Palau'amine: A Cytotoxic and Immunosuppressive Hexacyclic Bisguanidine Antibiotic from the Sponge *Stylotella agminata*[1]

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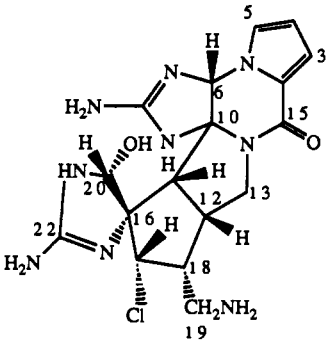
Guanidine is a familiar structural feature in marine natural products. Examples range from simple arginine derivatives to complex polycycles as, e.g., saxitoxin and tetrodotoxin.[3,4] We now report the isolation and structure determination of a hexacyclic bisguanidine, palau'amine (1)[5] from a sponge, *Stylotella agminata*, collected in the Western Caroline Islands. Aqueous extracts of the sponge, first collected in 1977 and recollected in November, 1991, at a depth of -5 to -50 m near Wonder Channel and Rock Islands, Republic of Belau,[6] had substantial activity against gram-negative and gram-positive organisms and showed remarkable resistance to fungal growth on prolonged storage.

Extraction of the lyophilized sponge[7] (600 g) with MeOH (6 L) and dissolution of the water-soluble residue after evaporation yielded 900 mL of aqueous extract. Ion-exchange chromatography of a portion (2/9) on Cellex CM with stepwise increasing concentrations of NaCl resulted in elution of the antibiotic activity[8] in the 0.5 M and, to a lesser extent, in the 1.0 M fractions. Repeated LH-20 chromatography (MeOH) of the 0.5 M fraction, after desalting by dissolving in EtOH, furnished essentially pure palau'amine, presumably as the hydrochloride (14 mg, 0.01% dry weight), as an optically active, off-white amorphous powder that decomposed prior to melting. Further purification of **1** could be effected by HPLC (YMC aqueous C18, H_2O/MeCN (90:10), 0.1% TFA). Although **1** is quite stable in acid, it decomposes rapidly >pH 6.5, so the free base could not be prepared.



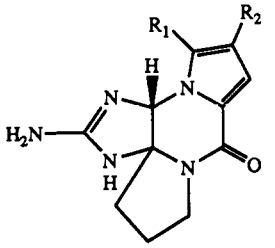
1

Monoprotonated palau'amine has composition $C_{17}H_{22}ClN_9O_2$, which is based on high-resolution mass spectral data (HRFABMS 420.1669 [MH^+]; Δ 0.6 mmu), on the isotopic cluster characteristic of one chlorine substituent, and on the ^{13}C NMR spectrum. The IR spectrum showed O-H and N-H bands (3350 cm^{-1}, broad), an amide (1658 cm^{-1}), and an absorption at 1700 cm^{-1} characteristic of a guanidine hydrochloride.[9] UV[10] and ^1H NMR data resembled those reported for phakellin (**2**, $R_1 = R_2 = H$).[11]

Table I. ^1H and ^{13}C NMR Data for Palau'amine (1) in D_2O

carbon	^{13}C, ppm[a]	multiplicity	^1H, ppm[b]	multiplicity
2	122.5	s		
3	115.6	d	6.85	dd, J = 3.9, 1.5
4	113.8	d	6.35	dd, J = 3.9, 2.8
5	125.2	d	6.99	dd, J = 2.8, 1.5
6	69.0	d	6.33	s
8	159.5	s		
10	80.8	s		
11	56.3	d	3.08	d, J = 14.1
12	41.8	d	2.52	dddd
13	46.1	t	3.96	dd, J = 7.3, 10.4
			3.28	dd, J = 10.3, 10.4
15	157.8	s		
16	72.1	s		
17	74.0	d	4.35	d, J = 7.9
18	48.6	d	2.47	dddd
19	41.9	t	3.32	dd, J = 13.2, 7.0
			3.24	dd, J = 13.2, 7.0
20	83.7	d	5.96	s
22	157.9	s		

[a] At 125 MHz referred to external dioxane. [b] At 500 MHz, HDO signal at 4.63 ppm.



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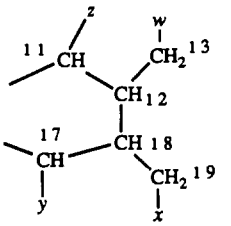

A

Full NMR data (Table I) revealed its characteristic guanidino pyrrolopyrazinone. Distinctly new features included a C_6H_8 portion (**A**), confirmed by COSY and decoupling experiments, a guanidine carbon (159.9 ppm, C22), a methine (83.5 ppm, C20), and a quaternary carbon (72.1 ppm, C16).

