted with MeOH (5 × 8 L) at room temperature. The MeOH extract (335.6 g) was dissolved in Et_2O (3 L) and extracted with 10% CH_3COOH (4 × 2 L). The pH of the acid layer was adjusted to 9 by the addition of NH_4OH. The basic layer was successively extracted with CH_2Cl_2 (5 × 3 L) and EtOAc (4 × 2.3 L). The CH_2Cl_2- and EtOAc-soluble fractions, weighing 4.8 and 2.1 g, respectively, were dried over anhydrous Na_2SO_4. Because of the similar pattern in TLC, these were combined to yield a 6.9 g extract. This combined extract was subjected to silica gel CC using mixtures of *n*-hexane−$CHCl_3$ and $CHCl_3$−MeOH of increasing polarity, to yield several crude fractions. The residue (286 mg) of a crude fraction eluted with $CHCl_3$−MeOH (98:1) was chromatographed over SiO_2, using CH_2Cl_2−MeOH mixtures, to yield four subfractions. Subfraction 2 (27.6 mg) was separated further by preparative TLC ($CHCl_3$−MeOH, 17:3) to give compound **3** (10.0 mg). The residue (348 g) of another crude fraction eluted with $CHCl_3$−MeOH (95:1) was successively rechromatographed by CC, using gradients of $CHCl_3$−MeOH, and by preparative TLC ($CHCl_3$−MeOH, 7:3), giving compounds **1** (7.0 mg) and **2** (5.0 mg).

Tabernine A (1): yellow oil; $[\alpha]^{22}_D$ +50 (*c* 0.1, MeOH); UV (MeOH) 214 (ε 32 000), 238 (ε 2000), 296 (ε 11 700) nm; IR (KBr) 3367, 2914, 1572, 1422, 1252, 1047, 746 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 251 $[M]^+$; HREIMS *m/z* 251.1413 (calcd for $C_{16}H_{17}N_3$ 251.1423).

Tabernine B (2): yellow oil; $[\alpha]^{22}_D$ +52 (*c* 0.1, MeOH); UV (MeOH) 238 (ε 2000), 298 (ε 11 700); IR (KBr) 3408, 2932, 1568, 1408, 1246, 1061, 743 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 251 $[M]^+$; HREIMS *m/z* 251.1428 (calcd for $C_{16}H_{17}N_3$ 251.1423).

Tabernine C (3): yellow oil; UV (MeOH) 238 (ε 2000), 296 (ε 11 700) nm; ^{1}H and ^{13}C NMR, see Table 1; SIMS 248 $[M + Na]^+$ (100), 233 (37), 204 (15); HRSIMS *m/z* 248.1151 (calcd for $C_{14}H_{15}N_3Na$ 248.1164).

Cell Cultures. The L5178 Y mouse T-lymphoma parental cell line was transfected with the pHa MDR1/A retrovirus as previously described.[24] The L5178 MDR cell line and the L5178 Y parental cell line (obtained from Prof. M. Gottesmann, NCI and FDA, USA) were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine, and antibiotics. MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. Cell viability was determined by trypan blue.

Assay for Reversal of MDR in Tumor Cells. The harvested cells were resuspended in serum-free McCoy's 5A medium and distributed into Eppendorf tubes at a density of 2×10^6 cell/mL. Then, 2 to 20 μL of the stock solution (1 mg/mL in DMSO) of the tested compounds was added, and the samples were incubated for 10 min at room temperature. Following the addition of 10 μL of rhodamine 123 to the samples (5.5 μM final concentration), the cells were further incubated for 20 min at 37 °C, washed twice, and resuspended in 0.5 mL of phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cells was measured by flow cytometry using a Beckton Dickinson FACScan instrument equipped with an argon laser. The fluorescence excitation and emission wavelengths were 488 and 520 nm, respectively. Verapamil was used as a positive control, and the influence of DMSO on the cells was monitored. The mean fluorescence intensity was calculated as a percentage of the control for the parental (PAR) and MDR cell lines as compared to untreated cells. An activity ratio (FAR) was calculated on the basis of the measured fluorescence values (FL-1) measured via the following equation.[25,26]

$$FAR = (FL\text{-}1_{MDR\ treated}/FL\text{-}1_{MDR\ control})/(FL\text{-}1_{parental\ treated}/FL\text{-}1_{parental\ control})$$

Assay for Antiproliferative Effect. The effects of increasing concentrations of the compounds alone on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μL of medium. Then, 1×10^4 cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h. At the end of the incubation period, 20 μL of MTT (thiazolyl blue, Sigma, St Louis, MO) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO) solution (10%) was measured into each well, and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula

$$100 - \left[\frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}}\right] \times 100$$

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Note Added after ASAP Publication: The footnotes were changed in Table 1 in the version posted on June 3, 2009.

Supporting Information Available: NMR spectroscopic data of new compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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