Makaluvamine N: A New Pyrroloiminoquinone from *Zyzzya fuliginosa*

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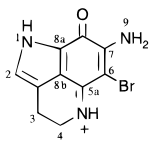
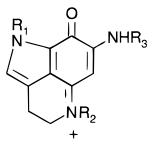
Makaluvamine N (**1**), a new pyrroloiminoquinone, was isolated from the Philippine sponge *Zyzzya fuliginosa*, together with the known compounds makaluvamines A, C, D, E (**2**−**5**), and I (**6**). The structure of **1** was determined by spectroscopic investigation. Makaluvamine N demonstrated an ability to inhibit the catalytic activity of topoisomerase II.

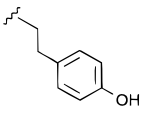
In our continuing search for novel, biologically active secondary metabolites isolated from marine organisms, we have carried out an extensive investigation of the sponge *Zyzzya fuliginosa* Carter, 1879 (order Poecilosclerida). Previously, we have reported the isolation of makaluvamines A−F, cytotoxic topoisomerase II-inhibiting pyrroloiminoquinones from specimens of *Z. fuliginosa* collected in the Fiji Islands.[1,2] Other reports have documented the isolations of makaluvamine G[3] from a *Histodermella* sponge collected in Indonesia and makaluvamines H−M[4] from specimens of *Z. fuliginosa* found in Micronesia. *Z. fuliginosa* has also been the source of a number of closely related pyrroloiminoquinone alkaloids: damirones B[1] and C,[4] discorhabdin A,[1] and makaluvone.[1] Here we report the isolation and structure determination of makaluvamine N (**1**), a new pyrroloiminoquinone isolated from a Philippine specimen of *Z. fuliginosa*.

The lyophilized sponge was extracted with MeOH. The filtered extract was then dried, resuspended in aqueous MeOH, and subjected to a modified Kupchan partitioning scheme.[5] Purification of the MeOH-soluble material was carried out on a reversed-phase C18 column. Final purification on Sephadex LH20 yielded the known compounds makaluvamines C (**3**), D (**4**), and E (**5**). The $CHCl_3$-soluble material was subjected to repeated chromatography on Sephadex LH20 to afford makaluvamines A (**2**), C (**3**), E (**5**), I (**6**), and the new pyrroloiminoquinone makaluvamine N (**1**).



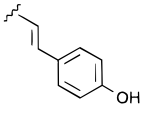
1





a

	R_1	R_2	R_3
2	Me	H	H
3	H	H	Me
4	H	H	a
5	Me	H	b
6	H	H	H



b

Makaluvamine N (**1**) was obtained as a brownish red solid. LRFABMS showed an $[M + H]^+$ ion at *m/z* 266, with an ion of equal intensity at *m/z* 268, indicating the presence of a single bromine atom. HRFABMS of the protonated molecular ion at *m/z* 265.9900 was consistent with the molecular formula $C_{10}H_9BrN_3O$ (Δ 2.9 mmu). ^{1}H-, ^{13}C-, and HMQC[6] NMR experiments confirmed the presence of five carbon-bound protons, four exchangeable protons, and seven quaternary carbons.

A basic pyrroloiminoquinone ring system was evident from ^{1}H-, ^{13}C-, HMQC, and HMBC[7] NMR spectral data and initial proton and carbon assignments made by comparison with the published data for makaluvamines A−M.[1,3,4] The ^{1}H-NMR spectrum contained a spin system comprising a pair of methylene triplets at 2.98 and 3.93 ppm (J = 7.5 Hz). Long-range ^{1}H−^{13}C correlations were observed from the methylene protons at 2.98 ppm to δ 45.11 (C-4), 120.99 (C-2a), 123.66 (C-8b), and 127.62 (C2) and from the protons at 3.93 ppm to δ 19.48 (C-3) and 120.99 (C-2a). These correlations unequivocally place these protons at positions 3 and 4 of the pyrroloiminoquinone ring system, respectively. An aromatic singlet at 7.15 ppm (MeOH-d_4) or 7.31 ppm (DMSO-d_6), attributable to H-2, and the presence of exchangeable NH protons at 8.93, 9.10, 10.38, and 13.26 ppm are consistent with spectral data reported for the known makaluvamines. The aromatic singlet at 7.15 ppm, attached to a carbon at 127.62 ppm, exhibits long-range coupling to the pyrrole carbons at δ 120.99 (C-2a), 123.66 (C-8b), and 125.07 (C-8a), confirming its assignment as H-2. The notable absence of the diagnostic H-6 proton, typically found between 5 and 6 ppm, fully supports bromination at position 6 of the makaluvamine ring.

