538 *J. Nat. Prod.* **2007,** *70,* 538−543

Cytotoxic Alkylpiperidine Alkaloids from the Brazilian Marine Sponge *Pachychalina alcaloidifera*[#]

Jaine H. H. L. de Oliveira,[†] Andréa M. Nascimento,[†] Miriam H. Kossuga,[†] Bruno C. Cavalcanti,[‡] Claudia O. Pessoa,[‡]
Manoel O. Moraes,[‡] Mario L. Macedo,[§] Antonio G. Ferreira,[§,⊥] Eduardo Hajdu,[⊥] Ulisses S. Pinheiro,[⊥] and
Roberto G. S. Berlinck*,[†]

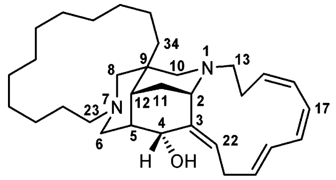
*Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, CEP 13560-970, São Carlos, SP, Brazil,
Laboratório de Oncologia Experimental, Universidade Federal do Ceará, Fortaleza, CE, Brazil, Departamento de Química,
Universidade Federal de São Carlos, São Carlos, SP, Brazil, and Museu Nacional, Universidade Federal do Rio de Janeiro,
Quinta da Boa Vista, s/n, 20940-040, Rio de Janeiro, RJ, Brazil*

Received September 12, 2006

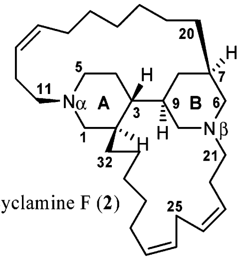
Four bis-piperidine alkaloids, madangamine F (**1**), haliclonacyclamine F (**2**). and arenosclerins D (**3**) and E (**4**), have been isolated from the marine sponge *Pachychalina alcaloidifera* and have been identified by analysis of spectroscopic data. The alkaloids displayed cytotoxic activity against different cancer cell lines. These results support the hypothesis of a common biogenetic origin for the Haplosclerida bis-piperidine and bis-pyridine alkaloids.

Marine sponges belonging to the order Haplosclerida are considered the richest natural source of alkylpiperidine alkaloids presenting diverse carbon skeletons. Recent examples of such alkaloids from *Cribrochalina* spp. include the cytotoxic bis-pyridine pyrinadine A[1] and cribochalines A and B, of which cribochaline A displays antifungal activity against both antibiotic-sensitive and -resistant strains of *Candida* spp.[2] Various *Xestospongia* species are the source of xestospongin and araguspongine alkaloids, which act on Ca^{2+} channels,[3,4] display cytotoxic[5] or antimalarial/antitubercular activity,[6] or are antifungal.[7] Other examples include the cytotoxic pyrinodemins from a marine sponge of the genus *Amphimedon*,[8] unusual oligomeric pyridinium alkaloids such as cyclohaliclonamines from *Haliclona* sp.[9] and viscosamine from *Haliclona viscosa*,[10] and macrocyclic dimeric haliclamines and the linear trimeric viscosaline also from *H. viscosa*.[11,12] Although they do not belong to the 3-alkylpiperidine group of alkaloids, the biogenetically related motuporamines, isolated from *Xestospongia exigua*, display potent anti-invasive activity[13] and are currently under investigation to better establish their mode of action.[14−17] These selected examples illustrate the structural diversity of the Haplosclerida alkylpiperidine alkaloids, which constitute a unique group of secondary metabolites.

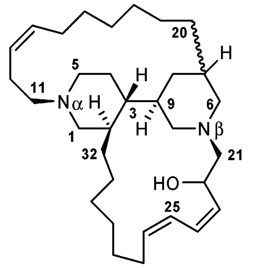
Crude extracts of Haplosclerida sponges very often display biological activities due to the occurrence of biogenetically related alkaloids. Such was the case of *Pachychalina alcaloidifera* (Pinheiro, Berlinck & Hajdu, 2005).[18] We have previously described the isolation of the antitubercular and cytotoxic ingenamine G, of new antibacterial and antifungal cyclostellettamines, and of a dibromotyrosine derivative from *Pachychalina* sp. (=*P. alcaloidifera*).[19] As the sponge taxonomy suggests, *P. alcaloidifera* crude extract presented a very complex mixture of alkaloids. Herein we report the results of the continuing isolation of compounds from this extract, which now include madangamine F (**1**), haliclonacyclamine F (**2**), and arenosclerins D (**3**) and E (**4**), as well as an evaluation of the cytotoxic activity of these compounds against different cancer cells lines.



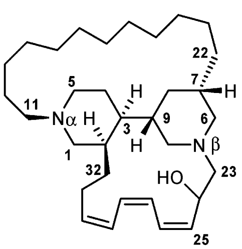
madangamine F (**1**)



haliclonacyclamine F (**2**)



arenosclerin D (**3**)



arenosclerin E (**4**)

Results and Discussion

The alkaloids present in the MeOH crude extract of *P. alcaloidifera* were difficult to separate. Pure alkaloids could be isolated by column chromatography on silica gel using small amounts (10 g or less) of stationary phase, with a gradient of either MeOH in CH_2Cl_2 or a gradient of 1:1 MeOH/MeCN in CH_2Cl_2. Using such

---

[#] Dedicated to Professor Francis J. Schmitz (University of Oklahoma) for his outstanding contributions in the discovery of bioactive natural products.

* To whom correspondence should be addressed. Tel: +55-16-33739954. Fax: +55-16-33739952. E-mail: rgsberlinck@iqsc.usp.br.

[†] Instituto de Química de São Carlos, Universidade de São Paulo.

[‡] Laboratório de Oncologia Experimental, Universidade Federal do Ceará.

[§] Departamento de Química, Universidade Federal de São Carlos.

[⊥] Museu Nacional, Universidade Federal do Rio de Janeiro.

10.1021/np060450q CCC: $37.00

separation conditions we have been able to isolate madangamine F (**1**) and arenosclerin E (**4**) from the basic alkaloid fraction of *P. alcaloidifera*. Haliclonacyclamine F (**2**) and arenosclerin D (**3**) were isolated from the acidic alkaloid fraction, using column chromatography on silica gel and gradients of MeOH in CH_2Cl_2, or 1:1 i-PrOH/MeOH in CH_2Cl_2, 1:1 MeCN/MeOH in CH_2Cl_2, or 3:7 MeOH/EtOAc in CH_2Cl_2 as eluents.

