Guanidine Alkaloids from *Monanchora arbuscula*: Chemistry and Antitumor Potential

by **Elthon G. Ferreira**[a][b], **Diego V. Wilke**[a], **Paula C. Jimenez**[a][b], **Julieta R. de Oliveira**[c], **Otília Deusdênia L. Pessoa**[c], **Edilberto R. Silveira**[c], **Francisco A. Viana**[d], **Cláudia Pessoa**[a], **Manoel Odorico de Moraes**[a], **Eduardo Hajdu**[e], and **Letícia Veras Costa-Lotufo***[a][b]

[a]) Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Rua Cel. Nunes de Melo, 1127, 60430-270 Fortaleza, Ceará, Brazil (phone: 55 85 33668255; fax: 55 85 33668333; e-mail: lvcosta@secrel.com.br)

[b]) Instituto de Ciências do Mar, Universidade Federal do Ceará, Fortaleza, CE, Brasil

[c]) Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará, Fortaleza, CE, Brasil

[d]) Departamento de Química, Universidade do Estado do Rio Grande do Norte, 59610-210, Mossoró-RN, Brasil

[e]) Museu Nacional do Rio de Janeiro, Universidade Federal do Rio de Janeiro, RJ, Brasil

Five guanidine alkaloids, mirabilin B (**1**), 8bβ-hydroxyptilocaulin (**2**), ptilocaulin (**3**), and a mixture of the 8β- and 8α-epimers, **4** and **5**, of 8-hydroxymirabilin (1,8a;8b,3a-didehydro-8-hydroxyptilocaulin), were isolated from *Monanchora arbuscula* colonies collected off the northeastern Brazilian coast. All structures were elucidated by spectroscopic analysis, including 1D (^{1}H-, ^{13}C- (BB), and ^{13}C-DEPT) and 2D (COSY, HSQC, and HMBC) NMR experiments, and comparison with the literature data. The cytotoxicity of the isolated compounds were evaluated against four tumor cell lines, showing that mirabilin B (**1**) and the two epimers were inactive, while 8bβ-hydroxyptilocaulin (**2**) and ptilocaulin (**3**) presented IC_{50} values in the range of 7.9 to 61.5 μM, and 5.8 to 40.0 μM, respectively. Further studies on the mechanism of action of ptilocaulin, using HL-60 leukemia cells, demonstrated that this guanidine compound induced apoptosis of the treated cells.

Introduction. – Ever since the discovery of the nucleosides isolated from the marine sponge *Cryptotethya crypta* in the 1950s by *Bergman* and *Feeney*, the marine environment has emerged as a promising source of unique molecules with high therapeutic potential [1][2]. Marine invertebrates are important sources of bioactive secondary metabolites [3], and the marine sponges, which stand out as the main beholders of such compounds, have already contributed with a wide variety of unusual structures and unique mechanisms of action. These organisms account for more than 15,000 compounds distributed in different chemical classes such as nucleosides, alkaloids, terpenes, and sterols. Ever since, these substances have served as templates for the synthesis of new biologically active molecules [4][5].

The genus *Monanchora*, as all genera of the Crambeidae family, is rich in cyclic guanidine alkaloids. This class of compounds is known to exhibit a wide range of biological activities such as cytotoxic, antiviral, antibacterial, and antifungal properties [6]. Moreover, it has been suggested that these compounds could be considered as taxonomic markers [7]. The genera *Crambe*, *Monanchora*, *Batzella*, and *Ptilocaulis* frequently present this group of compounds [8].

Brazil has the second largest coastal ocean in the world and the Northeast region accounts for more than half of the Brazilian coastline [9]. Studies conducted on the coast of Ceará State have shown the importance of the benthic fauna as a source of new molecules with biological prospective. The cytotoxicity of several species of ascidians, cnidarians, and sponges has been reported as a mean to carry on, predominantly, in the anticancer track [10–12].

In a prior study considering a bioactivity screening of the EtOH/H_2O extract obtained from 22 sponge species collected at the Marine State Park 'Pedra da Risca do Meio', located 10,000 nautical miles off the coast of Ceará, nine active extracts were found in at least one of the cytotoxicity inferences. Among these, the extract from *Monanchora arbuscula* stood out, mainly for its antiproliferative effect against cultured tumor cells [13]. This study, in turn, deals with a bioassay-guided fractionation approach to search for cytotoxic guanidine alkaloids in the marine sponge *M. arbuscula*.

Results and Discussion. – We identified five cyclic guanidine alkaloids in *M. arbuscula* extract: mirabilin B (**1**), 8bβ-hydroxyptilocaulin (**2**), ptilocaulin (**3**), and a mixture of the 8β- and 8α-epimers, **4** and **5**, respectively, of 8-hydroxymirabilin B, designated 1,8a;8b,3a-didehydro-8-hydroxyptilocaulin in [7] (see *Fig. 1*).

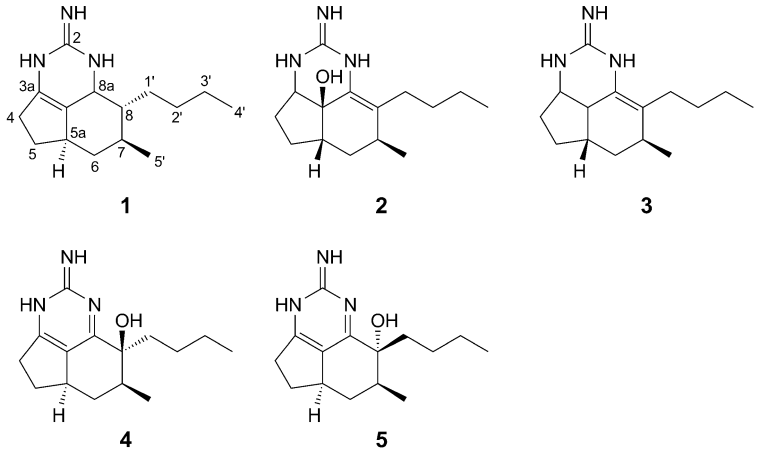

Fig. 1. *Structures of guanidine alkaloids* **1**–**5** *isolated from* Monanchora arbuscula

Other bioactivity screenings related to specimens of *M. arbuscula* collected widely around the globe have also led to the isolation of guanidine alkaloids [6][7][14–17]. *Muricy* and co-workers [14] analyzed a MeOH extract from *M. arbuscula*, collected further southeast on the coast of Rio de Janeiro, and reported for the first time the anti-infective activity of this species, revealing its antibacterial and antifungal activity. More recently, *Kossuga et al.* [15] carried out a more in-depth chemical and pharmacological characterization of this extract and isolated isoptilocaulin, which showed a strong antibacterial activity against chloramphenicol-resistant *Staphylococcus aureus.*

As previously mentioned, guanidine alkaloids are frequently found in sponge species of the genera *Monanchora*, *Crambe*, *Ptilocaulis*, and *Batzella* genera. However, some authors suggest that these genera, mainly the first three, could be merged to a single genus. Besides similar morphological and structural characters, the chemical correspondences among the species provide powerful arguments to support this suggestion [6][9][15][18].

