Secobatzellines A and B, Two New Enzyme Inhibitors from a Deep-Water Caribbean Sponge of the Genus *Batzella*

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Secobatzelline A (**1**), a new batzelline natural analogue, and secobatzelline B (**2**), a likely artifact formed during the isolation procedure, have been isolated from a deep-water marine sponge of the genus *Batzella*. Secobatzellines A and B inhibited the phosphatase activity of calcineurin, and secobatzelline A inhibited the peptidase activity of CPP32. Both compounds showed in vitro cytotoxicity against P-388 and A-549 cell lines. The isolation and structure elucidation of secobatzellines A (**1**) and B (**2**) are described.

In a continuing search for new protein phosphatase inhibitors from marine organisms,[1,2] we have isolated two new batzelline analogues, trivially named secobatzellines A and B, from a deep-water marine sponge of the genus *Batzella*, which inhibit calcineurin (CaN). Secobatzelline A (**1**) also inhibits the peptidase activity of CPP32. Secobatzellins A and B are probable precursors of simpler pyrroloiminoquinone alkaloids and have not been previously described in the literature. Secobatzelline A possesses an unusual pyrroloaminoiminoquinone moiety previously reported from isobatzellines A−D.[3] The structures were determined by a combination of NMR spectral studies and chemical interconversions.

Pyrroloiminoquinone alkaloids have been isolated from a few sponge genera and from a Fijian ascidian of the genus *Clavelina*.[4] The simplest of these pyrroloquinoline alkaloids are batzellines A−C[5] and isobatzellines A−D[3] from a deep-water Caribbean sponge of the genus *Batzella*. The related damirones A−B from Palau,[6] makaluvamines A−G from Fiji[7] and Indonesia,[8] and makaluvamines H−M and damirone C from Pohnpei[9] have been isolated from different sponges taxonomically assigned to the genus *Zyzzya*.[10] The related complex alkaloids, exemplified by discorhabdin C, were first reported from the sponge of the genus *Latrunculia* by a New Zealand group in 1986.[11] Since then, the same group[12−16] and others[17−19] have published the isolation of discorhabdins A−O from South Pacific sponges of the genus *Latrunculia* and Japanese sponge of the genus *Prianos*. Recently, we reported the isolation of discorhabdin P from a sponge of the genus *Batzella*.[2]

Calcineurin is a serine−threonine protein phosphatase involved in signal transduction and is recognized as being one of the principal signaling molecules that regulates the immune response.[20] Immunosuppressants such as FK506 and cyclosporin A have been shown to exert their effect through inhibition of CaN following their association with binding proteins.[21] This finding has prompted a search for small molecule inhibitors of CaN that might be expected to have useful pharmacological activity.

The caspases, which include CPP32, are a group of at least 10 cysteine proteases (also known as interleukin-2 converting enzymes or ICE_2), which play a major role in the programmed cell death mechanism known as apoptosis.[22] These enzymes are the mammalian homologues of the ced-3 gene product that modulates apoptotic processes in the nematode *Caenorhabditis elegans*.[23] Mutations in ced-3 prevent apoptosis during normal development of the nematode and in mammals, and inhibitors of caspase-3 (CPP32) have been shown to prevent apoptotic mediated death in a number of cell lines and in various tissues.[23,24] Caspase-3 has also been shown to be involved in the stimulation of IL-8 secretion of synoviocytes in rheumatoid arthritis, which serves to increase joint inflammation and progress of the disease.[25] Therefore, inhibitors of caspase enzymatic activities may serve to prevent the pathological damage induced by caspase-mediated apoptotic events.

Two samples of the sponge *Batzella* sp. were collected from the Bahamas in August 1994, and stored at −20 °C until extraction. The EtOH extracts of the two sponges gave an identical TLC pattern, and therefore the two extracts were combined for further studies. The combined EtOH extract was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was chromatographed over Si gel with CH_2Cl_2−MeOH and fractions monitored for inhibition of CaN. The fractions that showed inhibition of CaN were combined and subjected to HPLC to give secobatzellines A (**1**) and B (**2**).

