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Sebastianines A and B, Novel Biologically Active Pyridoacridine Alkaloids from the Brazilian Ascidian *Cystodytes dellechiajei*

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Abstract: Fractionation of the crude methanol extract of the ascidian *Cystodytes dellechiajei* collected in Brazil yielded two novel alkaloids, sebastianine A (**1**) and sebastianine B (**2**). The structures of both **1** and **2** were established by analysis of spectroscopic data, indicating an unprecedented ring system for both compounds, comprising a pyridoacridine system fused with a pyrrole unit in sebastianine A (**1**) and a pyridoacridine system fused with a pyrrolidine system condensed with α-hydroxyisovaleric acid in sebastianine B (**2**). Both alkaloids displayed a cytotoxic profile against a panel of HCT-116 colon carcinoma cells indicative of a p53 dependent mechanism.

Pyrido[4,3,2-*mn*]acridine alkaloids represent a group of natural products restricted to a selected group of marine invertebrates.[1,2] Amphimedine, the first pyridoacridine analogue from a marine organism, was isolated from an *Amphimedon* sp. sponge.[3] Subsequently, several other structurally related alkaloids were isolated from several species of ascidians and marine sponges.[1,2] Recent examples of naturally occurring pyridoacridine alkaloids include the mildly cytotoxic styelsamines isolated from the ascidian *Eusynstyela latericius*,[4] the topoisomerase inhibitor neoamphimedine isolated from *Xestospongia* sp.,[5] cycloshermilamine D from the ascidian *Cystodytes violatinctus*,[6] and deoxyamphimedine isolated from two different marine sponge specimens also belonging to the genus *Xestospongia*.[7] Although a microbial origin has been suggested for such alkaloids, because of their occurrence in phylogenetically unrelated taxa,[2] the biosynthesis of shermilamine was shown to be carried out in ascidian tissues.[8] Because of their relevant biological activities, not only as potentially useful anticancer leads[1,2,9] but also because of their ecological roles,[10] pyridoacridine alkaloids have been the target of a number of new synthetic approaches.[1,2,9,11]

During the 1997 Brazilian spring, we collected several species of marine invertebrates around São Sebastião Island. Among the ascidians collected, the crude methanol extract of *Cystodytes dellechiajei* displayed significant cytotoxic activity against T47D mammalian cancer cells and was selected for further investigation. As in the case of previous studies with this ascidian species,[12,13] the two alkaloids isolated in the present work, sebastianines A (**1**) and B (**2**), were shown to belong to the pyridoacridine family. Herein, we report their structure elucidation and biological activities.

Sebastianine A (**1**) was isolated as a yellow amorphous solid. The EIMS of **1** displayed a molecular ion at *m*/*z* 271, on which a high-resolution mass measurement indicated the formula $C_{17}H_9N_3O$, comprising 15 degrees of unsaturation. The UV spectrum showed maxima at 223, 238, 265, 301, and 390 nm indicating a polyheteroaromatic system. Analysis of ^1H, ^{13}C, and HSQC NMR spectra showed that compound **1** contained eight methines and nine quaternary sp^2 carbons (see Table 1), as well as one NH proton at δ 12.85 (bs). Analysis of the $^1H-^1H$ COSY spectrum and $^3J_{H-H}$ coupling constants indicated the presence of three spin systems in **1**: (a) a

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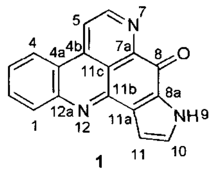

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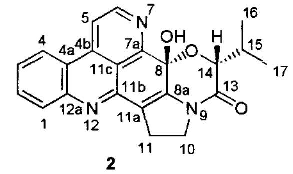

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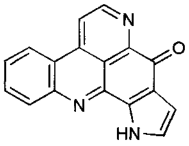

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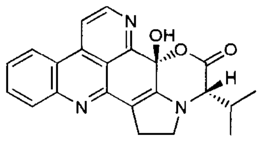

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four proton spin system was assigned to a *N*,*C*-1,2-disubstituted benzene ring; (b) a second spin system comprised two *ortho*-coupled protons in a pyridine ring with characteristic coupling constants and chemical shifts at δ 9.19 (d, 5.5 Hz) and 8.96 (d, 5.5 Hz); (c) the remaining spin system included the NH at δ 12.85 (bs) coupled with a proton at δ 7.48 (d, 2.6 Hz), which was further coupled with a proton at δ 7.01 (d, 2.6 Hz).

