Batzelline D and Isobatzelline E from the Indopacific Sponge *Zyzzya fuliginosa*

Leng Chee Chang,[†] Sarah Otero-Quintero,[†] John N. A. Hooper,[‡] and Carole A. Bewley*[,†]

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0820, and Queensland Museum, South Brisbane, Qld 4101, Australia

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Two new pyrroloquinoline alkaloids, isobatzelline E (**1**) and batzelline D (**2**), together with the known compounds batzelline C (**3**), isobatzelline C (**4**), and makaluvamine D (**5**), were isolated from an Indopacific collection of the marine sponge *Zyzzya fuliginosa*; the known compounds makaluvamines A (**9**), H (**10**), J (**7**), K (**8**), and P (**6**) were obtained from *Z. fuliginosa* collected in Papua New Guinea. The structures were elucidated by interpretation of 1D ^{1}H and ^{13}C NMR spectra and 2D HSQC and HSQC-LR spectra. Compounds **1**−**10** were isolated because the crude extracts of both *Zyzzya* species inhibited HIV-1 envelope-mediated cell fusion. However, the inhibition profile observed for the pure compounds **1**−**10** mirrors that reported for the inhibition of topoisomerase II by other pyrroloquinolines, leaving open the possibility that the activity results from interactions with DNA-modifying enzymes.

Marine sponges of the genus *Zyzzya* are notable for producing secondary metabolites that feature a pyrrolo-[4,3,2-*de*]quinoline skeleton. Previous studies of collections of *Zyzzya* yielded damirone C,[1] discorhabdin A,[2] makaluvamines A−F[2] and H−N,[1,3] and veiutamine.[4] In addition to *Zyzzya*, other pyrroloquinolines have been isolated from the sponges *Batzella* sp. (yielding batzellines A−C,[5] isobatzellines A−D,[6] and seco-batzellines A and B[7]), *Damiria* sp. (damirones A and B[8]), and *Histodermella* sp. (makaluvamine G[9]). Collections of *Latrunculia* sp.[10−12] and *Prianos melanos*[13,14] have yielded the more complex pyrroloiminoquinones, such as discorhabdins A−D. Outside of the phylum Porifera, the ascidian *Clavelina* sp. yielded the pyrroloiminoquinone wakayin,[15] and makaluvamine A was recently isolated from a laboratory culture of the myxomycete *Didymium bahiense*.[16]

In addition to the cytotoxicity reported for several of these compounds, interest in this series derives from the ability of some of the pyrroloquinolines to potently inhibit the enzyme topoisomerase II.[2] During the course of screening marine extracts for their ability to inhibit HIV-1 envelope-mediated fusion in vitro, crude extracts of two specimens of *Zyzzya fuliginosa* (collections C6733 and C9217, respectively, Natural Products Repository, NCI) were chosen for further investigation because the ethyl acetate-soluble extracts showed significant activity in an HIV-1 cell fusion assay. As a result, we have identified two new pyrroloquinolines, and we report the isolation, structure elucidation, and biological activity below.

The HRFAB mass spectrum of **1** showed two molecular ions at *m*/*z* 234.0441 and 236.0404 present in an approximate ratio of 3:1, indicating the presence of chlorine and a molecular formula of $C_{11}H_8ON_3Cl$. Eleven signals were observed in the ^{13}C NMR spectrum, consistent with the molecular formula; a DEPT spectrum revealed the presence of one methyl, three methines, and therefore seven quaternary carbons. In addition to these hydrogens, a broad singlet (δ_H 6.33, 2H) in the ^{1}H NMR spectrum suggested the presence of an amino group and when considered together with the molecular formula suggested that **1** was a pyrroloiminoquinone. A comparison of the ^{1}H and ^{13}C NMR data of **1** with that of the known compound isobatzelline C (**4**),[6] which was also present in this collection, indicated that they differed only by the presence of a double bond at C-3−C-4: the mutually coupled methylene signals (δ_C 17.8, δ_H 2.61, t, J = 7.8 Hz; δ_C 49.1, δ_H 3.97, t, J = 7.8 Hz) of **4** were replaced with a pair of coupled olefinic methines at δ_H 7.56 (d, J = 6 Hz) and 8.35 (d, J = 6 Hz) in **1** (Table 1). This ring system has been previously reported for makaluvamine B;[2] however, compared to makaluvamine B, the ^{1}H signal for H-6 is absent, indicating chlorination at C-6. Analysis of the HSQC-LR spectra (Table 1), run to observe J_{CH} couplings of 4 and 8 Hz, showed correlations from the *N*-methyl protons (δ_H 4.27) to C-2 and C-8a and from H-2 to C-2a, C-8a, and C-8b, confirming that **1** is a pyrroloiminoquinone. Compound **1** was given the trivial name isobatzelline E.

