Makaluvamine P, a New Cytotoxic Pyrroloiminoquinone from *Zyzzya* cf. *fuliginosa*

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A new pyrroloiminoquinone alkaloid (**1**) belonging to the makaluvamine family has been isolated from the sponge *Zyzzya* cf. *fuliginosa* collected in the waters off the Vanuatu Islands. The compound, designated makulavamine P, was characterized on the basis of its spectral data and displayed cytoxicity in the μM range on KB cells and antioxidant activity.

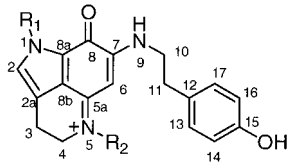
Makaluvamines, pyrroloiminoquinone alkaloids, represent an important class of marine metabolites isolated predominantly from sponges of the genera *Zyzzya*.[1−4] They were reported to show high *in vitro* and *in vivo* cytotoxic activity toward several tumor cell lines, and some of them demonstrate strong topoisomerase II inhibition.[5,6]

In our continuing search for cytotoxic marine metabolites,[7] we investigated the crude active extracts of the marine sponge *Zyzzya* cf. *fuliginosa* (order Poecilosclerida) collected off the coast of the Vanuatu Islands (preliminary cytotoxicity on KB cells of EtOH extract: 100% of inhibition of cell growth at 10 μg/mL). In this paper we report the isolation and spectroscopic characterization of the new cytotoxic makaluvamine P (**1**), which co-occurred with the known makaluvamines G[2] (**2**), J[3] (**3**), K[3] (**4**), and L[3] (**5**), which were identified by comparison with NMR spectral data reported in the literature. This compound exhibited moderate cytotoxicity to KB tumor cells: 64% inhibition of cell growth at a dose of 3.2 μg/mL. Moreover, makaluvamine P was tested for the evaluation of antioxidant activity in two different spectrophotometric analyses. It showed high inhibition of xanthine oxidase (IC_{50} = 16.5 μM), an important biological source of superoxide radicals involved in oxidative stress of the organism.[8] When analyzed on ABTS radical cation decolorating assay, compound **1** demonstrated moderate activity expressed as Trolox equivalent antioxidant activity (TEAC = 0.341).

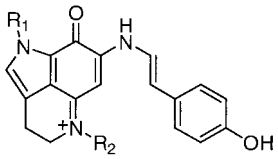
The freeze-dried organism (270 g) was initially extracted with MeOH, and the residue obtained after solvent evaporation was subsequently subjected to a modified Kupchan partitioning method,[9] affording *n*-hexane, CCl_4, $CHCl_3$, and *n*-butanol extracts. Fractionation of the bioactive $CHCl_3$-soluble material was performed by DCCC chromatography ($CHCl_3$/MeOH/H_2O, 7:13:8) followed by RP-HPLC on a C-18 μ-bondapak column (MeOH/H_2O, 45:55, with 0.1% TFA as eluent), affording pure makaluvamines G, J, K, L, and P (see Experimental Part).

Structural characterization of makaluvamine P (**1**) was achieved combining 1D and 2D NMR along with ESIMS and MS/MS data.

The ESIMS spectrum contained an intense molecular ion at 336 *m/z*, 14 atomic mass units greater than makaluvamine J (**3**) and K (**4**) ($C_{18}H_{20}N_2$) and 2 atomic mass units



