Minor Steroidal Alkaloids from the Marine Sponge Corticium sp.

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Minor Steroidal Alkaloids from the Marine Sponge *Corticium* sp.[#]

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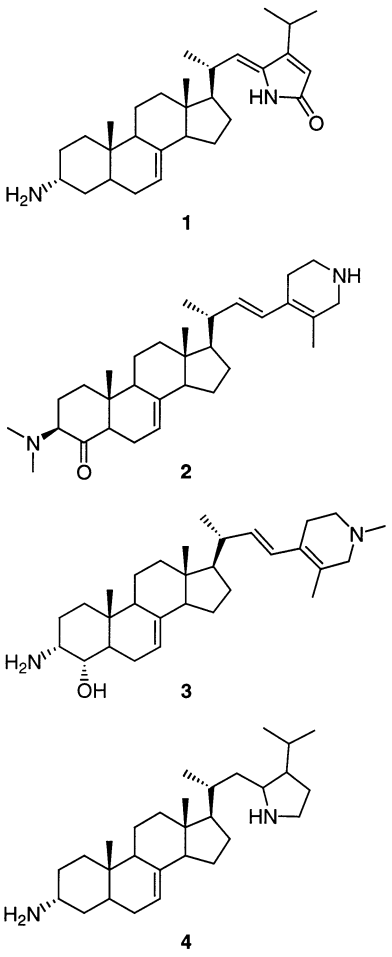
Four new steroidal alkaloids, plakinamine G (**1**), plakinamine H (**2**), 4α-hydroxydemethylplakinamine B (**3**), and tetrahydroplakinamine A (**4**), along with three known compounds, were isolated from the marine sponge *Corticium* sp. The structures of these metabolites were elucidated largely by 1D and 2D NMR methods and accurate mass measurements (HR-EIMS). Compounds **1**, **2**, and **4** show significant in vitro cytotoxicity.

Among marine organisms sponges are renowned for their ability to produce novel natural products with unique structures and biological activities.[1−3] These invertebrates contain a vast array of polyketides, terpenes, alkaloids, pigments, and cyclic peptides[4] but only a few steroidal alkaloids.[5−9] During our investigation of bioactive compounds from marine organisms, we previously reported the structural elucidation of seven steroidal alkaloids from a sample of *Corticium* sp. collected off Porth Havannah, Vanuatu, South Pacific.[7,8] A second collection of *Corticium* sp. contained a different array of metabolites that included the known steroidal alkaloids *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B, 24,25-dihydroplakinamine A, and *N*-methyltetrahydroplakinamine A.[8] Four new steroidal alkaloids, plakinamines G−H (**1**, **2**), 4α-hydroxydemethylplakinamine B (**3**), and tetrahydroplakinamine A (**4**), were isolated.

The freeze-dried sponge was extracted with methanol, and the methanolic extract was subjected to a modified Kupchan's partitioning procedure.[10] The $CHCl_3$- and BuOH-soluble materials were fractionated by droplet countercurrent chromatography (DCCC) and purified by HPLC to give pure plakinamines G (**1**) and H (**2**), 4α-hydroxydemethylplakinamine B (**3**), and tetrahydroplakinamine A (**4**), along with the known *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B,[8] 24,25-dihydroplakinamine A,[8] and *N*-methyltetrahydroplakinamine A.[8] The structures of these metabolites were determined by interpretation of the 1D and 2D NMR (^{1}H, ^{13}C, DEPT, COSY, TOCSY, HMQC, HMBC, and ROESY) spectra, UV, and accurate mass measurements (HR-EIMS).

Plakinamine G (**1**) corresponded to $C_{29}H_{44}N_2O$ on the basis of its combined HR-EIMS *m*/*z* 436.3458 (calcd 436.3454) and ^{13}C NMR spectral features. Analysis of its ^{1}H NMR, ^{13}C NMR, and COSY spectra (Table 1) revealed the tetracyclic system of **1** to be identical to that of 24,25-dihydroplakinamine A.[8] The main difference observed was in the substitution pattern of the side chain. The UV spectrum showed maximal absorption at 276 nm (log ϵ 4.11). The ^{13}C NMR spectrum showed seven low-field signals; among them, those due to one amide carbonyl (δ_C 174.5), one enamine (δ_C 137.1 and 122.3), and one trisubstituted olefin (δ_C 162.1 and 117.8) indicated the presence in the side chain of an α,β-unsaturated γ-lactam ring. Further confirmation of this structural assignment was obtained by an HMBC experiment (Table 2). The two methyl signals at δ_H 1.22 and 1.20 correlate to the tertiary



[#] Dedicated to the memory of Prof. Guido Sodano.