Makaluvamine N showed considerable in vitro cytotoxicity against the human colon tumor cell line HCT-116 with an LC_{50} of 0.6 μg/mL. Makaluvamine N also exhibited greater than 90% inhibition of topoisomerase II unwinding of pBR322 at 5 μg/mL.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were obtained at 500 and 125 MHz, respectively, on a Varian Unity 500 spectrometer. All chemical shifts were reported in parts per million relative to residual undeuterated solvent. The IR spectrum was recorded on a Perkin-Elmer 1600 FT spectrophotometer. The UV spectrum was obtained in

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MeOH on a Beckman DU-8 spectrophotometer. HR-FABMS and LRFABMS were run on a Finnigan MAT-95 high-resolution gas chromatograph−mass spectrometer with Finnigan MAT ICIS II operating system.

Animal Material. Specimens of the sponge *Zyzzya fuliginosa* (collection no. P96-4-57, University of The Philippines, Marine Sciences Institute) were collected at Cape S. Ildefonso, The Philippines.

Extraction and Isolation. The lyophilized sponge (202 g, dry wt) was extracted extensively with MeOH. The MeOH extract was then filtered through Celite and concentrated *in vacuo* (65.8 g). The dried extract was partitioned between 10% aqueous MeOH and hexane (1:1). The polarity of the MeOH fraction was then increased (40% aqueous MeOH) before extraction with $CHCl_3$. The aqueous MeOH fraction was dried and the material purified on a reversed-phase C18 VLC (vacuum liquid chromatography) column, eluting with MeOH−H_2O−0.1% TFA (0−100%), to afford a mixture of known compounds. These compounds were further purified by chromatography on Sephadex LH20, eluting with MeOH−0.1% TFA, to give makaluvamine C (**3**) (16.4 g, 8.1% dry wt), makaluvamine D (**4**) (42 mg, 0.02% dry wt), and makaluvamine E (**5**) (157 mg, 0.08% dry wt). The $CHCl_3$-soluble material was purified by repeated chromatography on Sephadex LH20, eluting with MeOH−0.1% TFA, to yield makaluvamine A (193 mg, 0.10% dry wt), makaluvamine C (800 mg, 0.4% dry wt), makaluvamine E (74 mg, 0.04% dry wt), makaluvamine I (123 mg, 0.06% dry wt), and the new compound makaluvamine N (21.5 mg, 0.01% dry wt).