Madangamine F (**1**) was isolated as an optically active glassy solid. The HRESIMS of **1** (479.4003, Δmmu 0.2) indicated the formula $C_{32}H_{51}N_2O$ for the quasi-molecular ion $[M + H]^+$. The presence of a hydroxyl group in the structure of madangamine F was evident from the analysis of the IR (3375 cm^{-1}), ^1H NMR (δ 4.12, m), and ^{13}C NMR (δ 70.1) spectra. Signals for 31 carbons were apparent in the ^{13}C NMR spectrum, indicating an overlap of two ^{13}C signals. The presence of four double bonds was evident by the analysis of the ^{13}C NMR spectra (BBD and DEPT) and included one trisubstituted (δ 141.1 quaternary and 129.3 methine) and three disubstituted double bonds (δ 124.4, 134.8, 140.1, 131.1, 133.5, and 121.2). Therefore, the carbon skeleton presented a pentacyclic system, similar to that in the madangamines[20] and ingamines/ingenamines[21] (herein referred as ingenamines).

The $^1H-^1H$ COSY spectrum showed few $^1H-^1H$ couplings, with a *W* long-range coupling between H-2 (δ 3.97) and H-10b (δ 3.34) as well as between H-2 and H-22 (δ 6.47). Other $^1H-^1H$ couplings were observed between the oxymethine proton H-4 (δ 4.12) and H-5 (δ 1.66), between H-11b (δ 1.30,^5J, long range) and H-21b (δ 2.17, homoallylic coupling), and between H-5 and H-11b. The limited number of $^1H-^1H$ long-range correlations observed in the COSY spectrum of **1** were similar to those observed for madangamine A.[20a]

The HMBC and HSQC-TOCSY spectra of **1** showed relevant $^1H-^{13}C$ long-range correlations between H-2 and C-3, C-5, C-9, C-11, C-12, C-13, C-20 and C-22; H-4 and C-11; H-5 and C-4 and C-24; CH_2-6 and C-5, C-8, C-9, C-23, and C-34; CH_2-8 and C-6, C-9, and C-34; CH_2-10 and C-2, C-8, C-13, and C-34; CH_2-11 and C-12 and C-34; H-12 and C-3, C-9, C-10, C-22, and C-34; H-13b and C-10, H-13a, and C-22; and H-22 and C-2, C-11, and C-12.

The comparison of ^1H and ^{13}C NMR data of **1** with data for the madangamines[20] and ingenamines[21] supported a madangamine central core for **1**. The chemical shift of H-2 (δ 3.97, s) shows a better agreement with the chemical shift of H-2 in the madangamines A−E[20] than the corresponding proton in the ingenamines.[21] The methylene pair CH_2-6 in **1** was observed at δ 2.06 and 3.73, in agreement with the corresponding assignments in madangamines. The β-pseudoaxial proton of the CH_2-6 methylene is strongly shielded in the ingenamines, with chemical shifts typically observed between 1.68 and 1.79.[21a] Moreover, we observed only one allylic methine in **1**, H-2 (δ 3.97), while the ingenamine skeleton has two allylic methine protons, H-2 and H-5.[21] Finally, the methine carbon C-12 (δ 35.2) of **1** shows a chemical shift similar to those of madangamines (δ < 40.1), upfield relative to C-8 in ingenamines (usually δ > 50.0). Considering the preceding arguments, we concluded that **1** had a madangamine skeleton.

The alkyl bridge connecting N-1 and C-3 was identified by analysis of the COSY, HMBC, and HSQC-TOCSY spectra (Table S1 in Supporting Information). Couplings were observed between the CH_2-13 methylene pair and C-10 and C-15, between CH_2-14 and H-15, H-16, C-3, C-15, and C-16, between H-15 and H-16, C-14, and C-16, between H-16 and CH-14, CH-15, and CH-17, between H-17 and H-16, CH-18, and C-19, between H-19 and H-18, C-17, and CH_2-21, and between H-20 and H-2, H-10b, H-21b, C-18, and C-19. The methylene pair CH_2-21 showed couplings with H-4, CH_2-13, H-19, H-20, C-3, C-18, C-19, and C-22. Finally, the proton H-22 showed couplings with H-2 and H-12. Therefore, we defined the N1−C3 bridge as an undeca-3,5,7,10-tetraene unit.

The stereochemistry of the double bonds was established by analysis of the ^1H and ^{13}C NMR and NOESY spectra. The chemical shift of C-14 (δ 28.0) indicated a *Z* stereochemistry of the $\Delta^{15,16}$ double bond. The $\Delta^{17,18}$ double bond was *Z* given the H-17 and H-18 coupling constant (J = 11.2 Hz). The NOESY spectrum indicated NOEs between H-15/H-18 and H-15/H-20, which also supported the proposed stereochemistries of $\Delta^{15,16}$ and $\Delta^{17,18}$. A change in the geometry of the C-3 exocyclic double bond in madangamine F (**1**) relative to previous madangamines[20] was evident, since in **1** the methylene CH_2-21 shows chemical shifts at δ 2.17/2.85 (^1H) and 35.8 (^{13}C), while in madangamines A−E the methylene group between the two double bonds $\Delta^{3,20}$ and $\Delta^{17,18}$ shows chemical shifts at δ 2.32/3.34 (^1H) and 26.8 (^{13}C). Considering that the stereochemistries at $\Delta^{15,16}$ and $\Delta^{17,18}$ were both assigned as *Z* in **1**, it follows that the stereochemistry at $\Delta^{19,20}$ must be *Z*, in order to account for the CH_2-21 ^{13}C chemical shift (δ 35.8).[25]

The relative stereochemistry at C-4 was established as *R** on the basis of the following criteria. Although the ^1H signals of H-4, H-5, and H-12 were observed as broad multiplets, and no information on their relative stereochemistry could be obtained from the analysis of coupling constants, the NOESY spectrum clearly showed NOEs between H-4/H-5, H-4/CH_2-11, and H-4/CH_2-21. Therefore, a *trans*-pseudoaxial relationship between H-4 and H-5 was ruled out. The NOE coupling between H-4 and the methylene CH_2-21 can be observed only if H-4 has a β-pseudoequatorial orientation while the $\Delta^{3,22}$ has the *E* geometry. These data clearly established the relative stereochemistry of madangamine F (**1**).