Compound **2** showed molecular ions at *m*/*z* 223.0276 and 225.0260, again in a 3:1 ratio, indicating a molecular formula of $C_{10}H_7O_2N_2Cl$. The ^{1}H NMR spectrum of **2** contained signals for an aromatic singlet at δ_H 7.15 (H-2), a pair of mutually coupled methylenes at δ_H 2.76 (H-3) and 3.58 (H-4), and two exchangeable NH protons at δ_H 8.33 (H-5) and 12.58 (H-1). Relative to **1**, the methyl group at N-1 and the amino group at C-7 were absent in compound **2**. Together with the molecular formula $C_{10}H_7O_2N_2Cl$, these data indicated that **2** was a pyrroloquinoline alkaloid similar to the damirones[1,8] and batzellines,[5] all of which contain a C-7 keto moiety in place of the C-7 amino group. Comparison of the ^{13}C NMR (Table 1), UV, and IR spectra of **2** with those reported for batzelline C (**3**),[5] which was also present in this collection, demonstrated that **2** differed only by the absence of the *N*-methyl group on the pyrrole nitrogen. Thus, compound **2** was given the trivial name batzelline D.

Once pure, compounds **1**−**10** were tested in a colorimetric vaccinia virus-based reporter gene assay that reproduces the sequence of events that lead to HIV-1 envelope-mediated cell fusion, or viral entry.[17] Surprisingly, only isobatzelline C (**4**) and makaluvamines A (**9**) and H (**10**) appeared to potently affect fusion as detected by the complete absence of β-galactosidase (β-gal) activity at concentrations less than 1 μg/mL. In contrast, no measurable activity was detected for compounds **1**−**3**, and greatly diminished activity was observed for compound **5**−**8**. Dose−response experiments yielded IC_{50}'s of ∼200 nM for iso-

* To whom correspondence should be addressed. Tel: (301) 594-5187. Fax: (301) 402-0008. E-mail: cb194k@nih.gov.
[†] National Institutes of Health.
[‡] University of Queensland.

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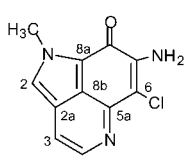
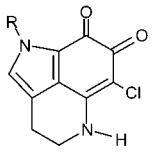
Table 1. ^{1}H and ^{13}C NMR Data (ppm) for **1** and **2** in DMSO-d_6[a]

	1			2		
position	δ_C (mult)	δ_H (mult, J (Hz))	HSQC-LR	δ_C (mult)	δ_H (mult, J (Hz))	HSQC-LR
1						
2	132.2 (d)	8.25 (s)	2a, 8a, 8b	124.0 (d)	7.15 (s)	2a, 8a, 8b
2a	123.4 (s)			117.8 (s)		
3	112.9 (d)	7.56 (d, 6)	4	19.8 (t)	2.76 (t, 7)	2a, 2, 4, 8b
4	142.0 (d)	8.35 (d, 6)	2a, 3, 5a	43.2 (t)	3.58 (dt, 7,2.4)	2a, 3, 5a
5a	143.8 (s)			149.0 (s)		
6	104.9 (s)			97.0 (s)		
7	146.0 (s)			168.6 (s)		
8	164.8 (s)			170.9 (s)		
8a	117.9 (s)			123.9 (s)		
8b	117.4 (s)			122.3 (s)		
1-NMe	37.3 (q)	4.27 (s)	2, 8a			
5-NH					8.33 (br s)	
7-NH_2		6.33 (br s)	6, 8			

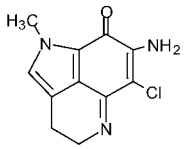
[a] Spectra were recorded at 500 MHz at 300 K and referenced to residual solvent signal of DMSO-d_6 (δ_H 2.50).



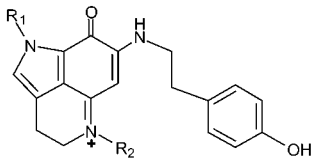
1



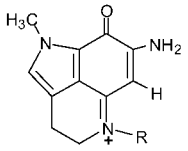
2 R = H
3 R = CH_3



4



5 R_1 = H R_2 = H
6 R_1 = CH_3 R_2 = CH_3
7 R_1 = H R_2 = CH_3
8 R_1 = CH_3 R_2 = H



9 R = H
10 R = CH_3

batzelline C (**4**) and ~5 μM for makaluvamines A (**9**) and H (**10**).