	R_1	R_2
1	CH_3	CH_3
3	H	CH_3
4	CH_3	H



	R_1	R_2
2	CH_3	CH_3
5	H	CH_3

greater than makaluvamine G (**2**) ($C_{19}H_{20}N_2$), suggesting for this product the molecular formula $C_{19}H_{22}N_2$. Interpretation of NMR data (^{1}H, ^{13}C, HSQC, and HMBC) of **1** strongly indicated the presence of pyrroloiminoquinone and *p*-hydroxyphenethyl moieties. Therefore, full ^{1}H and ^{13}C NMR assignments could be readily obtained by analysis of homonuclear and heteronuclear connectivities and by comparison with other known compounds of this series, particularly makaluvamines G (**2**), J (**3**), K (**4**), and L (**5**). The ^{1}H NMR spectrum, recorded in DMSO-d_6, contained a singlet resonance at δ 7.35 (H-2), attached to a carbon at 131.2 ppm, which exhibited long-range couplings with all the carbons of the pyrrole ring [117.3 ppm (C-2a), 122.4 ppm (C-8a), 123.1 ppm (C-8b)]. Two coupled methylenes at δ 2.92 and 3.89 (δ C 18.8 and 52.5, respectively) were assigned to positions 3 and 4 of the pyrroloiminoquinone skeleton on the basis of the HMBC correlations observed (H-3/C-2, H-3/C-2a, H-3/C-4, H-3/C-8b; H-4/C-2a, H-4/C-5a). Two downfield-shifted methyl signals at δ 3.91 and 3.39 (3H, s) were placed on N-1 and N-5, respectively, on the basis of observed HMBC correlations (N1-Me/C-2, N1-Me/C-8a; N5-Me/C-4, N5-Me/C-5a). The olefinic proton resonating at δ 5.62, which was attached to a carbon resonating at 83.5 ppm, was assigned to position 6 of the tricyclic skeleton due to its long-range ^{1}H−^{13}C correlations with the carbonyl carbon signal at 167.3 ppm (C-8) and the quaternary carbon at 123.1 ppm (C-8b).

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Furthermore, the NMR spectra contained signals for a *p*-hydroxyphenethyl unit that were superimposable with those reported for makaluvamines J (**3**) and K (**4**). Finally the connection between the pyrroloiminoquinone and *p*-hydroxyphenethyl moieties was established on the basis of the key HMBC correlation H_2-10/C-7. Additional support for the structural elucidation of **1** came from MS/MS data showing a fragment ion at *m*/*z* 229, corresponding to the loss of a *p*-hydroxyphenethyl residue from the molecular ion.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were determined on a Bruker DRX-600 Avance spectrometer, and the solvent was used as internal standard (CD_3-OD: ^{1}H δ 3.34, ^{13}C 49.0 ppm; DMSO-d_6: ^{1}H δ 2.50, ^{13}C 39.5 ppm). 2D experiments (COSY, HSQC, and HMBC) were recorded using conventional pulse sequences. The UV spectrum was obtained on a Beckman DU-70 spectrophotometer. Mass spectra were recorded on a Thermoquest LCQ-DECA spectrometer equipped with electrospray source.

Biological Material. The sponge *Zyzzya* cf. *fuliginosa* was collected along the coasts of Vanuatu Islands and taxonomically identified by Dr. John Hooper (voucher specimen located at Museum of Queensland, Brisbane, Australia; accession number G306878).

Extraction and Isolation. The lyophilized material (270 g) was extracted with MeOH (3 × 1.5 L) at room temperature. The combined methanolic extracts, filtered through paper and concentrated under reduced pressure, gave a dark red oil. The oily residue was partitioned using a modified Kupchan partition method:[9] the extract was dissolved in 0.7 L of a mixture of MeOH/H_2O containing 10% of H_2O and partitioned against 0.7 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 1 L of CCl_4 and 1 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH (0.5 L). The $CHCl_3$-soluble material (1 g) was then subjected to DCCC chromatography, using $CHCl_3$/MeOH/H_2O (7:13:8) as the eluting mixture. Fractions 70−83, purified by RP-HPLC (MeOH/H_2O, 45:55, with 0.1% TFA) afforded, as pure compounds, makaluvamine G (1.5 mg), makaluvamine J (1.7 mg), and makaluvamine K (6.7 mg), while fractions 84−98 gave makaluvamine L (2.2 mg) and the novel makaluvamine P (7.0 mg).

Makaluvamine P (1): violet solid; UV (MeOH) λ_{max} (ϵ) 222 (7470), 248 (7750), 361 (5075) nm; ^{1}H NMR (MeOH-d_4, 600 MHz) δ 7.10 (1H, s, H-2), 7.07 (2H, d, J = 8.2 Hz, H-13, H-17), 6.71 (2H, d, J = 8.2 Hz, H-14, H-16), 5.31 (1H, s, H-6), 3.95 (3H, s, N1-Me), 3.89 (2H, t, J = 7.7 Hz, H-4), 3.65 (2H, t, J = 6.7 Hz, H-10), 3.32 (3H, s, N5-Me), 2.95 (2H, t, J = 7.7 Hz, H-3), 2.91 (2H, t, J = 6.7 Hz, H-11);^{13}C (MeOH-d_4, 150 MHz): δ 168.6 (C-8), 157.5 (C-15), 157.3 (C-5a), 155.1 (C-7), 132.0 (C-2), 131.1 (C-13, C-17), 130.2 (C-12), 124.8 (C-8b), 124.3 (C-8a), 119.1 (C-2a), 116.5 (C-14, C-16), 85.5 (C-6), 54.2 (C-4), 46.6 (C-10), 39.8 (N5-Me), 36.5 (N1-Me), 34.8 (C-11), 20.3 (C-3); NMR data in DMSO-d_6: see Table 1; ESIMS *m*/*z* 336 $[M]^+$ (77), 229 $[M - C_7H_7O]^+$ (100).

