Makaluvic Acids A and B: Novel Alkaloids from the Marine Sponge *Zyzzya fuliginosus*

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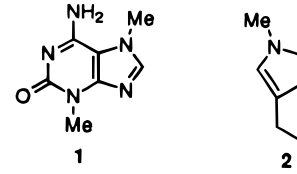
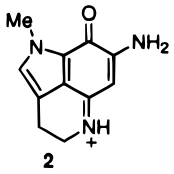
Two new substituted pyrrolecarboxylic acids, makaluvic acids A (**5**) and B (**6**), were isolated from the sponge *Zyzzya fuliginosus*, which was collected in Chuuk Atoll, Federated States of Micronesia. The known alkaloids 3,7-dimethylisoguanine (**1**) and makaluvamines A (**2**), E (**3**), and K (**4**) were also isolated.

A family of alkaloids characterized by a pyrroloquinone skeleton has been isolated in recent years from several sponges and an ascidian. Included in this family are the batzellines,[1] isobatzellines,[2] damirones,[3,4] makaluvamines,[4−6] discorhabdins,[7−10] prianosins,[11−13] and wakayin.[14] These alkaloids have shown a variety of biological activities including cytotoxicity against human tumor cell lines,[5] *in vivo* tumor inhibition,[5] inhibition of topoisomerase I[6] and II,[5] antifungal activity,[2] and stimulation of Ca^{2+} release from sarcoplasmic reticulum.[12] As part of our ongoing search for antitumor compounds from marine organisms, we examined an extract of the sponge *Zyzzya fuliginosus* (Carter), order Poecilosclerida, family Coelosphaeridae, collected in Chuuk Atoll, which exhibited cytotoxicity against murine leukemia P388 cells. Our work on the sponge has led to the discovery of two new compounds, makaluvic acids A (**5**) and B (**6**), along with the known compounds 3,7-dimethylisoguanine (**1**),[15] makaluvamines A (**2**), E (**3**),[5] and K (**4**)[4] and 4-hydroxybenzoic acid. We describe here the structure elucidation of the new compounds.



1



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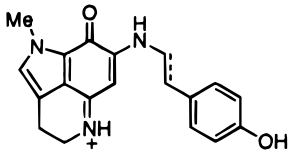
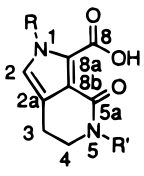


3 10, 11 double bond
4 10, 11 single bond



5 R=H, R'=Me
6 R=Me, R'=H

Specimens of *Z. fuliginosus* that had been frozen were thawed and extracted twice with MeOH and then twice with MeOH/CH_2Cl_2 (1:1). The combined extracts, after evaporation of solvents, were subjected to Kupchan partitioning[16] to give hexane, CH_2Cl_2, *n*-BuOH, and H_2O solubles. Both *n*-BuOH and H_2O fractions exhibited significant cytotoxicity and contained makaluvamine-like compounds and purine derivatives as judged from their ^{1}H NMR spectra. Chromatography of the *n*-BuOH fraction over SiO_2 using MeOH/CH_2Cl_2 as eluent yielded 3,7-dimethylisoguanine (**1**), and more **1** was obtained during desalting of the H_2O-soluble fraction on an HP-20 column. Compounds **2**−**6** were resolved by SiO_2 chromatography employing MeOH/CH_2Cl_2 (1:9) containing 1% TFA as eluent followed by reversed-phase HPLC.