HRFABMS of secobatzelline A (**1**) supported the molecular formula $C_{10}H_{10}ClN_3O_3$ [(M + H^+) *m*/*z* 256.0553, 258.0514, Δ 5 mmu]. The UV spectrum displayed characteristic absorptions for an aminoiminoquinone moiety at λ_{max} 203 (log ϵ 4.19), 233 (4.13), 322 (4.00) nm as reported for makaluvamine A.[7] IR spectral absorption at 1658 cm^{-1} indicated the presence of a conjugated carbonyl functionality. The ^1H NMR spectrum in DMSO-d_6 revealed the presence of two NH protons appearing as broad singlets (δ 12.50, 9.80), a 2H amino singlet (δ 6.55), a 1H olefinic singlet (δ 7.16), a 1H hydroxymethine triplet (δ 4.67), and a 2H hydroxymethylene doublet (δ 3.49). Analysis of the COSY spectrum indicated that the hydroxymethylene and the hydroxymethine groups constituted an isolated spin system. The ^{13}C NMR spectrum, analyzed together with the DEPT and HMQC spectra, revealed signals for seven quaternary carbons, a olefinic doublet, an oxymethine doublet, and an oxymethylene triplet. Comparison of the chemical shift values of the quaternary carbons (δ 141.2 and 103.9) with those reported for isobatzelline D (**5**, δ 143.8 and 103.9) supported the presence of an amino and a chlorine group bearing carbon atoms in the molecule, respectively.[3] These data suggested that the compound is

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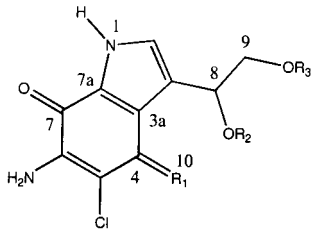
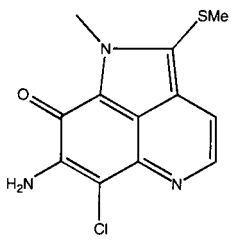
closely related to the isobatzellines previously reported from the same genus *Batzella*.[3] Acetylation of **1** with acetic anhydride in pyridine at 50 °C gave two diacetates **3** and **4**. The ^{1}H NMR spectrum of the diacetate **3** in DMSO-d_6 revealed the presence of two NH protons appearing as broad singlets (δ 12.65, 10.15), a 2H amino singlet (δ 6.41), a 1H olefinic singlet (δ 7.27), a 1H acetoxymethine triplet (δ 6.55), a 2H acetoxymethylene double doublet (δ 4.30), and two acetyl methyl singlets (δ 2.06, 1.98). The lowfield shift of the hydroxymethine proton (δ 4.67 to 6.55) and the hydroxymethylene protons (δ 3.49 to 4.30) indicated the acetylation of the two hydroxy groups. The latter lowfield shift established that the hydroxymethylene is not a part of a ring system. The hydrolysis of secobatzelline A in aqueous ethanol overnight yielded predominantly in secobatzelline B and thus established the presence of a readily hydrolyzable iminoquinone functionality in **1** and also confirmed the relationship between these two compounds. Hydrolysis of the iminoquinone to quinone functionality under acetylation conditions accounted for the formation of the diacetate **4**. The HMBC spectrum of secobatzelline A in DMSO-d_6 showed three-bond coupled ^{1}H−^{13}C correlations: H-2 (C-3a, C-7a), H-8 (C-2, C-3a), H-9 (C-3) and thus confirmed the position of the attachment of the dihydroxy side chain to the pyrrole ring system. Similarly, the HMBC spectrum of secobatzelline A diacetate **3** in DMSO-d_6 showed three-bond coupled ^{1}H−^{13}C correlations: H-1 (C-3a, C-7), H-2 (C-3a, C7a), H-8 (C-2), H-9 (C-3), H-10 (C-3a, C-5) and two-bond coupled ^{1}H−^{13}C correlations: H-2 (C-3), H-8 (C-9), H-9 (C-8), H-10 (C-4). These data confirmed the locations of the chlorine, imino, and quinone functionalities, and therefore the unaccounted amino group was conveniently placed at the remaining quaternary carbon atom. Combination of the above data established the structures for secobatzelline A (**1**) and secobatzelline A diacetate (**3**).

HRFABMS of secobatzelline B (**2)** supported the molecular formula $C_{10}H_9ClN_2O_4$ [(M + H$^+$) *m/z* 257.0433, 259.0409, Δ 1 mmu]. It showed an IR spectral absorption at 1667 cm^{-1} for the presence of a conjugated carbonyl functionality. The ^{1}H NMR spectrum in DMSO-d_6 indicated the presence of a NH proton appearing as a broad singlet (δ 12.63), a 2H amino singlet (δ 7.09), a 1H olefinic singlet (δ 7.11), a 1H hydroxymethine multiplet (δ 4.87), 2H hydroxymethylene multiplets (δ 3.49, 3.27), and two hydroxy doublets (δ 5.10, 4.57). Analysis of the COSY spectrum indicated that the hydroxymethylene, hydroxymethine, and the two hydroxyls constituted an isolated spin system. The ^{13}C NMR spectrum, analyzed together with the DEPT and HMQC spectra, revealed signals for seven quaternary carbons, an olefinic doublet, an oxymethine doublet, and an oxymethylene triplet. The ^{13}C NMR spectrum was very similar to that of secobatzelline A. The only notable exception was the replacement of the imino carbon signal (δ 158.0 in **1**) by a carbonyl carbon signal appearing at δ 175.8. Acetylation of secobatzelline B furnished a diacetate and its ^{1}H and ^{13}C NMR spectra were identical to those of diacetate **4,** the minor acetylated product of secobatzelline A. The ^{1}H NMR spectrum of the diacetate **4** in DMSO-d_6 showed the presence of a NH proton appearing as broad singlets (δ 12.80), a 2H amino singlet (δ 7.13), a 1H olefinic singlet (δ 7.28), a 1H acetoxymethine triplet (δ 6.30), a 2H acetoxymethylene multiplet (δ 4.27), and two acetyl methyl singlets (δ 2.04, 1.98). The ^{13}C NMR spectrum of **4** indicated two conjugated quinone carbonyls (δ 175.2, 169.6) and two acetoxy carbonyls (δ 170.0), together with two methyls (δ 20.8. 20.6), corresponding to two acetyl groups. Combination of the above data established the structures for secobatzelline B (**2**) and secobatzelline B diacetate (**4**). The absolute stereochemistry of the secondary hydroxyl group in **1** and **2** has not been determined.