The remaining $C_{12}H_{24}$ fragment accounted for a saturated chain between N-7 and C-9, similarly to that reported for madangamines D and E.[20b] Analysis of the COSY, HMBC, and HSQC-TOCSY spectra enabled the assignment of this chain (Tables 1 and 2; see also Table S1 in the Supporting Information). Therefore, we have been able to establish the structure of madangamine F as **1**. Madangamine F is the first member of the madangamine group of alkaloids with a C_{10} instead of a C_8 bridge between N-1 and C-3 and the first madangamine with a hydroxyl group at C-4.

Haliclonacyclamine F (**2**) showed signals in the ^{13}C NMR spectra (BBD and DEPT) of four sp^3 methine carbon signals at δ 36.7, 40.6, 36.3, and 42.3, as well as six sp^2 methine groups at δ 124.4, 134.6, 129.2, 126.8, 124.9, and 136.0. Therefore, the tetracyclic nature of **2** was deduced from the HRESIMS, which indicated the formula $C_{32}H_{55}N_2$ (measd 467.4364, Δmmu 0.3) for the quasi-molecular ion $[M + H]^+$. Typical N-bonded methylene resonances at δ 56.2, 51.7, 57.5, 59.6, 47.9, and 56.9 indicated a haliclonacyclamine/arenosclerin skeleton for haliclonacyclamine F (**2**).[22,23] The comparison of the ^1H and ^{13}C NMR data of **2** with the data reported for haliclonacyclamine E and arenosclerins A−C,[22] haliclonacyclamines A−D,[23b] halichondramine,[23c] and 22-hydroxyhalicyclamine A[23d] allowed us to verify that **2** was a new alkaloid.

The assignment of the ^1H and ^{13}C resonances of rings A and B in **2** was approached using a combined interpretation of COSY, HMBC, and HSQC-TOCSY spectra. For the construction of ring A, key long-range correlations were observed in the HSQC-TOCSY spectrum between H-11b and C-1, between H-4b and C-2, between H-1 and C-3, between H-2 and C-4, between H-3 and C-4, between H-9 and C-4, between both protons of CH_2-11 and C-5, and between H-4b and C-5. The HMBC spectra showed correlations between H-1 and C-2 and C-3, between H-5 and C-3, between H-4b and C-3, and between H-11b and C-5. Finally, the COSY spectrum showed correlations between CH_2-4 and CH_2-5 and CH_2-11. Ring B of **2** was constructed in a similar way. The HSQC-TOCSY spectrum showed couplings between H-8a and C-6 and C-7, between H-9 and C-7, between CH_2-10 and C-7, between CH_2-19 and C-7, between H-7 and C-8, between H-19b and C-8, between H-8a and C-9, and between both H-21b and H-22b and C-10. The HMBC spectra indicated couplings from C-6 to H-8a, H-10a, and

Table 1. ^1H NMR Data for Alkaloids **1−4** (CD_3OD)

position	**1**[a]	**2**[a]	**3**[b]	**4**[a]
1		3.25 (m)	2.91 (m); 2.89 (m)	3.30 (m)
2	3.97 (s)	1.86 (m)	1.65 (m)	1.93 (m)
3		2.03 (m)	1.89 (m)	2.12 (m)
4	4.12 (m)	2.12 (m); 2.05 (m)	1.99 (dd, 5, 10); 1.82 (m)	2.18 (m)
5	1.66 (m)	3.22 (m)	3.19 (t. 12.6); 2.95 (dd, 7, 11)	3.28 (m); 3.17 (t, 11.8)
6	3.73 (m); 2.06 (m)	3.27 (m); 2.66 (m)	2.83 (d, 10); 2.16 (dd, 6, 11)	3.44 (m); 2.64 (m)
7		1.91 (m)	1.52 (m)	1.98 (m)
8	3.77 (m); 2.09 (m)	2.32 (m); 1.26 (m)	2.31 (d, 11), 0.91 (dd, 7, 11)	2.33 (m); 1.30 (m)
9		2.18 (m)	1.76 (bt)	2.26 (m)
10	4.02 (m); 3.34 (m)	3.34 (m); 3.08 (m)	2.81 (t, 12); 2.62 (d, 11)	3.47 (m); 3.03 (t, 12.4)
11	1.68 (m); 1.30 (m)	3.44 (m); 3.26 (m)	3.01 (t, 8)	3.46 (m); 3.28 (m)
12	2.92 (m)	2.65 (m); 2.55 (m)	2.50 (dd, 6, 15); 2.44 (dd, 7, 15)	2.22 (m); 1.98 (m)
13	3.85 (m); 2.68 (m)	5.30 (m)	5.31 (ddd, 5.5, 6, 11)	1.38 (m)
14	2.24 (m); 1.88 (m)	5.70 (m)	5.59 (m)	1.52 (m)
15	5.34 (m)	2.21 (m); 1.94 (m)	2.12 (m)	1.48 (m)
16	5.79 (m)	1.58 (m); 1.14 (m)	2.03 (m)	1.40 (m)
17	7.38 (dd, 15, 11)	1.38 (m)	1.30 (m)[d]	1.44 (m)
18	6.24 (t, 11)	1.48 (m)	1.36 (m)[d]	1.56 (m)
19	5.88 (m)	1.46 (m); 1.32 (m)	1.45 (m)	1.60 (m)
20	5.84 (m)	1.44 (m)	1.98 (m)	1.58 (m); 1.14 (m)
21	2.85 (m); 2.17 (m)	3.46 (m); 3.38 (m)	3.08 (m); 2.90 (m)	1.44 (m)
22	6.47 (m)	2.49 (m); 1.31 (m)	4.90[c]	1.35 (m)
23	3.28 (m); 2.98 (m)	5.55 (m)	5.49 (t, 10)	3.37 (m)
24	1.40 (m)	6.46 (m)	6.46 (t, 11)	5.05 (m)
25	1.42 (m); 1.24 (m)	3.09 (m); 2.44 (m)	6.49 (t, 11)	5.62 (dd, 10, 11.4)
26	1.38 (m)	6.44 (m)	5.56 (m)	6.55 (q, 11.4)
27	1.52 (m)	5.57 (m)	2.56 (d, 11); 1.51 (m)	5.68 (m)
28	1.86 (m)	2.03 (m); 1.42 (m)	1.33 (m)[d]	5.32 (ddd, 6.2, 11.5, 11.4)
29	1.62 (m)	1.48 (m)	1.45 (m)[d]	5.71 (m)
30	1.63 (m)	1.42 (m)	1.33 (m)[d]	6.49 (q, 11.4)
31	1.70 (m)	1.57 (m)	1.29 (m)[d]	2.67 (m); 2.52 (m)
32	1.62 (m)	1.65 (m); 1.48 (m)	1.33 (m)	2.05 (m); 2.53 (m)
33	1.42 (m)			
34	1.94 (m); 1.80 (m)			

[a] 400 MHz. [b] 500 MHz. [c] Overlapped by the H_2O signal. [d] Assignments by comparison with literature data; in the text, a and b denote upfield and downfield resonances respectively of a geminal pair.