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Table 1. ^{1}H and ^{13}C NMR Chemical Shift Data of Compounds **1**−**3** (CD_3OD)[a]

	1			**2**			**3**		
position	δ_H[b]	δ_C	mult[c]	δ_H[b]	δ_C	mult[c]	δ_H[b]	δ_C	mult[c]
1	1.63, 1.38	32.8	CH_2	2.09, 1.71	38.0	CH_2	1.59, 1.34	32.1	CH_2
2	1.73, 1.30	30.6	CH_2	1.87	22.5	CH_2	1.85, 1.76	25.5	CH_2
3	3.25 br s	47.6	CH		72.2	CH	3.20 br s	53.0	CH
4	1.66, 1.45	35.1	CH_2		211.8	C	3.60 dd (11.0, 4.0)	71.5	CH
5	1.65	35.6	CH	2.38 dd (11.2, 4.3)	55.3	CH	1.48	42.5	CH
6	1.73	30.3	CH_2	2.17, 1.89	26.0	CH_2	2.27	26.3	CH_2
7	5.22	118.8	CH	5.22 br s	117.5	CH	5.25 br d (3.7)	118.5	CH
8		140.4	C		139.7	C		140.2	C
9	1.83	50.7	CH	2.06	50.7	CH	1.85	50.6	CH
10		35.9	C		42.6	C		37.0	C
11	1.65, 1.51	22.3	CH_2	1.71, 1.56	23.2	CH_2	1.66, 1.52	22.3	CH_2
12	2.08 br d (11.4), 1.35	40.7	CH_2	1.75, 2.07	40.6	CH_2	2.05 br d (12.8), 1.30	40.7	CH_2
13		44.4	C		44.6	C		44.5	C
14	1.90	56.0	CH	1.89	56.2	CH	1.88	56.3	CH
15	1.57, 1.45	23.9	CH_2	1.54, 1.46	23.9	CH_2	1.47	23.9	CH_2
16	1.87, 1.58	28.5	CH_2	1.72	29.1	CH_2	1.70	29.1	CH_2
17	1.50	57.7	CH	1.36	57.3	CH	1.34	57.3	CH
18	0.66 s	12.3	CH_3	0.59 s	12.6	CH_3	0.60 s	12.2	CH_3
19	0.83 s	12.7	CH_3	0.72 s	15.3	CH_3	0.87 s	14.2	CH_3
20	2.68 m	36.7	CH	2.18 m	42.4	CH	2.17 m	42.4	CH
21	1.13 d (6.6)	20.9	CH_3	1.07 d (6.6)	21.5	CH_3	1.07 d (6.6)	21.5	CH_3
22	5.36 d (10.7)	122.3	CH	5.49 dd (15.5, 9.0)	136.0	CH	5.48 dd (15.8, 8.8)	136.2	CH
23		137.1	C	6.40 d (15.5)	126.3	CH	6.39 d (15.8)	126.0	CH
24		162.1	C		127.7	C		127.2	C
25	2.85 m	26.6	CH		128.4	C		128.0	C
26	1.20 d[d]	24.0	CH_3	3.31	50.2	CH_2	2.91 s	60.8	CH_2
27	1.22 d[e]	22.8	CH_3	1.73 s	16.3	CH_3	1.73 s	16.4	CH_3
28	5.82 s	117.8	CH	2.22	25.8	CH_2	2.28 br t	27.0	CH_2
29		174.5	C	2.99 t (5.9)	43.6	CH_2	2.58 t (5.9)	53.2	CH_2
$N(CH_3)_2$				2.35 s	42.1	CH_3	2.33 s	45.5	CH_3

[a] The assignments were based on the COSY, TOCSY, HMQC, and HMBC experiments. [b] Coupling constants (in Hz) are given in parentheses. [c] Multiplicities were assigned from DEPT spectra. [d] Overlapped with CH_3-27. [e] Overlapped with CH_3-26.