Mirabilin B (**1**) was initially found among a series of six alkaloids (mirabilins A–F) isolated from the Australian sponge *Arenochalina mirabilis* in 1996, by *Barrow et al.* [19]. 8bβ-Hydroxyptilocaulin (**2**) was originally isolated from other specimens of *M. arbuscula* collected in Salvador, Brazil, by *Tavares et al.*, 1995 [16]. Ptilocaulin (**3**), obtained simultaneously with isoptilocaulin from the Caribbean sponge *Ptilocaulis spiculifer*, was described by *Harbour et al.* in 1981 for the first time [20]. 8β-Hydroxymirabilin B (**4**) and its isomer 8α-hydroxymirabilin B (**5**) were likewise identified as mixture by *Hua* and co-workers in 2004 [7].

In this investigation, compounds **1**, **4**, and **5** lacked any sizeable cytotoxicity against the four cell line panel, while **2** and **3** considerably affected HL-60 and MDA-MB-435 cell proliferation (*Table*). HCT-8 and SF-295 cells showed to be less sensitive to the toxic effects of **2** or **3**, since no significant differences in cell viability were observed when these cells were exposed to concentrations below 19 μM for 72 h. Nevertheless, on the normal cell model used herein, compounds **2** and **3** also lacked antiproliferative activity towards PBMC (*Table*). It is noteworthy that all five compounds have similar skeletons, but the active compounds show an unsaturation between C(8) and C(8a), which probably increased the cytotoxicity towards cancer cells (*Fig. 1*). Doxorubicin, the positive control, was much more active than compounds **2** and **3**, and was 2 to 40 times more potent against tumor cells than normal ones.

Additionally, compounds **2** and **3** were tested for hemolytic activity to assess potential damage to plasma membrane of mouse erythrocytes. The compounds exhibited effective concentrations (EC_{50}) of 352.91 and 577.95 μM, respectively. It is important to note that the hemolytic effect was observed at concentrations much higher than those applied for the cytotoxic activity on tumor cells detected by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay.

The absence of cytotoxic activity of **1**, isolated in this study, confirms the results of a study by *Hua et al.*, reporting that mirabilin B (**1**), isolated from the *M. unguifer*, was inactive against a 14 tumor cell line panel, regarding antimicrobial, anti-HIV, or antituberculosis effects, but, on the other hand, exhibited potent antifungal activity against *Cryptococcus neoformans,* with an IC_{50} value of 7.0 μg/ml and a pronounced antiprotozoal activity against *Leishmania donovani*, with an IC_{50} value of 17.0 μg/ml. In the same study, compounds **4/5** were also evaluated showing activity against *Plasmodium falciparum* with an IC_{50} value of 3.8 μg/ml, but no cytotoxic activity against tumor cell lines was observed [7].

It is worth mentioning that studies of *Harbour et al.* [20] and *Rinehart et al.* [21], both in the same year, 1981, demonstrated that ptilocaulin (**3**) of natural origin showed a mean IC_{50} value of 0.39 and 0.17 μg/ml, respectively, on the murine leukemic cells L1210. For this same line, *Ruben et al.* [22] obtained the average IC_{50} value of 0.13 μg/ml with a synthetic racemic ptilocaulin. The values determined in the present study, however, present a tenfold higher IC_{50} array (on HL-60, IC_{50} = 5.77 μM corresponds to

Table. *Cytotoxicity on Tumor Cell Lines: MTT Assay, Normal Human Cell* (PBMC), Alamer Blue™ *Test, and Hemolytic Activity in Mouse Erythrocytes for Guanidine Alkaloids Isolated from* Monanchora arbuscula

Samples	MTT: IC_{50} [μM] (CI 95%)[a])				*Alamar Blue*™: IC_{50} [μM] (CI 95%)	Hemolysis: EC_{50} [μM] (CI 95%)
	HL-60	HCT-8	MDA-MB-435	SF-295	PBMC	
Doxorubicin[b])	0.04 (0.02–0.05)	0.07 (0.05–0.09)	0.86 (0.62–1.10)	0.42 (0.35–0.46)	1.7 (0.9–3.1)	N.T.[c])
1	>20.4	>20.4	>20.4	>20.4	N.T.	N.T.
2	7.89 (6.24–7.66)	>19	11.34 (8.35–10.45)	>19	>19	577.95 (526.99–638.40)
3	5.77 (4.09–4.83)	17.69 (14.40–21.76)	7.58 (7.29–8.20)	>20.3	>20.3	352.91 (320.08–389.15)
4/5	>25 (μg/ml)	>25 (μg/ml)	>25 (μg/ml)	>25 (μg/ml)	N.T.	N.T.

[a]) The IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program (*Intuitive Software for Science*, San Diego, CA). [b]) Positive control. [c]) N.T.: Not tested.

ca. 1.1 μg/ml). *Ruben et al.* also verified that leukemic and melanoma cells presented a greater sensitivity to ptilocaulin (**3**), whereas the cytotoxic effects were less pronounced in lymphoid and cervical adenocarcinoma cells.

A second set of experiments was designed to assess the mechanism of action of ptilocaulin (**3**) in HL-60 leukemia cells. The evaluation of antiproliferative effects by the trypan blue exclusion assay and BrdU incorporation confirmed the results from the MTT analysis (*Fig. 2*, *a* and *b*). Additionally, fluorescence observations of (AO/EB) double-stained treated cells showed that **3** induced a concentration-dependent decrease in the number of viable cells, followed by an increase in cells with apoptotic appearance (*Fig. 2*, *c*). Morphological examination of ptilocaulin-treated cells corroborated these findings (*Fig. 3*), and features suggestive of apoptosis, such as membrane blebbing and cytoplasmatic vacuolization, were observed. Nuclear pyknosis, DNA fragmentation, and cell shrinkage occur at more advanced stages of apoptotic death, and were evident on cells exposed to 20 μM of **3** (*Fig. 3*).