The presence of a carbonyl group in **1** was established by analysis of the IR (1683 cm^{-1}) and ^{13}C NMR (δ 171.9) spectra. The chemical shifts of C-1 to C-8a and C-11a to C-12a (Table 1), as well as the number of unsaturations, led us to suspect that sebastianine A (**1**) presented the typical 8*H*-pyrido[4,3,2-*mn*]acridone skeleton as is present in the cystodytins.[12] This hypothesis was confirmed by extensive analysis of the HMBC, ACCORD-HMBC, and HSQC-TOCSY spectra (Table 1). Additional support for the structure of the pyridoacridine skeleton was obtained by NOE difference experiments that showed a dipolar coupling between H-4 (δ 8.88) and H-5 (δ 8.96) (Figure 1). Subtracting the formula of the pyridoacridine system of cystodytins ($C_{15}H_6N_2O$) from the molecular formula of sebastianine A (**1**) left a C_2H_3N fragment, which was assigned to a pyrrole ring fused with carbons C-8a and C-11a. The presence of the pyrrole moiety was confirmed by the small vicinal coupling (J = 2.5 Hz) between protons H-10 and H-11[14] and was further supported by NOE difference experiments that showed dipolar couplings between the NH (δ 12.85) and H-10 (δ 7.48), and between H-10 and H-11 (δ 7.01). Long-range correlations observed in the HMBC and ACCORD-HMBC spectra from C-11a (δ 130.9) to H-11, as well as from both C-11a and C-8a (δ 129.5) to H-10 supported the connection between the pyrrole nitrogen and C-8a and enabled us to propose structural isomer **1** rather than **3** for sebastianine A. Sebastianine A (**1**) is the first pyridoacridine to possess a 9*H*-7,9,12-triaza-benzo[*de*]cyclopenta[*a*]anthracen-8-one skeleton.

TABLE 1. NMR Data (400 MHz, DMSO-d_6) for Sebastianine A (1)

position	δ ^{13}C^a	δ ^{1}H (mult, J in Hz)	^{1}H−^{1}H COSY	HMBC^b and ACCORD-HMBC	HSQC-TOCSY
1	129.8	8.18 (d, 7.5)	H-2	H-3	H-4
2	131.6	7.94 (t, 7.1)	H-1, H-3	H-4	H-3, H-4
3	128.0	7.81 (t, 7.1)	H-2, H-4	H-1	H-2
4	124.0	8.88 (d, 7.6)	H-3	H-2	H-1
4a	121.3			H-1, H-3, H-5	
4b	137.0			H-4, H-6	
5	119.7	8.96 (d, 5.5)	H-6	H-6	H-6
6	149.1	9.19 (d, 5.5)	H-5	H-5	H-5
7a	148.5			H-6	
8	171.9				
8a	129.5			H-11	
NH-9		12.85 (s)	H-10, H-11		
10	128.9	7.48 (d, 2.6)	H-9, H-11	H-11	H-11
11	106.3	7.01 (d, 2.6)	H-9, H-10	H-10	
11a	130.9			H-10, H-11	
11b	148.5				
11c	117.1			H-5	
12a	145.2			H-2, H-4	

[a] Assignments by HSQC. [b] HMBC spectra were obtained with long-range coupling constants J = 3, 8, and 12 Hz.

In an attempt to unambiguously determine the orientation of the pyrrole ring, we tried to methylate the N-12 indole nitrogen. This methylation would hypothetically provide a NOE correlation from one of the pyrrole ring protons to the methyl and would distinguish between **1** and **3**. However, the reaction resulted in the methylation of the pyrrole nitrogen (N-9). The assignment was supported by ROESY data which showed a correlation between the methyl and H-10. The downfield shift of the methyl group (δ 4.11) indicates that it must be in the anisotropy deshielding cone of the carbonyl group, providing good support for structure **1** instead of **3**.