H-21a, from C-7 to CH_2-8, CH_2-6, and H-19b, from C-8 to CH_2-6 and H-20, from C-9 to H-4b, H-8b, H-10b, and H-19b (or H-22b), and from C-10 to H-8a, H-9, and H-21b. The COSY spectrum showed correlations between H-7 and both protons of CH_2-6 and H-8a, between H-8b and H-9, between H-19b and H-20, between H-9 and H-21b, and between H-10b and H-22a.

Analysis of COSY, HMBC, and HSQC-TOCSY spectra, as well as comparison with data of haliclonacyclamine E and arenosclerins A−C, indicated that the CH_2-11/CH_2-20 bridge of compound **2** was identical to the same moiety present in the alkaloids isolated from *Arenosclera brasiliensis*.[22] A *Z* double bond was positioned at C-13/C-14, and the remaining assignments of methylene groups were established by analysis of the COSY, HMBC, HSQC-TOCSY, and NOESY spectra (Table S2 in the Supporting Information).

The bridge between Nβ and C-2 also showed an N-substituted homoallylic spin system. Sequential couplings from CH_2-21 to CH-24 were clearly observed in the COSY, HMBC, and HSQC-TOCSY spectra. The methylene group at C-25 (^1H δ 2.44 and 3.09; ^{13}C δ 22.6) was located between the $\Delta^{23,24}$ double bond and a second double bond at CH-26 (δ 6.44) and CH-27 (δ 5.57). The $\Delta^{26,27}$ double bond was followed by a five methylene carbon chain, C-28 to C-32, which could be assigned by extensive analysis of COSY, HMBC, and HSQC-TOCSY spectra. A correlation observed between C-32 and H-4b (^1H δ 2.12) in the HSQC-TOCSY spectrum established the attachment of this chain with the bis-piperidine spin system. The stereochemistries of both $\Delta^{23,24}$ and $\Delta^{26,27}$ double bonds were assigned as *Z* considering the ^{13}C chemical shifts of C-22 (δ 26.6), C-25 (δ 22.6), and C-28 (δ 26.4). Therefore, the C_{12} chain of **2** consisted of a 1-amino-3(*Z*),6(*Z*)-dienedodecane moiety, which appears to be unprecedented in the literature based on searches in literature databases (MARINLIT and SciFinder).

The relative stereochemistry of the bis-piperidine moiety in haliclonacyclamine F could be established by analysis of the NOESY spectrum, which showed dipolar couplings between H-2 and H-4b, between H-2 and H-9, between H-2 and H-10a, between H-6b and H-10b, and between H-8b and H-10b. Therefore, the relative stereochemistry of the piperidine ring A in **2** is the same relative stereochemistry of haliclonacyclamine E and arenosclerin A.[22] A comparison of the ^{13}C chemical shifts of C-1 to C-5 in these alkaloids showed a good agreement. The relative stereochemistry of ring B in **2** could not be unambiguously established since we have not observed NOE dipolar couplings for H-7. However, since the ^{13}C chemical shifts of C-6−C-10 are practically identical to those of arenosclerin C,[22] the proposed relative stereochemistry of ring B in compound **2** is shown in Figure 1a.

Arenosclerin D (**3**) was obtained as a colorless, glassy solid, which displayed a quasi-molecular ion $[M + H]^+$ in the HRFABMS at *m*/*z* 483.43089 (calcd 483.43144, Δmmu 0.5), corresponding to the formula $C_{32}H_{55}N_2O$ with seven degrees of unsaturation. The presence of three double bonds (δ 126.3, 133.4, 135.0, 126.5, 125.1, and 136.0) and a carbinol carbon (δ 61.7) in the ^{13}C NMR spectrum indicated that **3** had a tetracyclic structure. The close relationship of arenosclerin D (**3**) with arenosclerins A−C[22] was evident by comparison of ^1H and ^{13}C NMR data. A careful analysis of the COSY, HMBC, and HSQC-TOCSY confirmed that compound **3** was a member of the arenosclerins and very similar to arenosclerin C (Tables 1 and 2 and Table S3 in the Supporting Information). However, minor differences observed for the ^{13}C NMR and ^1H NMR chemical shifts, in particular for H-2, H-7, H-9, CH_2-20, and C-3, suggested a different stereoisomer within the bis-piperidine moiety.

The relative stereochemistry of the bis-piperidine moiety in **3** was tentatively defined by analysis of the ^1H NMR, NOESY, and ROESY spectra. On the basis of the chemical shift and coupling constants of H-8b (δ 0.91, dd, 7 and 11 Hz), we inferred this proton was in a pseudoaxial orientation in compound **3**.[19a] Proton H-8a at

Table 2. ^{13}C NMR Data (CD_3OD) for the Alkaloids **1−4**

position	1^a	2^a	3^b	4^a
1		56.2	52.2	56.2
2	59.5	36.7	38.2	36.2
3	141.1	40.6	41.5	40.4
4	70.1	33.3	35.2	33.0
5	41.5	51.7	47.5	51.8
6	50.6	57.5	59.3	58.8
7		36.3	37.8	36.1
8	50.7	36.1	38.4	35.7
9	42.5	42.3	44.9	41.9
10	56.4	59.6	61.2	59.9
11	32.4	47.9	57.0	48.1
12	35.2	20.5	20.6	27.3
13	56.0	124.4	126.3	32.7
14	28.0	134.6	133.4	27.8
15	124.4	27.3	27.1	29.2
16	134.8	33.9	28.1	28.4
17	140.1	27.8	29.1^c	28.9
18	131.1	28.8	33.9^c	28.6
19	133.5	29.0	28.7	25.8
20	121.2	32.8	26.8	33.8
21	35.8	56.9	64.3	28.8
22	129.3	26.6	61.7	26.9
23	60.4	129.2	135.0	62.2
24	23.0	126.8	126.5	61.7
25	28.8	22.6	125.1	132.7
26	23.6	124.9	136.0	127.8
27	27.4	136.0	26.5	137.6
28	24.6	26.4	28.8^c	124.6
29	27.2	28.6	28.6^c	134.4
30	27.2	29.1	29.5^c	124.7
31	25.4	26.0	26.6^c	20.5
32	27.0	28.5	29.0	26.9
33	22.3			
34	38.9			