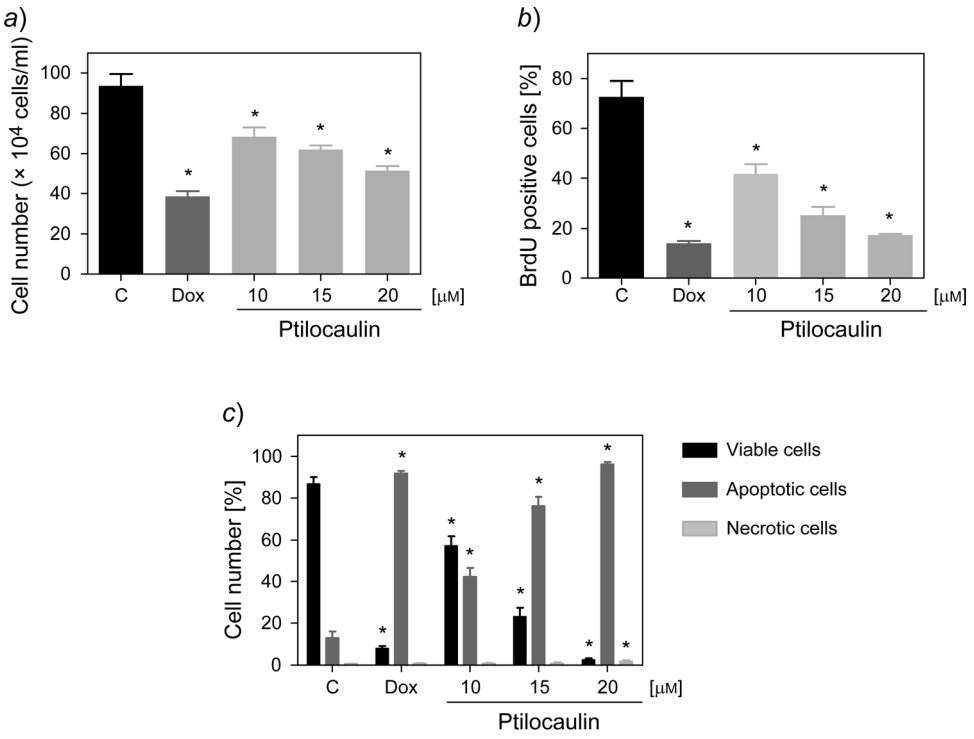

Fig. 2. *Antiproliferative activity of the alkaloid ptilocaulin* (10, 15, and 20 μM) *in HL-60 leukemic cells. a*) Effects of ptilocaulin on HL-60 cell viability determined by trypan blue staining. *b*) BrdU Incorporation by HL-60 cells treated with ptilocaulin. *c*) Effects of ptilocaulin on HL-60 cell viability AO/EB determined *via* fluorescence microscopy. Cells were treated for 24 h, and the negative control was incubated with the vehicle used to dilute the tested substance. Doxorubicin (Dox 0.5 μM) was used as the positive control. *: $p < 0.05$ compared to negative control by ANOVA followed by *Student Newman Keuls* test. Data are presented as mean values ± S.E.M. from three independent experiments performed in duplicate ($n = 3$).

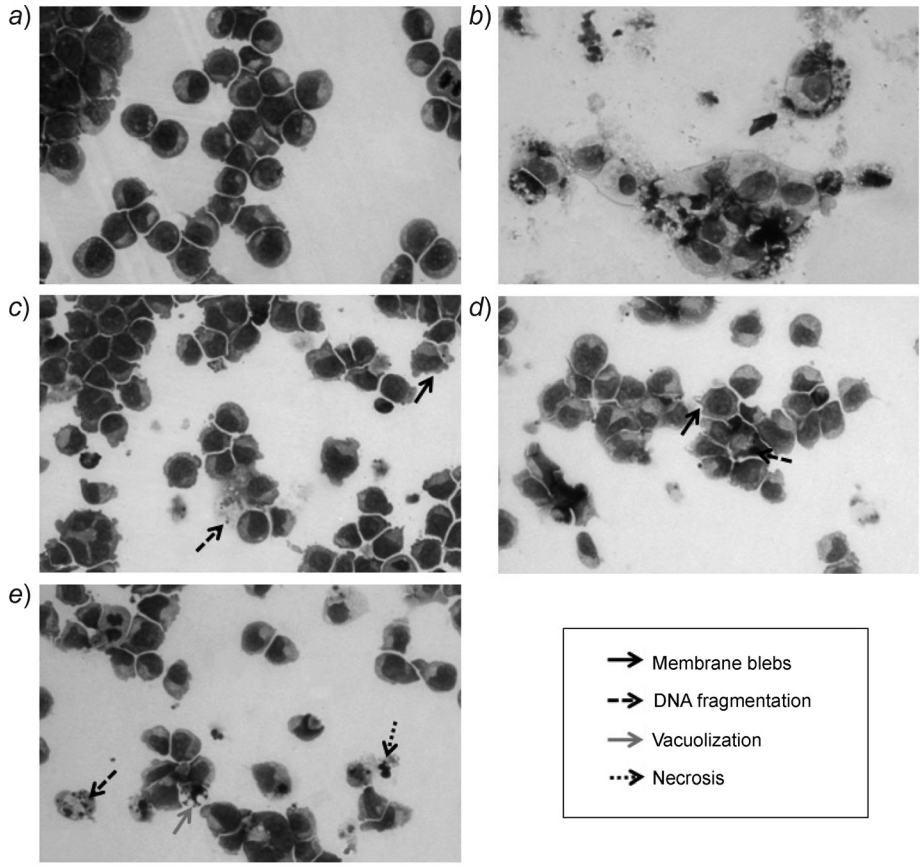

Fig. 3. *Ptilocaulin induces morphological changes in leukemia cells.* Microscopic analysis of *May–Grünwald–Giemsa*-stained HL-60 cells after 24 h of incubation with ptilocaulin at 10 (*c*), 15 (*d*), and 20 μM (*e*). The negative control was treated with the vehicle used to dilute the tested substance (*a*). Doxorubicin (*b*; 0.5 μM) was used as the positive control. The cells were analyzed by light microscopy (×400).

Flow-cytometry analysis of treated cells also showed a decrease in cell number for all concentrations of ptilocaulin (**3**; *Fig. 4,a*). Nevertheless, loss of membrane integrity was only observed for cells subjected to the 20 μM treatment (*Fig. 4,b*), which also agrees with the non-viable cells on the trypan blue count. Regarding the cell cycle, at 15 and 20 μM, **3** promoted a drop in cells undergoing S phase and increased the number of cells in G0/G1 (*Fig. 4,c*). DNA Fragmentation was detected on 15- and 20-μM ptilocaulin-treated cells (*Fig. 4,d*), which may well indicate, in accordance with the morphological findings, progression of apoptosis. Moreover, depolarization of the mitochondrial inner membrane was observed in cells treated with **3** at 15 and 20 μM (*Fig. 4,e*). To confirm whether ptilocaulin-treated cells were undergoing an apoptotic death, caspase 3 and 7 activation were measured by flow cytometry after 24 h of incubation. At all tested concentrations, ptilocaulin (**3**) led to the activation of effectors

caspases 3 and 7 (*Fig. 5*). Taken together, the results from morphological and flow cytometric analyses of ptilocaulin-treated HL-60 cells indicate cell death by apoptosis triggered by the intrinsic pathway.