Makaluvamine N (1): reddish brown solid; UV (MeOH) λ max (ϵ) 242 (52 966), 344 (35 116), 390 (18 806), 544 (2274) nm; IR (dry film) υ max 3096 (br), 1670, 1608, 1522, 1406, 1200, 1130, 720 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.87 (2H, t, J = 7.5 Hz, H-3), 3.84 (2H, t, J = 7.5 Hz, H-4), 7.31 (1H, s, H-2), 8.93 (1H, bs, NH-9), 9.10 (1H, s, NH-9), 10.38 (1H, br s, NH-5), 13.26 (1H, s, NH-1); ^1H NMR (MeOH-d_4) δ 2.98 (2H, t, J = 7.5 Hz, H-3), 3.93 (2H, t, J = 7.5 Hz, H-4), 7.15 (1H, s, H-2); ^{13}C NMR (DMSO-d_6) δ 18.13 (C-3), 43.90 (C-4), 81.88 (C-6), 119.27 (C-2a), 122.32 (C-8b), 123.25 (C-8a), 126.86 (C-2), 153.31 (C-5a), 154.26 (C-7), 165.40 (C-8); ^{13}C NMR (MeOH-d_4) δ 19.48 (C-3), 45.11 (C-4), 82.28 (C-6), 120.99 (C-2a), 123.66 (C-8b), 125.07 (C-8a), 127.62 (C-2), 155.37 (C-5a), 157.16 (C-7), 166.27 (C-8); HRFABMS m/z [M + H]$^+$ 265.9900 (calcd for $C_{10}H_9BrN_3O$, 265.9929).

Cell Culture. HCT-116 cells were obtained from the American Type Culture Collection (Rockville, MD). The HCT-116 cells were grown as a monolayer in McCoy's medium containing 10% fetal bovine serum (Atlanta Biological, Atlanta GA), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 5 units/mL of nystatin. All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Before drug treatment, cells grown as monolayers were detached with trypsin treatment. Media and antibiotics were obtained from Sigma Chemical Co.

Cytotoxicity Assays. Cytotoxicity was established in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using the human colon tumor cell line HCT-116. Cytotoxicity was assessed in an MTT-microtiter plate tetrazolium cytotoxicity assay (MTA). This assay was originally described by Mosmann[8] and modified by others.[9,10] Extracts and purified metabolites were dissolved in 100% DMSO at an initial concentration of 10 mg/mL and serially diluted. The final DMSO concentration in the cell culture wells was 1% or less. HCT-116 cells (20 000 cells/well) were seeded in 200 μL of growth medium in Corning 96-well microtiter plates. Four hours after seeding, cells were treated with 1 μL of drug and were refed on the third day of the assay with 100 μL of fresh complete McCoy's medium. This was followed by the addition of 11 μL of MTT solution (5 mg/mL in phosphate buffered saline, pH 7.4) and 4 h further incubation. The MTT is reduced by viable cells to a purple formazan product. The formazan product was solubilized by the addition, to each well, of 100 μL of 0.04 N HCl in 2-propanol. The absorbance at 540 nm for each well was measured using a BIO RAD MP450 plate reader. Average absorbance for each set of quadruplicate drug-treated wells was compared to the average absorbance of the control wells to determine the percentage of growth inhibition (fractional survival) at any particular drug dosage.

Topoisomerase II Catalytic Assay. Inhibition of the catalytic activity of topoisomerase II was measured *in vitro* using a DNA relaxation assay. The relaxation assay used supercoiled pBR322 DNA as a substrate. The production of relaxed plasmid species indicates catalytic activity. The in vitro inhibition of topoisomerase II relaxation of supercoiled pBR322 DNA, as described by Muller,[11] was performed as follows. Each reaction was carried out in a 0.5 mL microcentrifuge tube containing 19.5 μL of H_2O, 2.5 μL of 10× buffer (1× buffer contains 50 mM Tris−HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, and 30 μg of bovine serum albumin/mL), 0.5 μg of supercoiled pBR322, and 1 μL of DMSO-containing drug. This combination was mixed thoroughly and kept on ice. One unit of pure human topoisomerase II was added immediately before incubation in a H_2O bath at 34 °C for 30 min. After incubation, the relaxation assay was stopped by the addition of 5 μL of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and placed on ice. DNA was subjected to electrophoresis on a 1% agarose gel in TAE (trisacetate and ethylene diamine) buffer without ethidium bromide. The gel was subsequently stained with ethidium bromide before being photographed using a Polaroid Land camera. Pure human topoisomerase II_α, prepared from a yeast expression system (gift from Dr. J. A. Holden, University of Utah, Department of Pathology), was used in these experiments.

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