[a] Assignments by inverse detection at 100 MHz (HSQC). [b] Assignments by inverse detection at 125 MHz (HSQC). [c] Assignments by comparison with literature data.

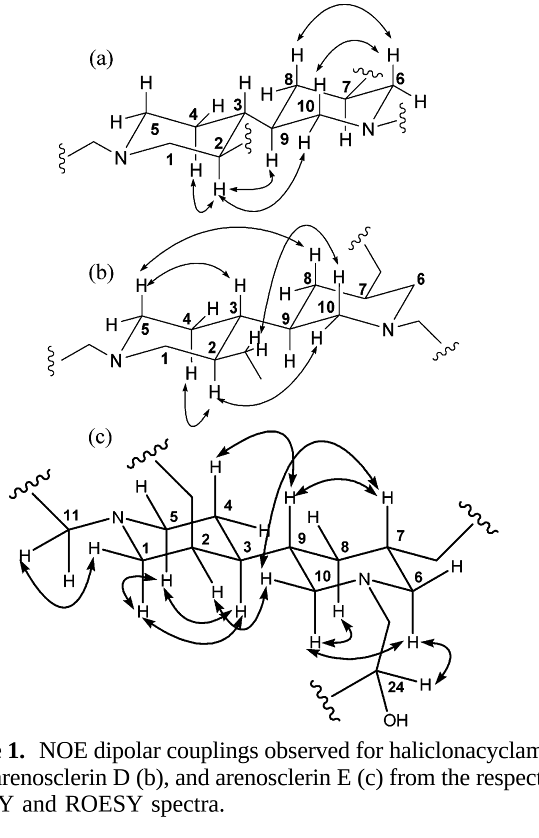


Figure 1. NOE dipolar couplings observed for haliclonacyclamine F (a), arenosclerin D (b), and arenosclerin E (c) from the respective NOESY and ROESY spectra.

δ 2.31 showed a geminal 11 Hz coupling constant; therefore the 7 Hz coupling of H-8b was to either H-7 (δ 1.52) or H-9 (δ 1.76), one of which was therefore in a pseudoequatorial position relative to H-8b. NOE couplings were observed between H-8b and H-5b (δ 2.95), between H-5b and H-3 (δ 1.89), between H-2 (δ 1.65) and H-10b (δ 2.62), and between H-10a (δ 2.81) and the methylene CH_2-32 (δ 1.33). A conformation and a relative stereochemistry at C-2, C-3, and C-9 that can accommodate such couplings is depicted in Figure 1. There were no observed NOE couplings for H-7 or CH_2-20; hence the relative stereochemistry at C-7 was not defined. Therefore, the structure of arenosclerin D (**3**) is tentatively proposed as a distinct stereoisomer of arenosclerins A−C.[22]

Arenosclerin E (**4**) was obtained as a colorless, glassy solid. The HRFABMS of **4** indicated a quasi-molecular ion $[M + H]^+$ at m/z 483.4305 (calcd 483.4314, Δmmu −1.9), corresponding to the formula $C_{32}H_{55}N_2O$. Since three double bonds were detected in the ^{13}C NMR spectrum of **4** (δ 132.7, 127.8, 137.6, 124.6, 134.4, and 124.7), the compound possessed a tetracyclic skeleton, clearly related to the arenosclerins due to the presence of a hydroxyl group observed in the IR (ν 3385 cm^{-1}), ^{1}H NMR (δ 5.05), and ^{13}C NMR spectra (δ 61.7). Analysis of the COSY, HMBC, and HSQC-TOCSY confirmed this hypothesis and enabled assignment of the bis-piperidine central core of compound **4** (Tables 1 and 2 and Table S4 in the Supporting Information).

The alkyl bridge connecting Nα to C-7 in **4** was shown to be completely saturated, and only a few correlations were observed for it, namely, from H-2, H-4a, and H-5a to C-11, between H-5a and C-12, between H-7 and C-22, between H-12a and C-14, between CH_2-13 and C-15, between CH_2-17 and C-16, between CH_2-22 and C-17, between CH_2-25 and C-17, and between H-22a and C-21.

In order to establish the structure of the Nβ−C-2 alkyl bridge, analysis of the COSY spectrum showed correlations between CH_2-23 and CH_2-6 as well as between CH_2-23 and H-10a, indicating a mutual connection of CH_2-6, CH_2-10, and CH_2-23 through Nβ. These assignments were confirmed by analysis of the HMBC spectra. Once we assigned the CH_2-23 methylene group, we observed in the COSY spectrum sequential ^{1}H−^{1}H couplings from CH_2-23 to CH_2-32 through a conjugated spin system composed of three double bonds, all of which were *Z* due to the coupling constants between H-25 and H-26 (10.0 Hz), between H-27 and H-28 (11.5 Hz), and between H-29 and H-30 (11.4 Hz). Moreover, the chemical shifts of C-24 (δ 61.7) and C-31 (δ 20.5) also indicated a *Z* geometry for the $\Delta^{25,26}$ and $\Delta^{29,30}$ double bonds. Several ^{1}H−^{1}H and long-range ^{1}H−^{13}C couplings were observed and enabled us to unambiguously assign all ^{1}H and ^{13}C resonances of this chain (see Tables 1, 2 as well as Table S4 in the Supporting Information). The connection of CH_2-32 to the bis-piperidine system was established by long-range correlations observed in the HMBC and HSQC-TOCSY spectra between CH_2-32 (δ 2.05 and 2.53) and C-2 (δ 36.2), between CH_2-31 (δ 2.52 and 2.67) and C-2, and between CH_2-32 and C-3. Therefore, this chain was defined as a (3*Z*,5*Z*,7*Z*)-1-aminodeca-3,5,7-trien-2-ol spin system, which is unprecedented in the literature. As far as we know, arenosclerin E (**4**) is the first arenosclerin/haliclonacyclamine bis-piperidine alkaloid with a C_{10} chain connecting Nβ to C-2. The hipsocromic UV absorption of **4** (λ_{max} 235 nm) is probably due to the fact that the *Z,Z,Z* triene chromophore is distorted due to angle strain within the chain.