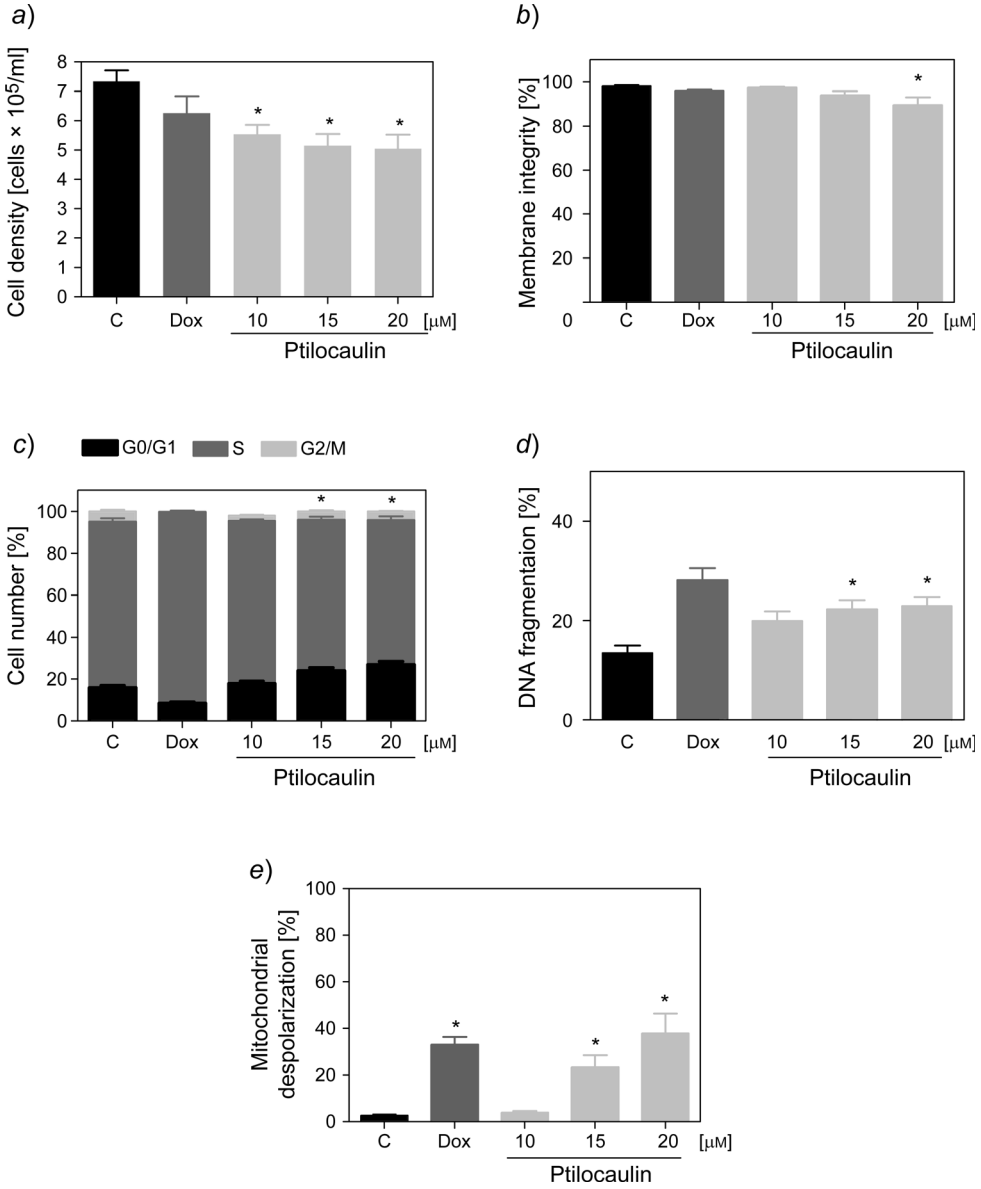

Fig. 4. *Effects of alkaloid ptilocaulin at concentrations of 10, 15, and 20* μM *evaluted for flow of cytometry in leukemic cells* (HL-60) *after 24 h of incubation*. C: Negative control; Dox: doxorubicin (0.5 μM) was used with positive control. *: $p < 0.05$ compared with the negative control by ANOVA, followed by *Dunnett*'s test. *a*) Cells density; *b*) membrane integrity; *c*) analysis of cell cycle; *d*) DNA fragmentation; *e*) mitochondrial despolarization [%].

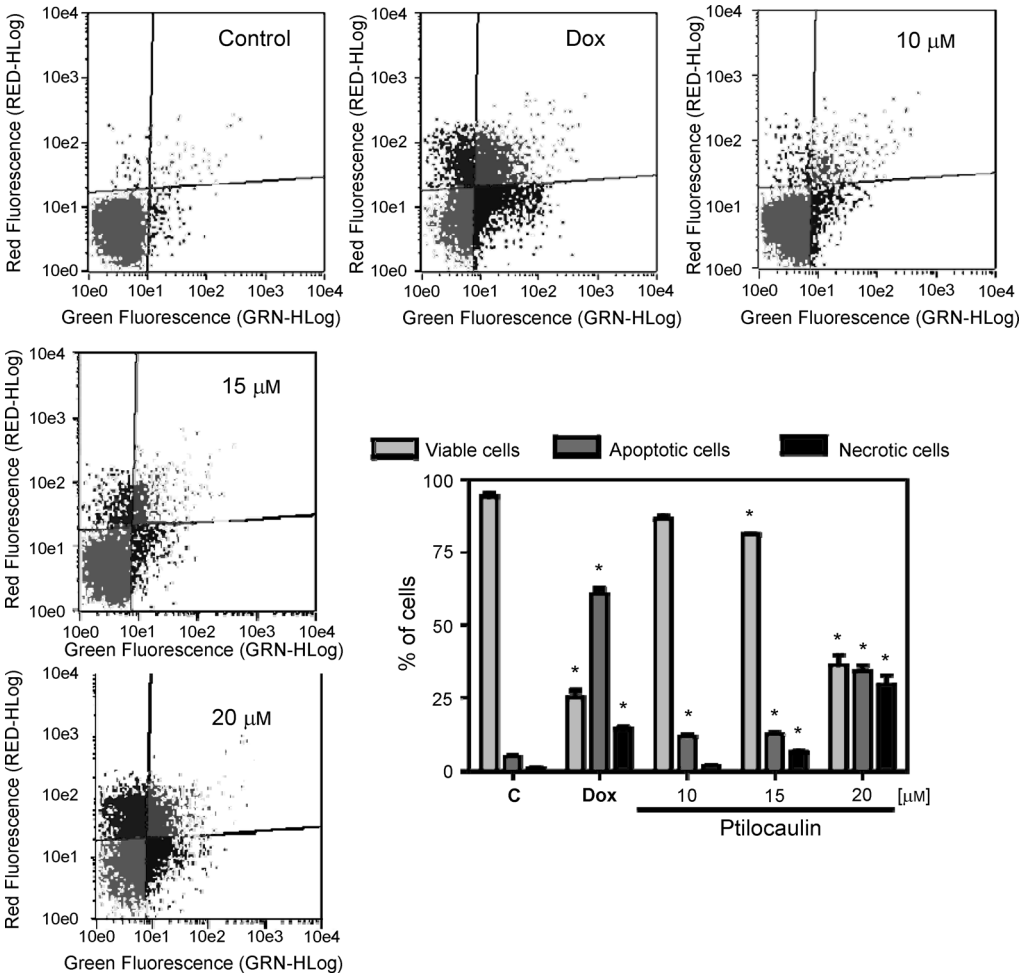

Fig. 5. *Ptilocaulin* (10, 15, and 20 μM) *induces caspase activation in HL-60 cells.* Activity of caspases 3 and 7 was determined by flow cytometry using propidium iodide and flica after 24 h of incubation. The negative control was the vehicle used to dilute the tested substance. Doxorubicin (Dox, 0.5 μM) was used as a positive control. Percentages of necrotic, early and late apoptotic, and viable cells are indicated. Data are presented as mean values ± S.E.M. from two independent experiments performed in triplicate.