Additional support came from ^{13}C NMR prediction using CS Windows chemNMR. The observed chemical shifts were more consistent with structure **1** versus **3** (data is provided in the Supporting Information). The relatively high-field chemical shift of the carbonyl suggests a β-nitrogen substituent, which through resonance gives the carbonyl more single bond character and hence an upfield shift. The chemical shifts of C-8a and C-11a are also more consistent with the predicted chemical shifts for **1** rather than **3**. The combination of predicted shifts and the chemical shift of the methyl on the pyrrole provide strong evidence for the proposed structure **1**.

Sebastianine B (**2**) was isolated as a pale yellow amorphous solid. The EIMS of **2** displayed a molecular ion peak at *m/z* 373, and the formula $C_{22}H_{19}N_3O_3$ was deduced from HREIMS. Analysis of the ^{13}C NMR and HSQC spectra of **2** indicated the presence of 15 sp^2 hybridized carbons: six methines, eight quaternary carbons, and one carbonyl group (δ 165.7). Additionally, seven sp^3 hybridized carbons were observed: one quaternary (δ 86.6), two methylenes (δ 25.7 and 45.4), two methines (δ 29.1 and 76.6), and two methyls (δ 16.4 and 19.2). Analysis of the ^{1}H NMR, HSQC, and ^{1}H−^{1}H COSY spectra enabled us to construct four spin systems: (a) a four proton spin system very similar to that of **1**, assigned to a *N*,*C*-1,2-disubstituted benzene ring; (b) a second spin system with two *ortho*-coupled protons in a pyridine ring, at δ 9.03 (d, 5.6 Hz) and 8.74 (d, 5.6 Hz); (c) two adjacent methylenes, at δ_C 25.7 (δ_H 3.14 and 3.24) and δ_C 45.4 (δ_H 4.04 and 4.20); (d) an isopropyl group comprising a deshielded methine at δ_C 76.6 (δ_H 4.72), an adjacent methine at δ_C 29.1 (δ_H 2.50), and two nonequivalent methyl groups at δ_C 19.2 (δ_H 1.12) and δ_C 16.4 (δ_H 0.82) (see Table 2).

Analysis of the long-range correlations observed in the HMBC spectra (3, 7, and 12 Hz) enabled us to assign the ^{13}C and ^{1}H resonances of the pyridoacridine aromatic

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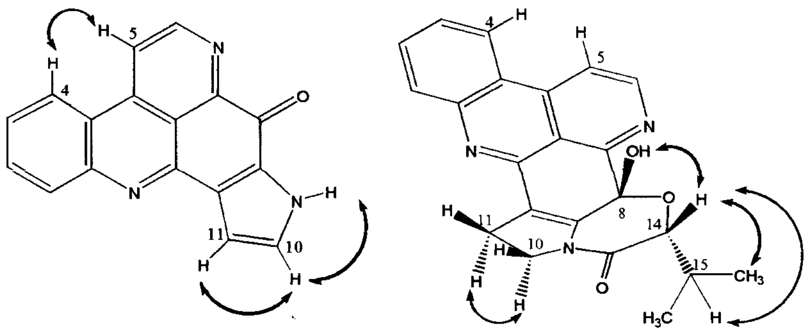

FIGURE 1. Dipolar couplings observed in NMR nuclear Overhauser effect difference experiments for **1** and **2**.