Table 2. HMBC[a] Correlations of the Side Chain in Compounds **1** and **2**

1			**2**		
δ_H	δ_C ($^2J_{HC}$)	δ_C ($^3J_{HC}$)	δ_H	δ_C ($^2J_{HC}$)	δ_C ($^3J_{HC}$)
H-21 (1.13)	C-20 (36.7)	C-17 (57.7) C-22 (122.3)	$N(CH_3)_2$ (2.35)		$N(CH_3)_2$ (42.1) C-3 (72.2)
H-26 (1.20)	C-25 (26.6)	C-24 (162.1) C-27 (22.8)	H-26 (3.31)	C-25 (128.4)	C-24 (127.7) C-29 (43.6)
H-27 (1.22)	C-25 (26.6)	C-24 (162.1) C-26 (24.0)	H-29 (2.99)		C-24 (127.7) C-26 (50.2)
H-28 (5.82)	C-24 (162.1) C-29 (174.5)	C-23 (137.1)			

[a] HMBC optimized for $^{2,3}J_{CH} = 10$ Hz.

carbon at δ_C 26.6 and to the quaternary carbon at δ_C 162.1, which required their placement in an isopropyl group linked to C-24. The C-21 methyl protons revealed ^2J coupling to C-20 and ^3J coupling to C-17 and C-22. The C-28 olefinic proton at δ_H 5.82 showed ^3J correlation to C-23 at δ_C 137.1 and ^2J correlations to C-24 and C-29 at δ_C 162.1 and 174.5, respectively, which revealed the connection between the γ-lactam and the tetracyclic nucleus.

The molecular formula, $C_{31}H_{48}N_2O$, of plakinamine H (**2**) was established by HR-EIMS *m*/*z* 464.3760 (calcd 464.3767). The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B[8] (Table 1) except for the disappearance of an *N*-methyl signal. In the HMBC spectrum (Table 2) two *N*-methyl signals at δ_H 2.35 showed ^3J correlations to each other and to C-3 at δ_C 72.2. The upfield ^{13}C NMR shifts of C-29 and C-26 (about 10 ppm compared to *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B) confirmed the absence of the *N*-methyl group in the side chain and allowed the structural elucidation of **2**.

HR-EIMS data (*m*/*z* 452.3758, calcd 452.3767) of compound **3** revealed the molecular formula $C_{30}H_{48}N_2O$. The ^{1}H and ^{13}C NMR data of **3** (Table 1) were superimposable on those of *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B[8] for C-22 to $-NCH_3$, but significantly different for the tetracyclic nucleus. The ^{13}C NMR spectrum featured a signal at δ_C 53.0, suggesting the presence of the α-amino group at C-3,[11] and a signal at δ_C 71.5 attributable to a hydroxyl group. This was subsequently located at C-4 by HMBC and COSY experiments. Its equatorial conformation was compatible with the chemical shift of CH_3-19 (δ_H 0.87).

The stereochemistry was confirmed by the small $H_{3,4}$ coupling constant ($J = 4.0$ Hz) and a ROESY experiment which showed correlation of H-4 to CH_3-19 (Figure 1). The low-field signals at δ_C 140.2 and 118.5 were assigned to a Δ^7 double bond, in close analogy with plakinamine G (**1**). Hence compound **3** is *N*-demethyl-4α-hydroxyplakinamine B.

Steroidal alkaloid **4** had the molecular formula $C_{29}H_{50}N_2$, as determined on the basis of ^{13}C NMR data and from its HR-EIMS spectrum, which showed a molecular ion peak at *m*/*z* 426.3980 (calcd 426.3974). The structure of **4** was readily established by comparison of the ^{1}H NMR, ^{13}C NMR, and COSY spectral data with those of 24,25-dihydroplakinamine A.[8] The chemical shifts of carbon

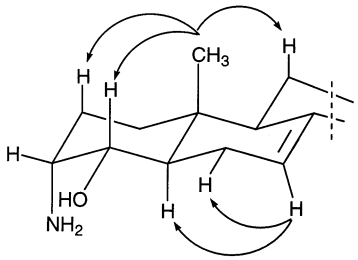

Figure 1. ROE correlations observed in the steroidal nucleus of **3**.