*: $p < 0.05$ compared to negative control by ANOVA, followed by *Student Newman Keuls* test.

In conclusion, five known guanidine alkaloids, **1**–**5**, were isolated from *Monanchora arbuscula* colonies collected from the northeastern Brazil coast. Studies on the cytotoxicity of **1**–**5** demonstrated that 8bβ-hydroxyptilocaulin (**2**) and ptilocaulin (**3**) are moderate by active, inducing apoptosis of the treated cells.

The authors are grateful to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), and *International Foundation for Science* (IFS) for support of the project. The authors also thank the *National Cancer Institute* (Bethesda, MD, USA) for donation of the tumor cell lines used in this study.

Experimental Part

General. Column chromatography (CC): silica gel *60* (SiO_2; 70–230 mesh; *Vetec*; or 230–400 mesh; *Merck*). TLC: Precoated SiO_2 aluminium sheets (*Kieselgel 60 F_{254}*, 0.20 mm; *Merck*); visualization by heating (100°) SiO_2 plates and spraying with vanillin/perchloric acid/EtOH soln. and/or *Dragendorff*'s reagent. Semi-prep. HPLC: *LC-10Atvp* (*Shimadzu*). Optical rotations: *Perkin-Elmer 341* digital polarimeter. IR Spectra (KBr pellets): *Perkin-Elmer FT-IR 1000* spectrometer. NMR Spectra: *Bruker DRX-500* spectrometer; ^{1}H: 500 and ^{13}C: 125 MHz). MS: *QP 5050* instrument.

Biological Material. The sponge studied was collected during a scientific expedition to the Marine State Park of 'Pedra da Risca do Meio', off the coast of Ceará State, Brazil, at a depth of 18 m, on July, 2004. A voucher specimen (MNRJ 8670) was deposited with the National Museum of the Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. The sponge was identified as *Monanchora arbuscula* (Duchassaing et Michelotti, 1864), order Poecilosclerida, class Demospongiae, family Crambeidae [13][23].

Extraction and Isolation. The marine material (1.5 kg) was stored in EtOH at low temp. The animal residue was separated from the hydroethanol soln. and blended with CH_2Cl_2/EtOH 1:1 (3 × 1.5 l). After filtration, the solvents were combined and distilled under reduced pressure to yield 17.8 g of crude extract, which was dissolved in MeOH/H_2O 6:4 and partitioned with petroleum ether (PE), followed by CH_2Cl_2 and AcOEt. The CH_2Cl_2 fraction (5.7 g) was chromatographed on a *Sephadex LH-20* column and eluted with MeOH to give three main fractions, *Frs. 1–3*, following TLC analysis. *Fr. 2* (1.94 mg) was rechromatographed over *Sephadex LH-20* and eluted with MeOH to yield four fractions, *Frs. 1′–4′*. *Fr. 2′* (1.45 mg) was subjected to CC (SiO_2; CH_2Cl_2/AcOEt 9:1, 8:2, 1:1; AcOEt; AcOEt/MeOH 9.5:0.5, 9:1, 8.5:1.5, 1:1; followed by MeOH) to yield eight fractions (8 ml each). *Mirabilin B* [18] (**1**, 26 mg) was isolated from the fraction CH_2Cl_2/AcOEt 1:1. The AcOEt fraction (2.56 g), obtained from the partition, was subjected to CC (SiO_2; CH_2Cl_2/AcOEt 1:1; AcOEt; AcOEt/MeOH 9.5:0.5, 9:1, 8.5:1.5, 8:2, 1:1; and finally MeOH) to yield 22 fractions (8 ml each). Combined *Frs. 11–14* (1.4 g), were rechromatographed over SiO_2 and eluted with AcOEt, followed by a gradient system of AcOEt/MeOH 9.5:0.5, 9:1, 8.5:1.5, 8:2, 1:1, and MeOH. *Frs. 9–10* (332 mg), obtained by elution with AcOEt/MeOH 8.5:1.5, was subjected to semi-prep. HPLC (MeOH/H_2O-0.1% 62:38 (*v*/*v*); 2 ml/min) to afford *8bβ-hydroxyptilocaulin* (**2**; 34.0 mg) [17] and *ptilocaulin* (**3**; 24.5 mg) [16]. *Frs. 13–15* (51 mg), obtained by elution with AcOEt/MeOH 8:2, was subjected to CC (SiO_2; CH_2Cl_2/AcOEt 3:7, AcOEt, and MeOH) to yield 22 fractions (4 ml each). From *Frs. 8–12*, eluted with AcOEt, the 8*β*- and 8*α*-epimeric mixture (6 mg) of mirabilin B, **4**/**5**, was isolated [7].

Mirabilin B (= *(5a*R*,7*S*,8*R*)-8-Butyl-3,4,5,5a,6,7,8,8a-octahydro-7-methylcyclopenta[*de*]quinazolin-2(1*H*)-imine*; **1**). Light yellow resin. $[\alpha]_D^{20} = +60$ (*c* = 0.085, MeOH). IR (NaCl): 3313, 3185, 2955, 2914, 2867, 1588, 1455, 1386, 762. ^{1}H-NMR ($CDCl_3$, 500 MHz): 2.92–2.90 (*m*, H_a–C(4)); 2.89–2.86 (*m*, H–C(5a)); 2.58 (*dd*, *J* = 16.7, 8.4, H_b–C(4)); 2.37–2.31 (*m*, H–C(5)); 2.21–2.19 (*m*, H–C(8)); 2.04–2.02 (*m*, H_a–C(1′)); 2.01–2.19 (*m*, H–C(6)); 1.86–1.83 (*m*, H–C(7)); 1.77–1.72 (*m*, H_b–C(1′)); 1.54–1.49 (*m*, H–C(5)); 1.32–1.29 (*m*, H–C(2′)); 1.28–1.25 (*m*, H–C(3′)); 1.11–1.09 (*m*, H–C(2′)); 1.08 (*d*, *J* = 6.6, Me), 0.94–0.88 (*m*, H–C(6)); 0.86 (*t*, *J* = 7.2, Me). ^{13}C-NMR ($CDCl_3$, 125 MHz): 174.9 (C(3a)); 166.1 (C(8a)); 163.2 (C(2)); 126.2 (C(8b)); 47.1 (C(8)); 39.9 (C(6)); 37.9 (C(5a)); 34.3 (C(7)); 33.8 (C(4)); 33.2 (C(5)); 30.5 (C(1′)); 27.9 (C(2′)); 23.4 (C(3′)); 21.1 (C(5′)); 14.2 (C(4′)). EI-MS: 245 ($[M+H]^+$), 202 (18.3), 189 (100), 174 (93.3), 160 (18.3).