In vitro cytotoxicity toward several tumor cell lines has been reported for some pyrroloiminoquinones. In addition, considerable interest in this class of compounds arose due to the ability of some of the pyrroloiminoquinones, exemplified by makaluvamines A and C, to potently inhibit the enzyme topoisomerase II (topo II).[2,18] Thus far, potent topo II activity in the pyrroloquinolines appears to correlate with the presence of (i) sp^3-hybridized carbons at positions 3 and 4 and (ii) the 7-amino,8-keto quinone moiety, illustrated here in isobatzelline C (**4**). In contrast, pyrroloquinolines bearing a double bond at C-3−C-4 (exemplified by isobatzelline E, **1**) and/or a 7,8-diketo system (featured in the damirones and batzellines such as **2** and **3**) have been shown to be inactive.[2,18] As a result of testing compounds **1**−**10**, we have observed a similar profile in our fusion assay; namely, isobatzelline C (**4**) and to a lesser extent makaluvamines A (**9**) and H (**10**) appear to inhibit fusion as detected by a complete absence of β-gal activity. In contrast, compounds **1**−**3** were inactive and compounds **5**−**8** modestly inhibited β-gal activity. Given these similar structure−activity profiles, we speculate that the "active" compound in the fusion assay, isobatzelline C, may be interfering with DNA-modifying enzymes (such as T7 polymerase) during the course of the assay. Additional studies are underway to discriminate between these different mechanisms.

Experimental Section

General Experimental Procedures. UV and IR spectra were obtained with a Beckman DU-600 spectrophotometer and a Bio-Rad FTS-45 FT-IR spectrophotometer. NMR experiments were recorded on a Bruker DMX500 spectrometer equipped with an *xyz*-shielded gradient triple resonance probe. FABMS was performed on a JEOL-SX102 and CIMS on a Finnigan 4500. Reversed-phase HPLC was carried out on a GBC system equipped with a photodiode array detector using a Waters μBondapak C_{18} column (7.8 × 300 mm) run with a flow rate of 3 mL/min and detected at 210 nm.

Animal Material. *Zyzzya fuliginosa* specimens Q66C0102 and Q66C6161 were collected respectively in September of 1990 on Abrohlos Island in the Indo-West Pacific and in October of 1991 in Papua New Guinea. Both sponges displayed massive, subspherical, partially burrowing bases with emergent fistules, were black in life, and produced a greenish-black mucus. While collection C6733 was originally identified as *Zyzzya massalis* (Dendy, 1922), we note that *Z. massalis and Z. fuliginosa* are synonymous, with the oldest identification being *Z. fuliginosa*. Although the chemistry of *Z. massalis* (also misspelled as *marsalis*) has been reported in the literature, this name is now accepted as a junior synonym of *Z. fuliginosa* (Van Soest et al., 1994), which now takes precedence.[19] The sponges were frozen immediately upon collection and shipped to NCI (National Cancer Institute, Frederick, MD). After aqueous extraction of the frozen sponges at 4 °C, the materials were lyopholized and extracted successively with CH_2Cl_2−MeOH (1:1) and MeOH. The combined organic extracts were evaporated in vacuo and stored at −30 °C. Voucher specimens were deposited at the Queensland Museum, Brisbane, and the Smithsonian Sorting Center, Suitland, MD.

Extraction and Isolation. The crude organic (1:1 CH_2Cl_2−MeOH) extracts of both *Zyzzya* specimens tested positive in a colorimetric HIV-1 fusion assay at concentrations less than 20 μg/mL and were therefore subjected to bioassay-guided fractionation and purification as follows. Two grams of *Z. fuliginosa* (Indo-Pacific collection) crude extract was partitioned with EtOAc−H_2O to give, respectively, 1.02 and 0.61 g of organic and aqueous extracts, both of which showed the same level of activity in the fusion assay. The EtOAc fraction was purified further by flash chromatography (Si gel) using a stepwise chloroform−methanol gradient. On the basis of activity, fractions 4, 6, and 8 (of 11 fractions total) were repurified individually over an LH-20 (Sephadex) column (1:1 $CHCl_3$−MeOH), affording compounds **1** (5.5 mg, 0.00055% wet weight), **3** (2.3 mg, 0.00023% wet weight), and **4** (50 mg, 0.005% wet weight), respectively. Similarly, the aqueous extract was purified using LH-20 (1:1 $CHCl_3$−MeOH), and the final purification of the active fractions was accomplished by reversed-phase HPLC (70% CH_3CN in 0.05% TFA) to yield compounds **2** (t_R 8.3 min, 1.8 mg, 0.00018% wet weight) and **5** (t_R 10.5 min, 2.0 mg, 0.0002% wet weight).