The known compounds 4-hydroxybenzoic acid, 3,7-dimethylisoguanine (**1**),[15] and makaluvamines A (**2**), E[5] (**3**), and K (**4**)[4] were identified by comparison of their MS and ^{1}H and ^{13}C NMR data with published values. Makaluvic acid A (**5**) crystallized from MeOH. A molecular formula of $C_9H_{10}N_2O_3$ was established for **5** by HRFABMS and corroborated by ^{13}C NMR data, suggesting six degrees of unsaturation. The presence of carboxylic acid and amide functionalities was indicated by IR bands at 1700 and 1680 cm^{-1} and ^{13}C NMR signals at δ 165.1 (s) and 159.8 (s). Further evidence for the existence of a carboxylic acid came from a strong EIMS fragmentation peak at *m*/*z* 150 due to loss of CO_2 from the molecule (Scheme 1). The ^{13}C NMR spectrum revealed the presence of two additional unsaturations: one sp^2 methine at δ 118.2 (d) and three sp^2 quarternary carbons at δ 123.3, 122.1, and 115.1. The remaining unsaturations required by the molecular formula must be satisfied by two rings. With the structures of the makaluvamines in mind, inspection of the ^{1}H NMR (DMSO-d_6) spectrum of **5**, which contained an exchangeable signal at δ 12.33 (H-1), a broad singlet at δ 6.89 (H-2), two 2-proton triplets at δ 3.61 (H-4) and 2.79 (H-3), and a methyl singlet at 3.04 (N-5-Me), led to the proposed structure **5** for makaluvic acid A. The *N*-methyl group was located at N-5 rather than at N-1, because the chemical shift of the methyl (δ 3.04) was similar to that of the N-5-methyl of damirones A (δ 3.01) and B (δ 3.05) and quite different from that of the *N*-1-methyl of damirone A[3] (δ 3.83) and makaluvamines A, D, and E[5] (δ 3.82−4.26).

The structure of makaluvic acid A (**5**) was confirmed by X-ray analysis. The pyrrole ring is perfectly planar. The carboxylic acid group lies close to the plane of the pyrrole ring, and the structure is stabilized by a strong intramolecular O···O hydrogen bond [O3-H···O1 = 2.481

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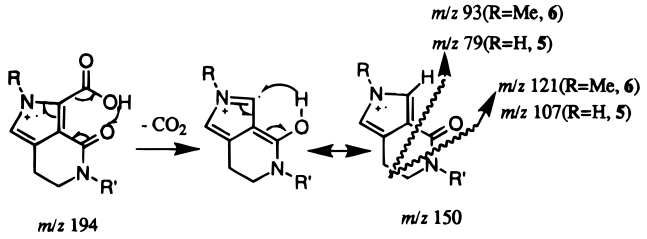
Scheme 1. Proposed EIMS Fragmentation Pathway for Makaluvic Acids A (**5**) and B (**6**)



Å]. The dihydropyridone ring is in a half-chair conformation. The molecules form unending chains through intermolecular N1−H···O2 hydrogen bonds.

High-resolution FABMS of makaluvic acid B (**6**) indicated a molecular formula of $C_9H_{10}N_2O_3$, the same as that found for **5**. The UV, IR, and ^1H and ^{13}C NMR spectra of makaluvic acid B were similar to those of makaluvic acid A (**5**), suggesting strongly that the two compounds are isomers, and hence, structure **6** was proposed for makaluvic acid B. A COSY experiment confirmed the proton spin system from H-3 → H-5. The methyl group was assigned to the N-1 position because its chemical shift, δ 3.91, resembled that of the N-1-methyl of damirone A and makaluvamine A. The downfield shift of C-2 and upfield shift of C-4 in **6** when compared to **5** also supports structure **6** as does it EIMS fragmentation pattern, especially the significant fragment ion peaks at *m*/*z* 93 and 121, which correspond to the peaks at *m*/*z* 79 and 107, respectively, for compound **5** (Scheme 1).