8bβ-Hydroxyptilocaulin (= *(5a*S*,7*S*,8b*S*)-8-Butyl-2,3,3a,4,5,5a,6,7-octahydro-2-imino-7-methylcyclopenta[*de*]quinazolin-8b(1*H*)-ol*; **2**). Yellowish resin. $[\alpha]_D^{20} = +69.3$ (*c* = 0.865, MeOH). IR (NaCl): 3289, 3222, 2957, 2867, 1676, 1603, 1458, 1400, 1372, 1341, 1115, 755. ^{1}H-NMR ($CDCl_3$, 500 MHz): 9.46 (*s*, NH); 9.02 (*s*, NH); 7.36 (*s*, NH); 3.70–3.61 (*m*, H–C(3a)); 2.44–2.41 (*m*, H–C(7)); 2.29–2.26 (*m*, H–C(5a));

2.18–2.14 (*m*, H_a–C(6)); 2.08–2.06 (*m*, H–C(1′)); 1.97–1.93 (*m*, H–C(4)); 1.83–1.77 (*m*, H_a–C(5)); 1.55–1.53 (*m*, H_b–C(5)); 1.52–1.48 (*m*, H_b–C(6)); 1.40–1.34 (*m*, H–C(3′)); 1.33–1.24 (*m*, H–C(2′)); 1.18 (*d*, *J* = 7.35, Me); 0.88 (*t*, *J* = 7.05, Me). ^{13}C-NMR ($CDCl_3$, 125 MHz): 151.9 (C(2)); 129.0 (C(8)); 122.9 (C(8a)); 69.8 (C(8b)); 59.0 (C(3a)); 40.3 (C(5a)); 30.8 (C(7)); 30.3 (C(2′)); 29.8 (C(4)); 29.1 (C(6)); 28.7 (C(1′)); 23.5 (C(5)); 23.1 (C(3′)); 21.8 (C(5′)); 14.1 (C(4′)). EI-MS: 263 ([*M* + H]$^+$), 248 (35), 220 (50), 206 (64), 204 (100),190 (41),178 (63).

Ptilocaulin (= *(5a*S*,7*S*)-8-Butyl-3,3a,4,5,5a,6,7,8b-octahydro-7-methylcyclopenta[*de*]quinazolin-2(1*H*)-imine*; **3**). Yellowish resin. $[\alpha]_D^{20} = +177$ (*c* = 0.055; MeOH). IR (NaCl): 3203, 2955, 2867, 1686, 1672, 1598, 1400, 1111, 617. ^{1}H-NMR ($CDCl_3$, 500 MHz): 9.53 (*s*, NH); 8.94 (*s*, NH); 7.53 (*s*, NH); 3.71–3.68 (*m*, H–C(3a)); 2.53–2.51 (*m*, H–C(8b)); 2.46–2.42 (*m*, H–C(5a)); 2.31–2.28 (*m*, H–C(7)); 2.27–2.25 (*m*, H–C(1′)); 1.96–1.94 (*m*, H–C(4)); 1.93–1.91 (*m*, H–C(6)); 1.71–1.65 (*m*, H–C(5)); 1.59–1.55 (*m*, H–C(5)); 1.50–1.43 (*m*, H–C(4)); 1.36–1.30 (*m*, H–C(3′/2′/6)); 1.13 (*d*, *J* = 7.15, Me); 0.88 (*t*, *J* = 6.05, Me). ^{13}C-NMR ($CDCl_3$, 125 MHz): 152.5 (C(2)); 126.7 (C(8)); 121.3 (C(8a)); 53.7 (C(3a)); 35.8 (C(8b)); 34.5 (C(6)); 34.3 (C(5a)); 32.1 (C(4)); 30.9 (C(2′)); 30.4 (C(7)); 27.8 (C(1′)); 27.5 (C(5)); 22.9 (C(3′)); 20.8 (C(5′)); 14.3 (C(4′)). EI-MS: 247 ([*M* + H]$^+$), 232 (33), 204 (100) 190 (50), 176 (37).

Mixture **4**/**5**. Amorphous yellowish solid. $[\alpha]_D^{20} = +25$ (*c* = 0.125; MeOH). IR (NaCl): 3330, 3196, 2953, 2928, 2859, 1692, 1606, 1587, 1460, 1380, 1330, 1178, 1143, 1104, 1045, 753, 527. EI-MS: 261 ([*M* + H]$^+$), 205 (100), 204 (68), 190 (50).

8β-Hydroxymirabilin B (= *(5a*R*,7*S*,8*S*)-8-Butyl-2,3,4,5,5a,6,7,8-octahydro-2-imino-7-methylcyclopenta[*de*]quinazolin-8-ol*; **4**). ^{1}H-NMR (C_5D_5N, 500 MHz): 2.85–2.81 (*m*, H–C(4), H–C(5a)); 2.56–2.50 (*m*, H–C(4)); 2.18–2.12 (*m*, H–C(5)); 2.04 (*td*, *J* = 12.9, 3.7, H–C(7)); 1.83–1.81 (*m*, H–C(1′)); 1.76–1.74 (*m*, H–C(6)); 1.53–1.51 (*m*, H–C(6)); 1.46–1.40 (*m*, H–C(5)); 1.32–1.30 (*m*, H–C(3′)); 1.32 (*d*, *J* = 7.0, Me); 1.24–1.18 (*m*, H–C(2′)); 0.83 (*J* = 7.0, Me). ^{13}C-NMR (C_5D_5N, 125 MHz): 176.4 (C(3a)); 165.9 (C(8a)); 165.7 (C(2)); 125.0 (C(8b)); 74.0 (C(8)); 39.0 (C(5a)); 37.9 (C(7)); 36.9 (C(1′)); 35.8 (C(6)); 34.4 (C(4)); 33.8 (C(5)); 28.0 (C(2′)); 24.1 (C(3′)); 16.0 (C(5′)); 14.6 (C(4′)).

8α-Hydroxymirabilin B (= *(5a*R*,7*S*,8*R*)-8-Butyl-2,3,4,5,5a,6,7,8-octahydro-2-imino-7-methylcyclopenta[*de*]quinazolin-8-ol*; **5**). ^{1}H-NMR (C_5D_5N, 500 MHz): 2.85–2.81 (*m*, H–C(5a)); 2.56–2.20 (*m*, H–C(4)); 2.65–2.59 (*m*, H–C(4)); 2.47–2.41 (*m*, H–C(7)); 2.39–2.33 (*m*, H–C(6)); 2.16–2.11 (*m*, H–C(5)); 2.07–2.00 (*m*, H–C(6)); 1.83–1.81 (*m*, H–C(1′)); 1.56–1.51 (*m*, H–C(2′)); 1.46–1.40 (*m*, H–C(1′), H–C(5)); 1.32 (*d*, *J* = 7.0, Me); 1.24–1.18 (*m*, H–C(2′), H–C(3′)); 0.77 (*t*, *J* = 7.2, Me). ^{13}C-NMR (C_5D_5N, 125 MHz): 175.8 (C(8a)); 165.9 (C(8a)); 165.7 (C(2)); 125.2 (C(8b)); 75.7 (C(8)); 43.7 (C(7)); 39.2 (C(5a)); 38.0 (C(6)); 37.8 (C(1′)); 34.7 (C(4)); 34.1 (C(5)); 27.9 (C(2′)); 24.4 (C(3′)); 16.6 (C(5′)); 14.4 (C(4′)).