TABLE 2. NMR Data (500 MHz, DMSO-d_6) for Sebastianine B (2)

position	δ ^{13}C^{a}	δ ^{1}H (mult, *J* in Hz)	^{1}H–^{1}H COSY	HMBC^{b,c}
1	129.2	8.08 (d, 7.8)	H-2	H-3
2	131.4	7.84 (t, 7.8)	H-1, H-3	H-4
3	127.0	7.69 (t, 7.8)	H-2, H-4	
4	123.9	8.76 (d, 7.8)	H-3	H-2
4a	120.8			H-1, H-3, H-5, H-6
4b	137.0			H-4, H-5, H-6
5	116.8	8.74 (d, 5.6)	H-6	H-6
6	148.5	9.03 (d, 5.6)	H-5	H-5
7a	156.0			H-6, OH
8	86.6			OH
8a	145.1*^{d}			OH, H-11′
10	45.4	4.04 (ddd, 12.0, 12.0, 7.5) 4.20 (ddd, 12.0, 12.0, 5.7)	H-11′, H-11″	
11	25.7	3.14 (ddd, 12.0, 10.1, 7.5) 3.24 (ddd, 12.0, 10.1, 5.7)	H-10′, H-10′	
11a	114.2			H-10′, H-11′
11b	149.7			
11c	114.5			H-5
12a	145.1*			H-2, H-4
13	165.7			
14	76.6	4.72 (s)	H-15	H-16, H-17
15	29.1	2.50 (m)	H-14, H-16, H-17	H-16, H-17
16	19.2	1.12 (d, 7.0)	H-15	H-17
17	16.4	0.82 (d, 7.0)	H-15	H-16
OH		7.92 (s)		

[a] Assignments by HSQC. [b] HMBC spectra were obtained with long-range coupling constants $J = 3$, 7, and 12 Hz. [c] ′and″ denote downfield and upfield resonances, respectively, of a geminal pair. [d] Asterisk indicates that assignments may be interchanged.

system and of the α-hydroxyisovaleric acid residue. The presence of a band at 1645 cm^{-1} in the IR spectrum, and a carbonyl group at δ 165.7 in the ^{13}C NMR spectrum, indicated the presence of an amide or a lactam in the structure of sebastianine B (**2**), ruling out the possibility that sebastianine B (**2**) presented the isomeric structure **4**.

Although no long-range correlations were observed between the protons of the methylene H-10 and the carbonyl group at δ 165.7 in any of the three HMBC spectra obtained, the chemical shift of the C-10 methylene protons strongly suggested that C-10 was attached to the remaining nonaromatic nitrogen, which was necessarily part of the lactam function. Additionally, the C-10 (δ 45.4) chemical shift supports the attachment to nitrogen. The downfield shift of the proton signals (H-10, δ 4.04 and 4.20) is consistent with *s-cis* amide geometry. We therefore considered that the remaining C-11 methylene was attached to C-11a, making a pyrrolidine ring in the place of the pyrrole ring of sebastianine A (**1**). Long-range couplings observed in the HMBC spectra between C-8a (δ 145.1) and OH (δ 7.92) and H-11′ (δ 3.24), as well as between C-11a (δ 114.2) and H-10′ (δ 4.20) and H-11′, gave support for the pyrrolidine moiety attached to C-8a and C-11a. The carbon chemical shift of the hemiketal group at δ 86.6 is upfield of the anticipated chemical shift; however, we observed HMBC correlations between the proton of the hydroxyl group at δ 7.92 (s) and the carbons C-7a (δ 156.0), C-8 (δ 86.6), and C-8a (δ 145.1). We propose that the deshielding effect of both C-7a and C-8a contributed to the unusual upfield chemical shift of C-8. The alternate proposal of a ketone-hydrate at C-8 and an open ring is in part discounted because of the positive ion ESI mass spectrum, which showed an $[M + H]^+$ at *m*/*z* 374 and the negative ion ESI mass spectrum, which showed an $[M - H]^-$ at *m*/*z* 372. We proposed that the ketone-hydrate would give an identical EIMS parent ion as the cyclic hemiketal but a different molecular ion for negative and positive ESI. Both positive and negative ESI mass spectral data support a molecular weight of 373 Da while the ketone-hydrate requires a molecular weight of 391 Da. Further confirmation for the structure of the nonaromatic moiety of sebastianine B (**2**) was obtained by analysis of several NOE difference experiments that showed key dipolar couplings between the hydroxyl proton at δ 7.92 with the H-14 methine proton at δ 4.72. Additional dipolar couplings were observed between the H-16 methyl (δ 0.82) and the H-14 methine, as well as between the H-15 (δ 2.50) methine and the H-14 methine. The vicinal coupling constants of the H-10 methylene (see Table 2) indicated that both H-10′ and H-10″ present an eclipsed conformation with the H-11 protons. The results of the NOE difference experiments and the analysis of the coupling constants indicated that sebastianine B (**2**) has the relative configuration shown in Figure 1. Sebastianine B (**2**) has an unprecedented ring system comprising a α-hydroxyisovaleric acid condensed with a pyrrolidine ring and a carbonyl group.