Fragment **A** replaces the trimethylene unit in phakellin. The C13 methylene is attached to the amide nitrogen (*w* in **A**). The chemical shifts of the methylene carbon and protons, which show HMBC contours to C10 and C15, are analogous to those in the phakellins. Terminus *z* is C10; correlations are observed from H11 to C10 and C6.

The correct regiochemistry of the remaining hetero functions was ascertained next. The carbon (74.0 ppm) and proton (4.35 ppm) shifts for C17 indicate that *y* is oxygen or chlorine. The shifts were unaffected by acetylation; nor was there any substantial change in that proton resonance when the ^1H NMR spectrum was determined in trifluoroacetic acid. Thus, *y* must be chlorine, and, since H17 is a doublet, C16 must be a quaternary carbon.

(1) A preliminary account of this work was presented at the Seventh International Symposium on Marine Natural Products, Capri, Italy, July 5-10, 1992, Abstract C7.
(2) On leave from Hamilton College, Clinton, NY 13323.
(3) Chevolot, L. In *Marine Natural Products*; Scheuer, P. J., Ed.; Academic: New York, 1981; Vol. 4, pp 53-91.
(4) Kobayashi, J.; Ishibashi, M. In *The Alkaloids*; Brossi, A., Cordell, G. A., Eds., Academic: San Diego, 1992; Vol. 41, pp 41-124.
(5) In addition to Palau, the geographical origin of the sponge, one meaning of the Hawaiian word palau is war club, which is an apt description of the characteristic shape of the spicules of *Stylotella* spp.
(6) Made possible through cooperation with the University of Guam Marine Laboratory personnel.
(7) The dust from the lyophilized sponge caused a powerful allergic reaction, which entailed severe shortness of breath for about 4 h; the effect largely disappeared within 24 h.
(8) Monitored by microbial assay against *Staphylococcus aureus*.
(9) Goto, T.; Nakanishi, K.; Ohashi, M. *Bull. Chem. Soc. Jpn.* **1957**, *30* 723-725.
(10) UV (MeOH) λ_{max} 272 (ε 7900), 224 nm (7800).
(11) Sharma, G.; Magdoff-Fairchild, B. *J. Org. Chem.* **1977**, *42*, 4118-4124. Sharma, G. M.; Burkholder, P. R. *J. Chem. Soc., Chem. Commun.* **1971**, 151-152. It is noteworthy that only brominated phakellins (**2**, $R_1 = R_2$ = H or Br) have been isolated to date; phakellin itself was produced by hydrogenation of the brominated derivatives.

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Acetylation of **1** with Ac_2O in pyridine resulted in a mixture from which no simple mono derivative was isolated. However, aqueous Ac_2O/NaOAc yielded a monoacetyl derivative. NMR experiments (COSY, HMQC, HMBC in D_2O, and DMSO-d_6) demonstrated that an acetamide had formed from a primary amine (x in **A**) attached to C19 methylene.

Presence of a hydroxyl was inferred by loss of water from the molecular ion in the MS-MS spectrum.[12] The hydroxyl proton (9.18 ppm)[13] shows COSY cross peaks with H20 and an NH, as well as an HMBC to C16; thus, the hydroxyl must be attached to C20.

The remaining structural features were elucidated with the aid of HMBC data. Both H11, a clean doublet, J = 14.1 Hz,[14] and H17 showed correlations to a quaternary carbon at 72 ppm (C16); thus, C16 must be attached to both carbons. H11 also correlates to a methine carbon at 83.5 ppm (C20)[15] and to five other carbons. The proton attached to C20 is a singlet in D_2O and a broadened doublet in DMSO-d_6. Thus, C20 is vicinal to an amide or guanidine NH group. Furthermore, H20 shows an HMBC to C16, C11, and C22; therefore, C20 must be a carbinolamine which is part of a ring containing the guanidine. Structure **1** of palau'amine is consistent with all of these data.

Relative stereochemistry can be deduced from NOEs and interproton coupling. H11 and H6 show positive NOE and ROESY correlations. The bicyclo[3.3.0]azaoctane ring is assuredly cis fused, while inspection of models and comparison of coupling constants suggests that the H12, H18, and H17 are all cis to one another. The NH proton that is coupled to H20 shows a clear ROESY correlation to H6. This is only possible if C20 is β-oriented. H20 and H17 show long-range COSY and ROESY correlations, which indicates syn geometry.[16]

Palau'amine is reasonably nontoxic;[17] it is quite active against P-388 and A549, less so against other cancer cell lines, and possesses antibiotic and antifungal activity.[18] It showed promise in an immunomodulatory assay[19] and is currently undergoing in vivo testing.