Table 3. IC_{50} (μg/mL) of Compounds **1**, **2**, **3**, and **4**

compound	C6[a]	RAW 264[b]
1	6.8	nc[c]
2	9.0	61.0
3	26.1	16.2
4	1.4	nc

[a] Rat glioma cell line. [b] Murine macrophage cell line. [c] Not cytotoxic.

atoms 1 to 19 are virtually identical in both compounds (see Experimental Section). The main difference observed in **4** is the replacement in the ^{13}C NMR spectrum of the imine signal of 24,25-dihydroplakinamine A by a carbon signal at δ_C 61.5. Interpretation of the COSY spectrum confirmed the presence of a spin system from C-20 to C-29, indicative of a saturated pyrrolidine ring. On this basis, the structure of **4** was established as tetrahydroplakinamine A.

Cytotoxicity was evaluated on rat glioma (C6) and murine monocyte/macrophages (RAW 264) cell lines (Table 3). Compounds **1** and **4** were the most active against C6 cells (IC_{50}'s 6.8 and 1.4 μg/mL, respectively), whereas they were without effect on RAW 264. Compounds **2** and **3** were cytotoxic against both cell lines, with compound **2** being more active against C6 cells (IC_{50} 9.0 μg/mL) than to RAW 264 (IC_{50} 61 μg/mL), while compound **3** exhibited higher cytotoxicity on RAW 264 (IC_{50} 16.2 μg/mL) than to C6 cells (IC_{50} 26.1 μg/mL).

Experimental Section

General Experimental Procedures. Specific rotations were measured on a Perkin-Elmer 243 B polarimeter. UV spectra were recorded on a Beckman DU-70 spectrophotometer. MS spectra were recorded on a Fisons VG Prospect spectrometer. NMR spectra were recorded on a Bruker AMX-500 spectrometer equipped with a Bruker X32 computer, using the UXNMR software package. ^1H and ^{13}C NMR spectra were recorded at 500.13 and 125.76 MHz, respectively. Chemical shifts are referenced to residual CHD_2OD (3.31 ppm) in CD_3OD; ^{13}C chemical shifts are referenced to the solvent (CD_3OD, 49.0 ppm).

Animal Material. Samples of the sponge *Corticium* sp. were collected at a depth of 12−18 m at Efatè, Porth Havannah, Vanuatu, South Pacific, in July 1996. The samples were frozen immediately after collection and lyophilized to yield 550 g of dry mass. The sponge was identified by Dr. John Hooper of the Queensland Museum, Brisbane, Australia, as *Corticium* sp. (Homosclerophorida, Plakinidae). A voucher specimen (R1718) has been deposited at the IRD Center in Nouméa, New Caledonia.

Extraction and Isolation. The lyophilized sponge (550 g) was extracted by blending with MeOH (3 × 2 L) at room temperature. The combined extracts (88 g) were concentrated and subjected to a modified Kupchan's partition as follows. The MeOH extract was dissolved in 10% aqueous methanol and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 20% and 40% and partitioned against CCl_4 and $CHCl_3$ respectively, yielding 4.9 g of $CHCl_3$ extract. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The $CHCl_3$ extract (1.2 g) was fractionated by DCCC using $CHCl_3$/MeOH/H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase). Fractions (6 mL each) were collected and examined by TLC on SiO_2 with $CHCl_3$/MeOH/H_2O (80:18:2) as eluent. Fractions 9−16 were pooled and purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.) eluting with MeOH/H_2O/TEA (95:5:0.5) to yield pure compound **2** (3.4 mg), pure *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B[8] (6.3 mg), and pure *N*-methyltetrahydroplakinamine A[8] (19.8 mg). Fractions 28−32 were purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.) with MeOH/H_2O/TEA (93:7:0.5) as eluent to yield pure compound **1** (1.1 mg), while fractions 38−78 under the same conditions gave pure 24,25-dihydroplakinamine A[8] (2.5 mg). The *n*-BuOH extract (6.6 g) was chromatographed on a Sephadex LH-60 column (3 × 80 cm) and eluted with MeOH/H_2O (2:1). Fractions 108−123 were pooled (1.49 g) and fractionated by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was used as stationary phase). Fractions (6 mL each) 81−98 and 107−115 were purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.) eluted with MeOH/H_2O/TEA (95:5:0.5) to give pure compounds **3** (2.4 mg) and **4** (2.5 mg), respectively.