Sebastianine A (**1**) and sebastianine B (**2**) were screened against four human colon tumor (HCT) cell lines comprised of p53 and p21 knockouts as well as the parental cell line of each. These isogenic cell lines offer a potential means to identify anticancer leads that may be useful in treating tumors with defined genetic mutations.[15] Specifically, a majority of tumors have a mutated p53 gene that can lead to genetic instability and a loss of checkpoint

TABLE 3. Cytotoxicity of Sebastianine A (1) and Sebastianine B (2)

	$p53^{+/+}$	$p53^{-/-}$	$p21^{+/+}$	$p21^{-/-}$
1	5.1[a]	9.7	4.6	1.3
2	0.92	2.9	1.3	1.1
doxorubicin	0.0087	0.0057	0.0089	0.0068

[a] The numbers represent the IC_{50} value as determined from four replicates expressed in micromolar.

response.[16] At present, many anticancer agents are less cytotoxic against $p53^{-/-}$ tumor cell lines as compared to $p53^{+/+}$ tumor cell lines consistent with a p53 dependent mechanism.[17] Both alkaloids showed a similar pattern (see Table 3) indicating a p53 dependent mechanism. Like other pyridoacridine alkaloids, the sebastianines may be DNA damaging agents, which is typically a p53 dependent mechanism of cell death. Both **1** and **2** showed a slightly lower IC_{50} in the $p21^{-/-}$ versus the $p21^{+/+}$. In the same assay, doxorubicin showed a small increase in cytotoxicity against the $p21^{-/-}$ cell line, which is consistent with previously published results.[15]

In conclusion, two cytotoxic pyridoacridine alkaloids with new ring systems have been isolated from the ascidian *C. dellechiajei*. Their structures were established by analysis of spectroscopic data. The compounds displayed cytotoxic activity against HCT cells in a p53 dependent manner.

Experimental Section

General Experimental Procedures. TLC analyses were performed with precoated TLC sheets of silica gel on polyester, eluting with different mixtures of MeOH in CH_2Cl_2 and were observed under UV light (λ_{max} 254 nm). The ^1H and ^{13}C NMR spectra for sebastianine A (**1**) were obtained at 400 and 100 MHz, respectively. The ^1H and ^{13}C spectra for sebastianine B (**2**) were obtained at 500 and 125 MHz, respectively. All NMR spectra were obtained in DMSO-d_6 at 28 °C using the residual proton signals of DMSO as reference (δ 2.49 for ^1H; δ 40.6 for ^{13}C).

Collection and Extraction of *C. dellechiajei*. Deep blue morphs of the ascidian *C. dellechiajei* (85 g wet wt) were collected during the Brazilian spring of 1997 at depths between 15 and 20 m near São Sebastião Island (Ilhabela). Freshly collected animals were stored in ethanol at −20 °C. A voucher specimen (POLY46) is deposited in the Ascidian collection of the Zoological Department of Federal University of Paraná. Animals were separated from the ethanol, blended, and extracted twice with methanol. The ethanol and methanol extracts were combined, filtered, and evaporated in vacuo. The organic material was dissolved in 95% methanol (600 mL) and partitioned against hexanes (3 × 600 mL). After evaporation, we obtained 0.31 g of the hexanes extract and 0.57 g of the methanol extract.