Makaluvic acids A and B could arise from the batzellines, isobatzellines, or makaluvamines by oxidation. 4-Hydroxybenzoic acid, an oxidation product of the tryamine-derived portion of compounds **3** or **4**, was also found in the sponge. Makaluvamines A (**2**), E (**3**), and K (**4**) showed *in vitro* cytotoxicity against murine leukemia P388 cell lines with ED_{50} of 0.4, 0.6, and 0.7 μg/mL, respectively, but compound **1** and makaluvic acid A (**5**) were inactive in this assay. The lack of cytotoxicity for **5** supports the earlier suggestion that the iminoquinone moiety may be responsible for the observed biological activities, since no activity was reported for damirones and batzellines, which lack this structural feature.[6]

Experimental Section

General Methods. All solvents were redistilled. Merck Si gel 60 (230−240 mesh) was used for vacuum flash chromatography. HPLC was conducted using a UV detector and Spherex 5 C-18 columns. IR spectra were taken on a Bio-Rad 3240-SPC FT instrument and UV spectra on a Hewlett-Packard spectrophotometer. NMR experiments were conducted with Varian XL-300 and VXR-500 instruments; signals are reported in parts per million (δ), referenced to the solvent used. EIMS and FABMS were measured on Hewlett-Packard and VG ZAB-E mass spectrometers, respectively. Diaion HP-20 is a macroporous styrene-based polymer bead resin providing an aromatic nonpolar surface (Mitsubishi Kasei America, Inc., White Plains, NY).

Sponge Material. Samples of sponges were collected at −20 M in July 1992, within the lagoon of Kuop atoll and Nov 6, 1992, at ∼−20 M at the Northeast pass of Chuuk lagoon, Chuuk State, Federated States of Micronesia. The sponge forms a thick encrustation with short broad surface fistules and a detachable papery surface that is usually heavily laden with slit. The texture is brittle and slightly compressible. The color in life is black, dark reddish-black in alcohol. The skeleton consists of large irregular compact tracts of acanthotylotes, between which are scattered smaller, faintly acanthose, strongyles. The sponge is similar to *Zyzzya massalis* (Dendy, 1992) (order Poecilosclerida, family Coelosphaeridae) as described by Hooper and Krasochin[17] but differs in the smaller size of the megascleres and the lack of a strongly acanthose condition. However, there is a considerable range of variation in specimens reported to be the same species,[18] and our specimen could represent one extreme of this range. Van Soest *et al.*[18] regard the holotype of *Zyzzya massalis* (Dendy, 1922) from Mauritius to be conspecific with the holotype of *Z. fuliginosus* (Carter, 1879), and thus the latter name should be the preferred name to use in the future. The specimen identified as *Histodermella* sp (cat. no. 003:00879) in Carney *et al.*[6] has been reidentified and is morphologically very close to the specimen under consideration here. A voucher specimen has been deposited at The Natural History Museum, London (BMNH 1996:5:8:1), and another voucher is maintained at the University of Oklahoma (48-T-92).

Extraction and Isolation. The sample (90 g wet wt; 18.5 g dry wt after extraction) was stored frozen until workup. Thawed specimens were extracted twice with MeOH overnight and then twice with MeOH/CH_2Cl_2 (1:1) overnight. The combined concentrated extracts, after removal of solvents, were dissolved MeOH/H_2O (9:1) (300 mL), and the solution was extracted with hexane (2 × 300 mL). The resulting aqueous MeOH solution was diluted with H_2O (86 mL) to 30% of H_2O in MeOH and partitioned against CH_2Cl_2 (2 × 300 mL). The aqueous MeOH solution was concentrated *in vacuo*, and the aqueous residue was diluted with H_2O to 150 mL and then extracted with *n*-BuOH (150 mL × 2). The *n*-BuOH layer was evaporated *in vacuo* to dryness, and the H_2O solution was lyophilized. Purification of the *n*-BuOH fraction by flash chromatography over Si gel using a MeOH/CH_2Cl_2 step gradient (0−20%) as eluent afforded compound **1** (35 mg, 0.19% of the dry weight sponge). Compound **1** was also obtained from the H_2O fraction by passing it through an HP-20 column using H_2O and then MeOH as eluent. The fractions that contained makaluvamine-like compounds based on ^1H NMR spectra were pooled and rechromatographed over Si gel eluting with MeOH/CH_2Cl_2 (1:9) containing 1% TFA. This yielded makaluvamine A as a major component from fractions 6−8 after removal of the solvents. Reversed-phase HPLC of fraction 1 and 2 over a C-18 column using MeOH/H_2O (7:3) containing 0.5% TFA as eluent afforded makaluvic acid A (**5**) and a mixture of 4-hydroxybenzoic acid and makaluvic acid B (**6**), which was resolved over the same C-18 column but using MeOH/H_2O (13:7) containing 0.5% TFA as eluent. Makaluvamine E and makaluvamine K were obtained from fractions 3 and 4 by C-18 HPLC eluting with MeOH/H_2O (3:1) containing 0.5% TFA.