The relative stereochemistry in the bis-piperidine moiety was established by analysis of ROESY and NOESY spectra. Several dipolar couplings were observed, most importantly between CH_2-1, H-3, and H-5b, positioning these hydrogens α-axially. Dipolar couplings observed between H-4b and H-9, between H-9 and H-7, and between H-7 and H-10a, in addition to NOEs observed between H-6b and H-10b and between H-10b and H-8b, indicated a conformation of the B ring where H-9 and H-7 are β-axially oriented while H-6b, H-8b, and H-10b are α-axially oriented. Further dipolar couplings observed between H-2 and H-10a as well as between H-32b and H-9 indicated the relative configuration at C-2 and enabled us to establish the relative stereochemistry of arenosclerin

Table 3. Cytotoxic Activity of Alkaloids **1**−**4** Aginst Cancer Cell Lines (μg/mL)

	cell lines[a]			
alkaloid	SF 295	MDA-MB435	HCT8	HL60
1	19.8	16.2	>25	16.7
2	4.5	1.0	8.6	2.2
3	5.9	1.2	6.2	2.1
4	8.7	3.1	>25	6.9

[a] Cell lines: SF 295 (human CNS), MDA-MB435 (human breast), HCT8 (colon), and HL60 (leukemia).

E (**4**) as depicted in Figure 1c. Several other NOEs observed within the C_{10} polyunsaturated chain confirmed the positioning and the stereochemistry of the double bonds.

The alkaloids **1**−**4** were tested in cytotoxic assays against SF 295 (human CNS), MDA-MB435 (human breast), HCT8 (colon), and HL60 (leukemia) cancer cell lines using the MTT method (Table 3). Haliclonacyclamine F (**2**) and arenosclerin D (**3**) were the most active alkaloids, followed by arenosclerin E (**4**) and madangamine F (**1**). The results suggest that each of these alkaloids may have a distinct cytotoxicity mode of action depending on the three-dimensional structure of each compound.

The isolation of madangamine F (**1**), haliclonacyclamine F (**2**), and arenosclerins D (**3**) and E (**4**), along with ingenamine G and several cyclostellettamines[19] from *P. alcaloidifera* is strong support for a common biogenetic pathway for these alkaloids. The occurrence of a hydroxylated madangamine in *P. alcaloidifera* is noteworthy, considering that the madangamine skeleton is supposed to be biogenetically derived from an ingenamine skeleton.[20a] The hydroxyl position in **1** corresponds to the C-4 position in the putative ingenamine precursor, which is commonly unsaturated at $\Delta^{3,4}$ and, therefore, is susceptible to an enzyme-mediated addition of H_2O. The sponge *P. alcaloidifera* presents a unique chemical profile, composed of alkaloids belonging to four distinct structural classes. To the best of our knowledge, no other Haplosclerida sponge presents such a variety of bis-piperidine and bis-pyridine alkaloids. Although several other alkaloid-containing fractions have been obtained from the MeOH extract of *P. alcaloidifera*, the availability of only small amounts (0.5−2 mg) made their isolation and identification difficult.

Experimental Section

General Experimental Procedures. The general experimental procedures have been previously described,[19a] except for ^{1}H NMR spectra recorded at 500 MHz and ^{13}C NMR spectra recorded at 125 MHz on a Bruker DRX500 11.7 T NMR spectrometer, referenced relative to the signal of TMS.

Animal Material. Same as previously reported.[19a]

Isolation of Compounds 1−4. The CH_2Cl_2 fraction (0.87 g) obtained from the crude extract upon alkaline partitioning, as previously described,[19a] was subjected to flash column chromatography on cyanopropyl-bonded SiOH Waters Sep Pak (10 g) eluted with a gradient of MeOH in CH_2Cl_2. Two fractions were obtained from this separation, F1 (0.797 g) and F2 (0.075 g). Fraction F1 was subjected to flash column chromatography on a SiOH Waters Sep Pak column (10 g) eluted with a gradient of MeOH in CH_2Cl_2. This separation resulted in four fractions, F1A (0.097 g), F1B (0.295 g), F1C (0.165 g), and F1D (0.232 g). Fraction F1C was further separated by flash column chromatography on a SiOH Waters Sep Pak column (10 g) and eluted with a gradient of MeOH in CH_2Cl_2, to give four fractions, F1C1 (0.0105 g), F1C2 (0.0336 g), F1C3 (0.0376 g), and F1C4 (0.0635 g). Fraction F1C3 was purified by flash column chromatography on a SiOH Waters Sep Pak (5 g) eluted with a gradient of 1:1 MeOH/MeCN in CH_2Cl_2, to give 0.0040 g (2.0×10^{-4}%, wet) of madangamine F (**1**). Fraction F1D was subjected to flash column chromatography on a SiOH Waters Sep Pak (10 g) eluted with a gradient of MeOH in CH_2Cl_2. The major fraction obtained, F1D5 (0.0410 g), was further purified by flash column chromatography on a Waters Sep Pak (5 g) eluted with a gradient of 1:1 MeOH/MeCN in CH_2Cl_2, resulting in the isolation of 0.0184 g (9.2×10^{-4}% wet) of arenosclerin E (**4**).