The crude organic extract (1.9 g) of *Z. fuliginosa* (Papua New Guinea) was partitioned following a similar scheme as described above to yield 1.36 and 0.47 g of EtOAc and aqueous-soluble extracts, respectively. The ethyl acetate extract was chromatographed on a Sephadex LH-20 (MeOH) column to obtain 10 major subfractions. Further purification focused on active fractions 3−6. Thus, fractions 3 and 4 were combined and purified by reversed-phase HPLC eluting with 75% aqueous CH_3CN in 0.05% TFA to give compounds **6** (t_R 10.2 min; 4.5 mg; 0.00056% wet weight), **7** (t_R 15.0 min; 6.0 mg; 0.0008% wet weight), **8** (t_R 18.4 min; 3.5 mg; 0.00043% wet weight), and **10** (t_R 21.0 min; 5.2 mg; 0.00065% wet weight). Fraction 6 was subjected to Sephadex LH-20 chromatography using 1:2 choloroform−methanol as eluent and afforded 7.8 mg of compound **9** (0.00097% wet weight).

Isobatzelline E (1): red solid; UV (MeOH) λ_{max} (log ϵ) 224 nm (3.72), 341 nm (2.26), 422 nm (3.43); IR (film) ν_{max} 3250, 2960, 2927, 1680, 1620, 1550, 1196 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (75 or 125 MHz, DMSO-d_6) data appear in Table 1; HRFABMS (NBA) *m/z* 234.0441 [$(M + H)^+$], $C_{11}H_8ON_3Cl$ requires *m/z* 234.0445.

Batzelline D (2): purple solid; UV (MeOH) λ_{max} (log ϵ) 248 nm (4.0), 328 nm (3.73); IR (film) ν_{max} 3290, 2927, 2879, 1670, 1605, 1545, 1205, 1120 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (75 or 125 MHz, DMSO-d_6) data appear in Table 1; HRFABMS (NBA) *m/z* 223.0276 [$(M + H)^+$], $C_{10}H_7O_2N_2Cl$ requires *m/z* 223.0280.

Batzelline C (3): purple solid; UV, IR, and ^1H and ^{13}C NMR spectral data are in agreement with previously published data.[5]

Isobatzelline C (4): dark brown solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[6]

Makaluvamine D (5): red solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[2]

Makaluvamine P (6): violet solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[20]

Makaluvamine J (7): red-brown solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[1]

Makaluvamine K (8): red-brown solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[1]

Makaluvamine A (9): red solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[1]

Makaluvamine H (10): red-brown solid; UV, IR, and ^1H and ^{13}C NMR spectra data are in agreement with previously published data.[1]

Bioassays. Cell fusion assays were conducted as previously described[17] using soluble CD4 (NIH AIDS Research and Reference Repository Program) and recombinant vaccinia viruses expressing genes for HIV-1 Env, the T-cell tropic coreceptor CXCR4, *E. coli* T7 polymerase (T7), and β-galactosidase (β-Gal). Briefly, BS-C-1 and NIH3T3 cells were used as effector and target cell populations, respectively. The effector cells were co-infected with recombinant vaccinia viruses encoding HIV-1 Env and β-Gal, and target cells were co-infected with recombinant vaccinia viruses encoding CXCR4 and T7 at a multiplicity of infection (MOI) of 5. For inhibition studies, extracts or pure compounds were added to an appropriate volume of DMEM (supplemented with 2.5% serum) and PBS (to yield identical buffer compositions (100 μL), followed by addition of 1×10^5 effector cells (in 50 μL of media) per well; 1×10^5 target cells (in 50 μL) and soluble CD4 were then added to each well. Following a 2.5 h incubation, β-Gal activity of cell lysates was measured (A_{570}, Molecular Devices 96-well spectrophotometer) upon addition of chlorophenol-red-β-D-galactopyranoside (CPRG).

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