3,7-Dimethylisoguanine (1): amorphous powder; LRFABMS *m*/*z* $[M + H]^+$ 180 (100), $[M + Na]^+$ 202 (46); ^1H NMR (CF_3COOD), δ 4.00 (3H, s), 4.23 (3H, s), 8.51 (1H, s, H-8); ^1H NMR (CD_3OD), δ 3.62 (3H, s), 3.97 (3H,

s), 7.81 (1H, s, H-8); ^{13}C NMR (DMSO-d_6) δ 30.6 (q, N-3-Me), 32.8 (q, N-7-Me), 110.8 (s, C-5), 140.4 (d, C-8), 147.8 (s, C-4), 154.0 (s, C-2), 162.0 (s, C-6); ^{13}C NMR (DMSO-d_6/TFA-d) δ 31.7 (q, N-3-Me), 33.7 (q, N-7-Me), 109.2 (s, C-5), 144.2 (d, C-8), 148.5 (s, C-4), 152.0 (s, C-2), 152.6 (s, C-6).

Makaluvic acid A (5) (2.3 mg; 0.012%): UV (MeOH) λ_{max} 268 (ϵ 12 684), 285 (ϵ 11 086); IR (neat) ν_{max} 3500 (br), 2950, 2925, 2855, 1700, 1680, 1583, 1530, 1457, 1443, 1410, 1200, 1135 cm^{-1}; ^{1}H NMR (DMSO-d_6), δ 12.33 (1H, br, H-1), 6.89 (1H, br s, H-2), 3.61 (2H, t, J = 7.0 Hz, H-4), 3.04 (3H, s, N-5-Me), 2.79 (2H, t, J = 7.0 Hz, H-3); ^{1}H NMR (CD_3OD), δ 6.85 (1H, s, H-2), 3.67 (2H, t, J = 7.0 Hz, H-4), 3.14 (3H, s, N-5-Me), 2.89 (2H, t, J = 7.0 Hz, H-3); ^{13}C NMR (DMSO-d_6), δ 165.1 (s), 159.8 (s), 123.3 (s), 122.1 (s), 118.2 (d, C-2), 115.1 (s), 49.9 (t, C-4), 34.1 (q, N-5-Me), 19.6 (t, C-3); HRFABMS m/z $[M + H]^+$ 195.0768, calcd for $C_9H_{11}N_2O_3$ 195.0770; LRFABMS m/z $[M + H]^+$ 195 (100), $[M + Na]^+$ 217 (27), $[M + K]^+$ 233 (4); LREIMS m/z $[M]^+$ 194 (40), 178 (7), 150 (87), 148 (27), 133 (37), 121 (19), 107 (100), 93 (14), 79 (85), 69 (23).