Cell Line and Cell Culture. The cell lines used in this investigation were acute promyeloblastic leukemia (HL-60), melanoma (MDA-MB-435), colon (HCT-8), and glioblastome (SF-295) cells, all obtained from the National Cancer Institute in Bethesda, MD, USA. The cells were maintained in *RPMI 1640* medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37° with 5% CO_2. The cultures were split on the 3rd d and diluted 1 d before each experiment. For all experiments, cells were seeded at 3×10^5 cells/ml.

Heparinized blood (from healthy, non-smoker donors who had not taken any drug at least 15 d prior to sampling) was collected, and peripheral blood mononuclear cells were isolated by a standard method of density-gradient centrifugation over *Ficoll-Hypaque*.

MTT Assay. For the MTT (= 9-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay, HL-60, MDA-MB-435, HCT-8, and SF-295 cells were incubated with the assayed compounds and mixture (0.019–5 μg/ml) for 72 h in a 96 well plate. Doxorubicin was used as a positive control (0.02–8.6 μM). At the end of the incubation period, the medium of each well was replaced with fresh media containing 0.5 μg/ml of the MTT soln. and left for another 3 h. Then, the cells were centrifuged, and the supernatant was discarded. The formazan was diluted in DMSO, and the absorbance was read in a plate spectrometer at 550 nm. The 0% effect was considered as the absorbance found at the wells with non-treated cells, and the IC_{50} value (concentration of tested compound causing 50% inhibition of cell growth) was obtained on that basis [24].

Alamar Blue™ *Assay.* To investigate the selectivity of guanidine alkaloids toward a normal proliferating cell, the *Alamar Blue™* assay was performed with PBMC (periphral blood mononuclear

cell) after 72 h drug exposure. PBMCs were washed and resuspended at a concentration of 3×10^5 cells/ml in *RPMI 1640* medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37° with 5% CO_2. Phytohemagglutinin (3%) was added at the beginning of the culture. After 24 h, the samples dissolved in DMSO 1% were added to each well and incubated for 72 h. Doxorubicin (0.02–8.6 μM) was used as positive control. Control groups received the same amount of DMSO. Four h before the end of the total incubation time, 10 μl of stock soln. (0.312 mg/ml) of the *Alamar Blue* (*Resazurin*, *Sigma–Aldrich* Co.) was added to each well. The absorbance was measured using a multiplate reader (*DTX 880 Multimode Detector*, *Beckman Coulter*®), and the drug effect was quantified as the percentage of control absorbance at 570 and 595 nm, and the percent of reduction was obtained as described in [25][26].

Analysis of the Mechanisms Involved in the Cytotoxic Activity. All subsequent experiments were performed with HL-60 cells (3×10^5 cells/ml) after 24 h of incubation using different concentration of ptilocaulin (**3**; 10, 15, and 20 μM). Doxorubicin (0.5 μM) was used as a positive control.

Trypan Blue Assay. Cell viability was determined by the trypan blue dye exclusion test. Aliquots were removed, and viable cells and non-viable cells were estimated after 24 h of incubation. After each incubation period, a growth curve was established. Cells that excluded trypan blue were counted in a *Newbauer* chamber [27].

Measurement of DNA Synthesis. Ten μl of 5-bromo-2′-deoxyuridine (BrdU, 10 mM) was added to each well and incubated for 3 h at 37°, before completing the 24-h period of drug exposure. To assay the amount of BrdU incorporated into DNA, cells were harvested, spun on to glass slides, and allowed to dry for 2 h at r.t. Cells that had incorporated BrdU were labeled by direct peroxidase immunocytochemistry utilizing the chromogen diaminobenzidine (DAB). Slides were counterstained with hematoxylin, mounted, and cover-slipped. Evaluation of BrdU positivity was performed by light microscopy (*Olympus*, Tokyo, Japan). Two hundred cells were counted per sample to determine the percentage of positive cells [28].

Morphological Analysis with May–Grünwald–Giemsa *Staining.* Untreated or treated cells were examined for morphological changes by light microscopy (*Olympus*, Tokyo, Japan). To evaluate nuclear morphology, cells were harvested, transferred to cytospin slides, fixed with MeOH for 50 s, and stained with *May–Grünwald–Giemsa* [29].

Morphological Analysis with Fluorescence Microscopy. At the end of the 24 h incubation period, cells were resuspended in 25 μl of phosphate buffered saline (PBS). Then, 1 μl of an aq. soln. of acridine orange/ethidium bromide (AO/EB; 100 μg/ml) was added to each vial, and the cell events were observed under a fluorescence microscope with a 470/440 nm filter (*Olympus*). Three hundred cells were counted per sample and classified as follows: viable cells, apoptotic cells, and necrotic cells [30][31].

Cell Membrane Integrity. HL-60 Cell membrane integrity was evaluated by the exclusion of propidium iodide (2 μg/ml). Cell fluorescence was then determined by flow cytometry in the *Guava EasyCyte Mini*, using *Guava Express Plus* software. Five thousand events were evaluated per experiment and, cellular debris was omitted from the analysis.

Internucleosomal DNA Fragmentation. HL-60 Cells were incubated at 25° for 30 min, in the dark, in a lysis soln. which contained 0.1% citrate, 0.1% *Triton X-100*, and 50 μg/ml propidium iodide. Cell fluorescence was then determined by flow cytometry in the *Guava EasyCyte Mini* using *Guava Express Plus* software. Five thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

Measurement of Mitochondrial Transmembrane Potential. Mitochondrial transmembrane potential was determined by retention of the rhodamine 123 dye in HL-60 cells. About one million cells were washed with PBS, incubated with rhodamine 123 (5 μg/ml) at 37° for 15 min in the dark, and washed twice. The cells were then incubated in PBS at 37° for 30 min in the dark, and fluorescence was then measured as described above.

Caspase 3/7 Activation. The activity of caspase 3/7 in HL-60 cells treated with ptilocaulin (**3**) at the concentrations 10, 15, and 20 μM, and positive control doxorubicin (0.5 mM) was measured by flow cytometry using caspase 3/7 FAM kit after 24 h of incubation. The cells (3.0×10^5 cells/ml) were incubated with *Fluorescent Labeled Inhibitor of Caspases* (*FLICA*™) for 1 h at 37° in a CO_2 incubator. After incubation, the cells were added to 80 μl of wash buffer and then centrifuged at 2000 rpm for 5 min.

The cell pellet precipitated was resuspended in 200 µl of wash buffer and centrifuged again. The cells were then re-suspended in the working soln. (1:200 propidium iodide/wash buffer 1X) and analyzed immediately using flow citometry.