The CH_2Cl_2 fraction (5.03 g) obtained from the crude extract upon acidic partitioning, as described precedingly,[19a] was subjected to chromatographic separation on a cyanopropyl-bonded SiOH column (Waters Sep Pak, 10 g) eluted with a gradient of MeOH in CH_2Cl_2. Five fractions were obtained, E1 (3.050 g, mostly pigments), E2 (108 mg), E3 (150 mg), E4 (1.150 g), and E5 (280 mg). The E4 fractions was divided in two portions, E4 and E4′. The E4 fraction (0.328 g) was subjected to flash column chromatography on a cyanopropyl-bonded SiOH Waters Sep Pak (10 g) eluted with a gradient of MeOH in CH_2Cl_2. Four fractions were obtained: E4A (0.107 g), E4B (0.150 g), E4C (1.150 g), and E4D (0.280 g). Fraction E4C was separated by flash column chromatography on a SiOH Waters Sep Pak (10 g) eluted with a gradient of MeOH in CH_2Cl_2, resulting in seven fractions, E4C1 to E4C7. Fraction E4C5 (0.051 g) was separated by flash column chromatography on a Waters Sep Pak column (5 g) eluted with a gradient of 1:1 MeOH/i-PrOH in CH_2Cl_2 and resulted in five fractions, E4C5A to E4C5E. Fraction E4C5E (0.0150 g) was purified by flash column chromatography on a Waters Sep Pak column (5 g) eluted with a gradient of 1:1 MeOH/i-PrOH in CH_2Cl_2 and yielded 0.0044 g (2.2×10^{-4}% wet) of haliclonacyclamine F (**2**). The E4′ fraction (0.820 g) was subjected to flash column chromatography on a SiOH Waters Sep Pak (10 g) eluted with a gradient of MeOH in CH_2Cl_2. Four fractions have been obtained from this separation: E4′A to E4′D. The fraction E4′C (0.465 g) was subjected to column chromatography on a SiOH Merck Lobar column (A size, 240 × 10 mm) eluted with a gradient of MeOH in CH_2Cl_2, resulting in three fractions: E4′C1 (0.216 g), E4′C2 (0.220 g), and E4′C3 (0.027 g). Fraction E4′C2 was subjected to flash column chromatography on a Waters Sep Pak (10 g) eluted with a gradient of 7:3 EtOAc/MeOH in CH_2Cl_2. Six fractions were obtained, E4′C2A to E4′C2F. Fraction E4′C2F (0.0557 g) was separated by flash column chromatography on a SiOH Waters Sep Pak (2 g) eluted with a gradient of 7:3 EtOAc/MeOH in CH_2Cl_2. This separation resulted in three fractions, E4′C2E1 to E4′C2E3. Fraction E4′C2E3 (0.0200 g) was purified by flash column chromatography on a Waters Sep Pak (2 g), resulting in the isolation of 0.0143 g of arenosclerin D (**3**) (7.1×10^{-4}%, wet).

Madangamine F (1): colorless, glassy solid; $[\alpha]_D^{25}$ −32.5 (*c* 0.004, MeOH); UV (MeOH) λ_{max} 237 nm (ϵ 22 480); IR (film) 3375, 2932, 2861, 1654, 1463, 1007 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; positive ESIMS *m/z* 479.5 $[M + H]^+$ (93%), 399.4 (100%), 370.4 (17%), 338.4 (63%); HRESIMS *m/z* found 479.4003 $[M + H]^+$, calcd for $C_{32}H_{51}N_2O$ 479.4001 $[M + H]^+$.

Haliclonacyclamine F (2): colorless, glassy solid; $[\alpha]_D^{25}$ +5.4 (*c* 0.0041, MeOH); UV (MeOH) λ_{max} 226 nm (ϵ 2,450); IR (film) 3385, 2928, 2854, 1634, 1460, 992 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; positive ESIMS *m/z* 467.3 $[M + H]^+$ (100%); HRESIMS *m/z* found 467.4364 $[M + H]^+$, calcd for $C_{32}H_{55}N_2$ 467.4365 $[M + H]^+$.

Arenosclerin D (3): colorless, glassy solid; $[\alpha]_D^{25}$ +6.9 (*c* 0.014, MeOH); UV (MeOH) λ_{max} 236 nm (ϵ 28 456); IR (film) 3294, 2925, 2855, 1649, 1599, 1455, 1358, 1272, 1018, 733 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR (CD_3OD, 125 MHz), see Table 2; positive FABMS *m/z* 483 $[M + H]^+$ (trace); HRESIMS *m/z* found 483.43089 $[M + H]^+$, calcd for $C_{32}H_{55}N_2O$ 483.43144 $[M + H]^+$.

Arenosclerin E (4): colorless, glassy solid; $[\alpha]_D^{25}$ +14.5 (*c* 0.015, MeOH); UV (MeOH) λ_{max} 235 nm (ϵ 23 780); IR (film) 3385, 2931, 2861, 1641, 1460, 997 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; positive ESIMS *m/z* 483.7 $[M + H]^+$ (100%); HRESIMS *m/z* found 483.4305 $[M + H]^+$, calcd for $C_{32}H_{55}N_2O$ 483.4314 $[M + H]^+$.

Acknowledgment. The authors thank M. J. Garson (Department of Chemistry, The University of Queensland, Brisbane, Australia) for a careful revision of the manuscript. The authors also thank D. E. Williams and R. J. Andersen (Departments of Chemistry and Earth and Ocean Sciences, University of British Columbia, Vancouver, Canada) and N. Pearce and B. R. Copp (Department of Chemistry, University of Auckland, Auckland, New Zealand) for their assistance in obtaining MS analyses, as well as V. Glass and G. V. J. da Silva (Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo) for providing NMR analyses at 500 MHz. Financial support was provided by FAPESP Grant 01/03095-0 to R.G.S.B., by FAPESP scholarships 00/07457-6 to

J.H.H.L.O. and 03/10805-4 to A.M.N., by a CAPES scholarship to M.H.K, and by CNPq scientific research awards to A.G.F., E.H., and R.G.S.B.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kariya, Y.; Kubota, T.; Fromont, J.; Kobayashi, J. *Tetrahedron Lett*. **2006**, *47*, 997−998.

(2) Nicholas, G. M.; Molinski, T. F. *Tetrahedron* **2000**, *56*, 2921−2927.

(3) Ta, T. A.; Feng, W.; Molinski, T. F.; Pessah, I. N. *Mol. Pharm*. **2006**, *69*, 532−538.

(4) Jaimovich, E.; Mattei, C.; Liberona, J. L.; Cardenas, C.; Estrada, M.; Barbier, J.; Debitus, C.; Laurent, D.; Molgo, F. *FEBS Lett*. **2005**, *579*, 2051−2057.

(5) Liu, H.; Mishima, Y.; Fujiwara, T.; Nagai, H.; Kitazawa, A.; Mine, Y.; Kobayashi, H.; Yao, X.; Yamada, J.; Oda, T.; Namikoshi, M. *Mar. Drugs* **2004**, *2*, 154−163.