Makaluvic acid B (6) (0.8 mg; 0.004%): UV (MeOH) λ_{max} 268 (ϵ 12 497), 288 (ϵ 11 386); IR (neat) ν_{max} 3500 (br), 2953, 2930, 2850, 1695, 1680, 1585, 1558, 1536, 1453, 1438, 1410, 1200, 1180, 1130 cm^{-1}; ^{1}H NMR (DMSO-d_6), δ 8.72 (1H, br, H-5), 7.06 (1H, s, H-2), 3.91 (3H, s, N-1-Me), 3.41 (2H, m, H-4), 2.70 (2H, t, J = 6.9 Hz, H-3); ^{1}H NMR (CD_3OD), δ 6.90 (1H, s, H-2), 3.98 (3H, s, N-1-Me), 3.52 (2H, t, J = 7.0 Hz, H-4), 2.79 (2H, t, J = 7.0 Hz, H-3); ^{13}C NMR (DMSO-d_6), δ 166.4 (s), 159.9 (s), 125.1 (d, C-2), 122.7 (s), 121.0 (s), 116.0 (s), 40.7 (t, C-4), 37.2 (q, N-1-Me), 19.4 (t, C-3); HRFABMS m/z $[M + H]^+$ 195.0761, calcd for $C_9H_{11}N_2O_3$ 195.0770; LRFABMS m/z $[M + H]^+$ 195 (100), $[M + Na]^+$ 217 (39), $[M + K]^+$ 233 (10); LREIMS m/z $[M]^+$ 194 (8), 150 (89), 148 (33), 121 (91), 93 (100), 69 (55).

X-ray Crystal Structure for Makaluvic Acid A (5). The compound was crystallized from methanol. A small plate of size 0.20 × 0.14 × 0.03 mm was selected for all crystallographic measurements. Cell dimensions were obtained by least-squares fit to $\pm 2\theta$ values of 25 reflections measured at 223 K using Cu Kα_1 radiation. All X-ray measurements were carried out on an Enraf-Nonius CAD-4 diffractometer equipped with a liquid N_2 low-temperature device. Crystal Data: $C_9H_{10}O_3N_2$, MW = 194.2, monoclinic, $P2_1/c$, a = 7.287(2) Å, b = 7.703(3) Å, c = 14.454(2) Å, β = 90.53(2)°, V = 867.4 Å^3, Z = 4, D_x = 1.487 gm cm^{-3}, $F(000)$ = 408, λ(Cu Kα = 1.541 78 Å, μ(Cu Kα) = 8.5 cm^{-1}.

The intensity data of all the unique reflections within 2θ range 0−140° were collected at 223 ± 2 K using Cu K$\bar{\alpha}$ radiation and employing the $\theta - 2\theta$ scan technique with a variable scan width of (0.90 + 0.20 tan θ)° and horizontal aperture of (3.0 + 0.86 tan θ)mm. Three standard reflections were monitored every 2 h of X-ray exposure, and they showed maximum variation of 1.5%. The crystal orientation was checked regularly by three control reflections. A total of 1633 unique reflections were recorded, of which 959 reflections were considered observed on the basis, $I \geq 2\sigma(I)$. The intensities were corrected for Lorentz and polarization factors but no absorption correction was made. The structure was solved by direct methods and the use of program SHELXS-86[19] and refined by a full-matrix least-squares routine SHELX76[20] in which the quantity $\Sigma\omega(F_o - F_c)^2$ is minimized, where $\omega = 1/\sigma^2(F_o)$. All of the hydrogen atoms (except one C4 hydrogen) were located from difference Fourier maps, and hydrogen parameters were refined. In the final stages of refinement, non-hydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final R = 0.047, R_w = 0.057 for 959 observations and 164 parameters, S = 1.8, Δ/σ = 0.05, electron density in the final difference map ±0.3 e/Å.[21]

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(21) Positional and equivalent isotropic thermal parameters of the non-hydrogen atoms, bond distances, bond angles, selected torsion angles, anisotropic thermal parameters, and hydrogen atom parameters of **5** have been deposited with the Cambridge Crystallographic Data Centre and can be obtained upon request from Dr. Olga Kennard, 12 Union Road, Cambridge CB2 1EZ, U.K.

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