Cytotoxicity Tests. C6 and RAW 264 cells (3.5×10^3 cells) were plated on 96-well plates in 50 μL and allowed to adhere at 37 °C in 5% CO_2/air for 2 h. Thereafter, 50 μL of 1:4 (v/v) serial dilution of the test compounds was added and incubated with the cells for 24 h. Cell viability was assessed through an MTT conversion assay.[12] After 24 h, 25 μL of MTT (5 mg/mL) was added, and the cells were incubated for an additional 3 h. After this time, the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v/v) *N*,*N*-dimethylformamide and 20% (w/v) SDS with an adjusted pH of 4.5.[13] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titerteck Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

Plakinamine G (1): colorless gum; $[\alpha]^{22}_D$ −24.4° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 276 (4.11); ^1H and ^{13}C NMR data (CD_3OD), see Table 1; HREIMS *m*/*z* 436.3458 (calcd for $C_{29}H_{44}N_2O$, 436.3454).

Plakinamine H (2): colorless gum; $[\alpha]^{22}_D$ +29.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 250 (3.77); ^1H and ^{13}C NMR data (CD_3OD), see Table 1; HREIMS *m*/*z* 464.3760 (calcd for $C_{31}H_{48}N_2O$, 464.3767).

4α-Hydroxydemethylplakinamine B (3): colorless gum; $[\alpha]^{22}_D$ +6.7° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 241 (3.80); ^1H and ^{13}C NMR data (CD_3OD), see Table 1; HREIMS *m*/*z* 452.3758 (calcd for $C_{30}H_{48}N_2O$, 452.3767).

Tetrahydroplakinamine A (4): colorless gum; $[\alpha]^{22}_D$ +23.2° (*c* 0.19, MeOH); ^1H NMR (CD_3OD, 500.13 MHz) δ 5.22 (1H, br s, H-7), 3.25 (1H, br s, H-3), 3.09 (1H, m, H-23), 2.97 (2H, m, H_2-29), 2.09 (1H, br d, J = 12.1 Hz, H-12), 1.96 (1H, H-28), 1.88 (1H, H-14), 1.83 (1H, H-9), 1.81 (1H, H-22), 1.78 (1H, H-24), 1.76 (1H, H-16), 1.73 (1H, H-25), 1.73 (1H, H-2), 1.73 (1H, H-6), 1.69 (1H, H-6), 1.66 (1H, H-4), 1.65 (1H, H-5), 1.65 (1H, H-11), 1.63 (1H, H-1), 1.58 (1H, H-16), 1.57 (1H, H-15), 1.52 (1H, H-20), 1.51 (1H, H-11), 1.48 (1H, H-15), 1.45 (1H, H-4), 1.39 (1H, H-1), 1.31 (1H, H-17), 1.30 (1H, H-2), 1.29 (1H, H-22), 1.29 (1H, H-12), 1.06 (3H, d, J = 6.6 Hz, CH_3-21), 0.99 (3H, d, J = 5.9 Hz, CH_3-26), 0.93 (3H, d, J = 5.9 Hz, CH_3-27), 0.84 (3H, s, CH_3-19), 0.61 (3H, s, CH_3-18); ^{13}C NMR (CD_3OD, 125.76 MHz) δ 140.4 (C-8), 118.8 (C-7), 61.5 (C-23), 57.9 (C-17), 56.0 (C-14), 52.0 (C-24), 50.7 (C-9), 47.6 (C-3), 45.6 (C-29), 44.5 (C-13), 42.1 (C-22), 40.6 (C-12), 35.9 (C-20), 35.9 (C-10), 35.6 (C-5), 35.1 (C-4), 32.8 (C-1), 30.6 (C-2), 30.5 (C-25), 30.3 (C-6), 28.8 (C-16), 27.1 (C-28), 23.7 (C-15), 22.3 (C-11), 21.7 (C-26), 19.4 (C-21), 18.3 (C-27), 12.7 (CH_3-19), 12.3 (CH_3-18); HREIMS *m*/*z* 426.3980 (calcd for $C_{29}H_{50}N_2$, 426.3974).

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