Statistical Analysis. For cytotoxicity assays, the IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the Graphpad program (*Intuitive Software for Science*, San Diego, CA). Data obtained are presented as mean ± SEM from at least three independent experiments. To determine statistical differences, data were compared by one-way analysis of variance (ANOVA), followed by *Newman–Keuls* test ($P < 0.01$).

REFERENCES

[1] W. Bergman, R. J. Feeney, *J. Org. Chem.* **1951**, *16*, 981.
[2] L. V. Costa-Lotufo, D. V. Wilke, P. C. Jimenez, R. de A. Epifânio, *Quím. Nova* **2009**, *32*, 703.
[3] B. Haefner, *Drug Discovery Today* **2003**, *8*, 536.
[4] D. Sipkema, M. C. R. Frassen, R. Osinga, J. Tramper, R. H. Wijffels, *Mar. Biotechnol.* **2005**, *7*, 142.
[5] E. H. Belarbi, A. C. Gómez, Y. Chisti, F. G. Camacho, E. M. Grima, *Biotechnol. Adv.* **2003**, *21*, 585.
[6] R. Tavares, D. Daloze, J. C. Braekman, E. Hajdu, G. Muricy, R. W. M. Van Soest, *Biochem. Syst. Ecol.* **1994**, *22*, 645.
[7] H.-M. Hua, J. Peng, F. R. Fronczec, M. Kelly, M. T. Hamann, *Bioorg. Med. Chem.* **2004**, *12*, 6461.
[8] L. C. Chang, N. F. Whittaker, C. A. Bewley, *J. Nat. Prod.* **2003**, *66*, 1490.
[9] R. W. M. Van Soest, J. C. Braeckman, D. J. Faulkner, E. Hajdu, M. K. Harper, J. Vacelet, *Bull. Inst. R. Sci. Nat. Belg.* **1996**, *66* suppl., 89.
[10] P. C. Jimenez, G. L. S. Teixeira, D. V. Wilke, N. A. P. Nogueira, E. Hajdu, C. Pessoa, M. O. Moraes, L. V. Costa-Lotufo, *Arq. Ciên. Mar* **2004**, *37*, 85.
[11] P. C. Jimenez, D. V. Wilke, R. Takeara, T. M. C. Lotufo, C. Pessoa, M. O. de Moraes, N. P. Lopes, L. V. Costa-Lotufo, *Comp. Biochem. Physiol.* **2008**, *151*, 391.
[12] D. V. Wilke, P. C. Jimenez, C. Pessoa, M. O. de Moraes, R. M. Araújo, W. M. B. da Silva, E. R. Silveira, O. D. L. Pessoa, R. Braz-Filho, N. P. Lopes, L. V. Costa-Lotufo, *J. Braz. Chem. Soc.* **2009**, *20*, *1455*.
[13] E. G. Ferreira, D. V. Wilke, P. C. Jimenez, T. A. Portela, E. R. Silveira, E. Hajdu, C. Pessoa, M. O. Moraes, L. V. Costa-Lotufo, in 'Porifera Research: Biodiversity, Innovation and Sustainability', Eds. G. Muricy, G. Lobo-Hajdu, E. Hajdu, M. R. Custodio, Museu Nacional, Rio de Janeiro, 2007, p. 313.
[14] G. Muricy, E. Hajdu, F. V. Araujo, A. N. Hagler, *Sci. Mar.* **1993**, *57*, 427.
[15] M. H. Kossuga, S. P. de Lira, A. M. Nascimento, M. T. P. Gambardella, R. G. S. Berlinck, Y. R. Torres, G. G. F. Nascimento, E. F. Pimenta, M. Silva, O. H. Thiemann, G. Oliva, A. G. Tempone, M. S. C. Melhem, A. O. de Souza, F. C. S. Galetti, C. L. Silva, B. Cavalcanti, C. O. Pessoa, M. O. Moraes, E. Hajdu, S. Peixinho, R. M. Rocha, *Quím. Nova* **2007**, *30*, 1194.
[16] R. Tavares, D. Daloze, J. C. Braekman, E. Hajdu, R. W. M. Van Soest, *J. Nat. Prod.* **1995**, *58*, 1139.
[17] R. Laville, O. P. Thomas, F. Berrué, D. Marquez, J. Vacelet, P. Amade, *J. Nat. Prod.* **2009**, *72*, 1589.
[18] A. D. Patil, A. J. Freyer, P. Offen, M. F. Bean, R. K. Johnson, *J. Nat. Prod.* **1997**, *60*, 704.
[19] R. A. Barrow, L. M. Murray, T. K. Lim, R. J. Capon, *Aust. J. Chem.* ***1996****, 49*, 767.
[20] G. C. Harbour, A. A. Tymiak, K. L. Rinehart Jr., P. D. Shaw, R. G. Hughes Jr., S. A. Mizsak, J. H. Coats, G. E. Zurenko, L. H. Li, S. L. Kuentzel, *J. Am. Chem. Soc.* **1981**, *103*, 5604.
[21] K. L. Rinehart Jr., P. D. Shaw, L. S. Shield, J. B. Gloer, G. C. Harbour, M. E. S. Koker, D. Samain, R. E. Schwartz, A. A. Tymiak, D. L. Weller, G. T. Carter, M. H. G. Munro, R. G. Hughes Jr., H. E. Renis, E. B. Swynenberg, D. A. Stringfellow, J. J. Vavra, J. H. Coats, G. E. Zurenko, S. L. KuentzeI, L. H. Li, J. G. Bakus, R. C. Brusca, L. L. Craft, D. N. Young, J. L. Counor, *Pure Appl. Chem.* **1981**, *53*, 795.
[22] R. L. Ruben, B. B. Snider, F. W. Hobbs Jr., P. N. Confalone, B. A. Dusak, *Invest. New Drugs* **1989**, *7*, 147.
[23] E. L. Esteves, V. P. Santos, L. F. S. Machado, E. Hajdu, in 'Livro de Resumos do XII Congresso Latino-Americano de Ciências do Mar', Aoceano, Florianópolis, 2007, p. 1.
[24] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.

[25] J. E. Springer, R. D. Azbill, S. L. Carlson, *Brain Res. Protoc.* **1998**, *2*, 259.
[26] Y. Zhi-Jun, N. Sriranganathan, T. Vaught, S. K. Arastu, S. A. Ahmed, *J. Immunol. Methods* **1997**, *210*, 25.
[27] D. Renzi, M. Valtolina, R. Foster, *ATLA* **1993**, *21*, 89.
[28] F. Pera, P. Mattias, K. Detzer, *Cell Tissue Kinet.* **1977**, *10*, 255.
[29] G. Rovera, D. Santoli, C. Damsky, *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2779.
[30] M. F. Cury-Boaventura, C. Pompéia, R. Curi, *Clin. Nutr.* **2004**, *23*, 721.
[31] C. X. Geng, Z. C. Zeng, J. Y. Wang, *World J. Gastroenterol.* **2003**, *9*, 696.

Received June 10, 2010