(6) Orabi, K. Y.; El Sayed, K. A.; Hamann, M. T.; Dunbar, D. C.; Al-Said, M.S.; Higa, T.; Kelly, M. *J. Nat. Prod*. **2002**, *65*, 1782−1785.

(7) Moon, S.-S.; MacMillan, J. B.; Olmstead, M. M.; Ta, T. A.; Pessah, I. N.; Molinski, T. F. *J. Nat. Prod*. **2002**, *65*, 249−254.

(8) (a) Tsuda, M.; Hirano, K.; Kubota, T.; Kobayashi, J. *Tetrahedron Lett*. **1999**, *40*, 4819−4820. (b) Hirano, K.; Kubota, T.; Tsuda, M.; Mikami, Y.; Kobayashi, J. *Chem. Pharm. Bull*. **2000**, *48*, 974−977.

(9) Teruya, T.; Kobayashi, K.; Suenaga, K.; Kigoshi, H. *J. Nat. Prod*. **2006**, *69*, 135−137.

(10) Volk, C. A.; Köck, M. *Org. Lett*. **2003**, *5*, 2567−3569.

(11) Volk, C. A.; Lippert, H.; Lichte, E.; Koeck, M. *Eur. J. Org. Chem*. **2004**, 3154−3158.

(12) Volk, C. A.; Köck, M. *Org. Biomol. Chem*. **2004**, *2*, 1827−1830.

(13) (a) Williams, D. E.; Lassota, P.; Andersen, R. J. *J. Org. Chem*. **1998**, *63*, 4838−4841. (b) Williams, D.E.; Craig, K. S.; Patrick, B.; McHardy, L. M.; van Soest, R.; Roberge, M.; Andersen, R. J. *J. Org. Chem*. **2002**, *67*, 245−258.

(14) Roskelley, C. D.; Williams, D. E.; McHardy, L. M.; Leong, K. G.; Troussard, A.; Karsan, A.; Andersen, R. J.; Dedhar, S.; Roberge, M. *Cancer Res*. **2001**, *61*, 6788−6794.

(15) McHardy, L. M.; Sinotte, R.; Troussard, A.; Sheldon, C.; Church, J.; Williams, D. E.; Andersen, R. J.; Dedhar, S.; Roberge, M.; Roskelley, C. D. *Cancer Res*. **2004**, 1468−1474.

(16) (a) Kaur, N.; Delcros, J.-G.; Martin, B.; Phanstiel, O. *J. Med. Chem*. **2005**, *48*, 3832−3839. (b) Breitbeil, F., III; Kaur, N.; Delcros, J. G.; Martin, B.; Abboud, K. A.; Phanstiel, O., IV. *J. Med. Chem*. **2006**, *49*, 2407−2416.

(17) To, K. C. W.; Loh, K. T.; Roskelley, C. D.; Andersen, R. J.; O'Connor, T. P. *Neuroscience* **2006**, *139*, 1263−1274.

(18) Pinheiro, U. S.; Berlinck, R. G. S.; Hajdu, E. *Contrib. Zool*. **2005**, *74*, 271−278.

(19) (a) Oliveira, J. H. H. L.; Grube, A.; Köck, M.; Berlinck, R. G. S.; Macedo, M. L.; Ferreira, A. G.; Hajdu, E. *J. Nat. Prod*. **2004**, *67*, 1685−1689. (b) Oliveira, J. H. H. L.; Seleghim, M. H. R.; Timm, C.; Grube, A.; Köck, M.; Nascimento, G. G. F.; Martins, A. C. T.; Silva, E. G. O.; Souza, A. O.; Galetti, F. C. S.; Minarini, P. R. R.; Silva, C. L. L.; Hajdu, E.; Berlinck, R. G. S. *Mar. Drugs* **2006**, *4*, 1−8. (c) Oliveira, M. F.; Oliveira, J. H. H. L.; Galetti, F. C. S.; Souza, A. O.; Silva, C. L.; Hajdu, E.; Peixinho, S.; Berlinck, R. G. S. *Planta Med*. **2006**, *72*, 437−441.

(20) (a) Kong, F.; Andersen, R. J.; Allen, T. M. *J. Am. Chem. Soc*. **1994**, *116*, 6007−6008. (b) Kong, F.; Graziani, E. I.; Andersen, R. J. *J. Nat. Prod*. **1998**, *61*, 267−271.

(21) (a) Kong, F.; Andersen, R. J. *Tetrahedron* **1995**, *51*, 2895−2906. (b) Kong, F.; Andersen, R. J.; Allen, T. M. *Tetrahedron* **1994**, *50*, 6137−6144.

(22) Torres, Y. R.; Berlinck, R. G. S.; Magalhães, A.; Schefer, A. B.; Ferreira, A. G.; Hajdu, E.; Muricy, G. *J. Nat. Prod*. **2000**, *63*, 1098−1105.

(23) (a) Charan, R. D.; Garson, M. J.; Brereton, I. M.; Willis, A. C.; Hooper, J. N. A. *Tetrahedron* **1996**, *52*, 9111−9120. (b) Clark, R. J.; Field, K. L.; Charan, R. D.; Garson, M. J.; Brereton, I. M.; Willis, A. C. *Tetrahedron* **1998**, *54*, 8811−8826. (c) Chill, L.; Yosief, T.; Kashman, Y. *J. Nat. Prod*. **2002**, *65*, 1738−1741. (d) Matsunaga, S.; Miyata, Y.; van Soest, R. W. M.; Fusetani, N. *J. Nat. Prod*. **2004**, *67*, 1758−1760.

(24) See for example: (a) Fontana, A.; González, M. C.; Gavagnin, M.; Templado, J.; Cimino, G. *Tetrahedron Lett*. **2000**, *41*, 429−432. (b) Durán, R.; Zubía, E.; Ortega, M. J.; Naranjo, S.; Salvá, J. *Tetrahedron* **2000**, *56*, 6031−6037.

(25) In fact, the madangamines A−E have the C-3/C-20 double bond with *Z* stereochemistry, which is the inverse geometry of the C-3/C-22 double bond in madangamine F (**1**), since in this case the presence of the hydroxyl group at C-4 changes the nomenclature priority.

NP060450Q