Isolation. The methanol extract was subjected to chromatography on a C-18 reversed-phase Sep Pak column (Waters, 10 g), with a gradient of MeOH in H_2O, to give five fractions (CyDe-1 to CyDe-5). TLC analysis (CH_2Cl_2−MeOH 9:1, visual inspection under UV at 254 nm) showed that the hexanes extract and CyDe-5 were similar, and these fractions were combined. The combined fraction was applied to a Sephadex LH20 column (170.0 cm × 2.0 cm) and eluted with methanol, to give 8.0 mg of pure sebastianine A (**1**).

The fraction CyDe-4 (~110 mg) was applied to a silica gel column (Waters Sep Pak, 2 g) and eluted with a gradient of MeOH in CH_2Cl_2 yielding five fractions (CyDe-4A to CyDe-4E). Fraction CyDe-4B (~58 mg) was separated by HPLC on a C-18 μ-Bondapak Waters column (10 μm, 7.8 cm × 300 mm) with MeOH−H_2O 7:3. Four fractions were obtained (CyDe-4BI to CyDe-4BIV). Fraction CyDe-4BII (~3 mg) was further purified by HPLC with a Waters μ-Porasil amorphous silica column (10 μm, 7.8 mm × 300 mm) using a mixture of *i*-PrOH−MeOH 7:3. Pure sebastianine B (**2**, 0.8 mg) was obtained after a final purification on a Rainin Dynamax C-18 column (8 μm, 10 mm × 250 mm) with the following conditions: H_2O−MeOH 6:4, 4 mL/min, for 10 min, and then a 40 min linear gradient up to 80% MeOH.

Sebastianine A (1). UV (MeOH) λ_{max} (log ϵ) 223 (4.56), 238 (4.49), 265 (4.36), 301 (4.11), and 390 (3.85) nm; IR (neat) 1683, 1590, 1208, 1139, 844, 803, 724 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC NMR data, see Table 1; EIMS *m*/*z* 271.0752 $[M]^+$ (calcd for $C_{17}H_9N_3O$, 271.0745).

Sebastianine B (2). UV (MeOH) λ_{max} (log ϵ) 206 (4.57), 252 (4.58), 292 (4.23), 366 (4.19) nm; CD_{MeOH} (*c* 107 μM) $[\theta]_{239}$ +15 900, $[\theta]_{264}$ −1160, $[\theta]_{278}$ +5290, $[\theta]_{297}$ −4508, $[\theta]_{322}$ +559, $[\theta]_{341}$ −1640, $[\theta]_{363}$ −1020, $[\theta]_{386}$ −1922.5; IR (film) 3400 (br), 1650, 1595, 1411, 1366, 1037, 756 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC NMR data, see Table 2; EIMS *m*/*z* 373.1428 $[M]^+$ (calcd for $C_{22}H_{19}N_3O_3$, 373.1426).

Methylation of 1. A 1.7 mg portion of sebastianine A was suspended in 750 μL of acetone. A 5 μL portion of iodomethane was added, and the reaction mixture was refluxed for 24 h. The reaction mixture was analyzed by TLC, but no reaction had occurred. To the sample were added 50 μL of iodomethane and 5 mg of K_2CO_3, and the mixture refluxed for 16 h. The solvent was evaporated under a stream of argon, and the sample was analyzed by NMR, indicating the formation of N-12 methyl sebastianine A. ^1H NMR (DMSO-d_6, 500 MHz) δ 9.19 (1H, bd, $J = 6.0$ Hz, H-6), δ 8.93 (1H, bd, $J = 6.0$ Hz, H-5), δ 8.84 (1H, d, $J = 8.0$ Hz, H-4), δ 7.92 (1H, t, $J = 8.0$ Hz, H-2), δ 7.78 (1H, t, $J = 8.0$ Hz, H-3), δ 7.47 (1H, d, $J = 2.5$ Hz, H-11), δ 6.95 (1H, d, $J = 2.5$ Hz, H-10), δ 4.11 (3H, s, N9−CH_3).

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Supporting Information Available: MS, IR, ^1H NMR, and ^{13}C NMR spectra of sebastianine A (**1**) and sebastianine B (**2**), and ^{13}C NMR chemical shift prediction of sebastianine A (S1−S14). This material is available free of charge via the Internet at http://pubs.acs.org.

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