Cytotoxicity Assay. A defined number of tumor cells were placed in microtiterplates (100 μL) and incubated at 37 °C/5% CO_2 for 24 h. The samples were dissolved in DMSO (333-fold more concentrated than the highest test concentration), and 50 μL of the solutions/concentrations or 50 μL of pure DMSO (controls) was pipetted to the cell cultures. The cell cultures were incubated with the samples for an additional 45 h. After this incubation period, 75 μL of XTT solution was added to the culture, and incubation continued for 3 h at 37 °C/5% CO_2. The extinction of all cell cultures was measured at 490 nm and compared to the extinction of the corresponding control cultures. This assay was run on a screening robot (Biomek 2000, Beckman).[10] The percentage of inhibition found for **1** by this procedure was 64% on KB tumor cell line, at 3.2 μg/mL.

Table 1. NMR Data for Makaluvamine P (**1**) in DMSO-d_6 (600 MHz)

	^{1}H, d, *J* (Hz)	^{13}C, ppm	HMBC
2	7.35, s	131.2	C-8a; C-2a; N1-CH_3
2a		117.3	
3	2.92, t, *7.2*	18.8	C-8b; C-2; C-2a; C-4
4	3.89, t, *7.2*	52.5	C-5a; C-2a; N5-CH_3; C-3
5a		154.8	
6	5.62, s	83.5	C-8; C-8b
7		153.1	
8		167.3	
8a		122.4	
8b		123.1	
9-NH	9.07		
10	3.63, t, *7.2*	45.0	
11	2.84, t, *7.2*	32.7	C-12; C-13; C-10
12		128.3	
13	7.07, d, *7.7*	129.7	C-15; C-17; C-11
14	6.71, d, *7.7*	115.1	C-15; C-12; C-16
15		156.0	
16	6.71, d, *7.7*	115.1	C-15; C-14; C-16
17	7.07, d, *7.7*	129.7	C-15; C-13; C-11
N1-CH_3	3.91, s	35.9	C-2; C-8a
N5-CH_3	3.39, s	39.0	C-5a; C-4
15-OH	9.33		C-14; C-15; C-16

Xanthine Oxidation Inhibition Assay. Xanthine oxidase activity was evaluated by the spectrophotometric measurement of the formation of uric acid from xanthine.[11] A 100 μM solution of xanthine in 0.1 M phosphate buffer pH 7.8 with 0.04 units/mL of xanthine oxidase was incubated for 10 min at room temperature and read at 295 nm against a control sample, which did not contain the enzyme.

Various concentrations of testing compound (5, 10, 15, 25 μg/mL) were added to samples before the enzyme had been instilled, and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} value. The IC_{50} value calculated for **1** was 16.5 μM.

ABTS Radical Cation Decolorization Assay. Evaluation of free radical scavenging activity was performed with the TEAC assay. TEAC value is based on the ability of the antioxidant to scavenge the $ABTS^+$, the preformed radical monocation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), with spectrophotometric analysis, according to Re et al.[12]

Samples were diluted with methanol to have 0.3, 0.5, 1.0, 1.5, and 2.0 mM solutions. The reaction was enhanced by the addiction of 1.0 mL of diluted ABTS to 10 μL of each solution of sample or Trolox (standard) or 10 mL of methanol (control). The determination was repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration as a function of the control's absorbance, 1 min after initial mixing. The antioxidant activity was expressed as TEAC (Trolox equivalent antioxidant activity), which is the concentration of standard Trolox solution with equivalent percentage inhibition to a 1 mM solution of the tested compound. The TEAC value obtained for **1** was 0.341.

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