We have also identified the following known compounds in extracts of this sponge: sceptrin, hymenidin, oroidin, dibromophakellin, hymenialdisine, hymenin, and "the yellow compound."[20] There are also at least three less active, brominated derivatives of palau'amine present, as well as the analog to dibromoisophakellin.[21] Studies of the constituents of this sponge are continuing, and details will be reported in a full paper.

Acknowledgment. We thank Valerie Paul, Karen and Larry Meyer, and Steve Pennings for recollection of the sponge, Professor P. R. Bergquist for identification, Kay Larsen, Faith Caplan, and Glynn Faircloth for bioassays, Wesley Yoshida for measuring 500-MHz NMR spectra, Walter Niemczura for helpful discussions, Ryuichi Sakai (University of Illinois) for mass spectral determinations, and Marion Ho for able assistance. We appreciate financial support from PharmaMar, S. A., the National Science Foundation, and the Sea Grant College Program. R.B.K. is grateful to Hamilton College for generous sabbatical leave support.

Supplementary Material Available: 125-MHz ^{13}C NMR and 500-MHz ^1H NMR spectra for **1** in D_2O and DMSO-d_6; HMBC spectrum for **1** in D_2O; 125-MHz ^{13}C NMR and 500-MHz ^1H NMR spectra for its acetamide in D_2O; 500-MHz ^1H NMR spectrum for the acetamide in DMSO-d_6; and HMBC spectra for the acetamide in both solvents (10 pages). Ordering information is given on any current masthead page.

(12) MH+ - 18, 26%. Also observed were peaks at MH^+ - 59, -83, and -142; a peak for guanidinium (m/z 60, 51%) and for acylpyrrolium (m/z 94, 35%) were the other strong peaks.

(13) This signal is broadened slightly and presumably is still exchanging too fast to show coupling to the proton on C20. In DMSO-d_6 containing a trace of D_2O, the carbon spectrum of C20 shows three peaks, which demonstrates that it is close to two exchanging protons. See Christofides, J. C.; Davies, D. B. *J. Am. Chem. Soc.* **1983**, *105* 5099–5105.

(14) This coupling constant seems large, but comparable values are observed in similarly rigid, spiroannulated five-membered rings. See, for example: Lowry, J. B.; Riggs, N. V. *Tetrahedron Lett.* **1964**, 2911–2914.

(15) The shifts of C20 and H20 are comparable to those found for the hemilactal in tetrodotoxin. See: Yasumoto, T.; Yotsu, M.; Murata, M.; Naoki, H. *J. Am. Chem. Soc.* **1988**, *110*, 2344–2345. The carbon shift for C16 is consistent with strained bicyclo[3.3.0] systems, as in modhephene. See: Zalkow, L. H.; Harris, R. N.; Van Derveer, D. *J. Chem. Soc., Chem. Commun.* **1978**, 420–421.

(16) The absolute stereochemistry is unknown. Palau'amine is levorotatory (α^{24}_D -45.2° (c = 3.0, MeOH)). Its CD spectrum showed a broad, weakly positive peak centered at 270 nm, a negative shoulder at 230 nm, and a strong negative peak at 208 nm, very similar to those observed for monobromophakellin hydrochloride.

(17) LD_{50} 13 mg/kg (i.p. mice).

(18) Cytotoxicity in tumor cells (IC_{50} in parentheses): P-388 (0.1 μg/mL; A549 (0.2 μg/mL); HT-29 (2 μg/mL); KB (10 μg/mL). Antibiotic: active against *Staphylococcus aureus* and *Bacillus subtilis* at 10 μg/disk. Antifungal: 24-mm zone against *Penicillium notatum* at 50 μg/disk.

(19) In the mixed lymphocyte reaction (MLR) palau'amine showed an IC_{50} < 18ng/mL, while the cytotoxicity assay against a primary culture of murine lymphocytes showed an IC_{50} of 1.5 μg/mL.

(20) These have all been reviewed: Ireland C. M. ; Molinski, T. F.; Roll, D. M; Zabriskie, T. M.; McKee, T. C.; Swersey, J. C.; Foster, M. P. In *Bioorganic Marine Chemistry*; Scheuer, P. J., Ed.; Springer-Verlag: New York, 1989; Vol. 3, pp 1–48.

(21) Fedoreyev, S. A.; Utkina, N. K.; Ilyin, S. G.; Reshetnyak, M. V.; Maximov, O. B. *Tetrehedron Lett.* **1986**, *27*, 3177–3180.