	R_1	R_2	R_3
1	NH	H	H
2	O	H	H
3	NH	Ac	Ac
4	O	Ac	Ac

5

Secobatzelline A (**1**) inhibited CaN and CPP32 with IC_{50} values of 0.55 and 0.02 μg/mL, respectively.[26] Similarly, secobatzelline B (**2**) inhibited CaN with IC_{50} values of 2.21 μg/mL, and CPP32 analysis was not determined. Secobatzelline A diacetate (**3**) and secobatzelline B diacetate (**4**) inhibited CPP32 with IC_{50} values of 0.80 and 8.80 μg/mL, respectively. CaN activity for **3** and **4** have not been determined. The activities observed are highly significant as there are presently very few compounds in the literature that demonstrate nM potency for inhibition of either calcineurin or CPP32 activity.

Compounds **1**, **2**, **3**, and **4** exhibited in vitro cytotoxicity against the cultured murine P-388 tumor cell line, with IC_{50} values of 0.06, 1.22, 0.01, and 3.83 μg/mL and against human lung carcinoma A-549 cell line, with IC_{50} values of 0.04, 2.86, 0.12, 4.68 μg/mL, respectively.

Experimental Section

General Experiment Procedures. IR spectra were obtained on a Midac FT−IR M 1200 instrument. UV spectra were taken with a Perkin−Elmer Lambda 3B UV/vis spectrophotometer. 1D and 2D NMR spectra were measured on a Bruker AMX-500 instrument. The ^{1}H NMR chemical shifts were assigned using a combination of data from COSY and HMQC experiments. Similarly, ^{13}C NMR chemical shifts were assigned on the basis of DEPT and HMQC experiments. The HRMS were obtained on a Finnigan MAT95Q mass spectrometer at the Spectroscopic Services Group, University of Florida.

Collection and Taxonomy. One sponge sample (HBOI # 25-VIII-94-1-001) was collected on August 25, 1994, by the *Clelia*, a manned submersible, at a depth of 152 m, from the western Great Bahama Bank, Bahamas (latitude 25° 23.921′ N; longitude 79° 14.104′ W). The second sponge sample (HBOI # 26-VIII-94-4-003) was collected on August 26, 1994, by the *Clelia*'s manned submersible at a depth of 138 m, from North Bimini west of Alice Town, Bahamas (latitude 25° 44.288′ N; longitude 79° 18.981′ W). The two sponges are identical and have been assigned to the genus *Batzella* (class Demospongia, order Poecilosclerida, family Desmacididae), as described and discussed by Van Soest et al.[27] The sponge has a detachable ectosome and a spicule skeleton of strongyles of one size category. Some of the strongyles have malformed tips. The sponge incorporates sediment into its skeleton. There are numerous papillae scattered over the surface of the sponge. The sponge is dark brown to black when alive, brown when

preserved in EtOH. Taxonomic reference samples have been deposited in the Harbor Branch Oceanographic Museum, catalog numbers 003:00923 (25-VIII-94-1-001) and 003:00924 (26-VIII-94-4-003).

Extraction and Isolation. The sponges (25-VIII-94-1-001, wet wt 101 g) and (26-VIII-94-4-003, wet wt 37 g) were extracted separately in EtOH and concentrated to give 3.1 and 1.2 g of the EtOH extracts, respectively. The two extracts gave an identical TLC pattern and hence were combined for further studies. The combined EtOH extract was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction (0.2 g) was then column chromatographed over Si gel (230−400 mesh) using a CH_2Cl_2−MeOH step gradient, and were monitored for inhibition of the CaN protein phosphatase. The CaN inhibitory fractions were combined and further purification by reversed-phase HPLC (C_{18}-amino, 5 μm, 250 × 10 mm) with 40% H_2O−MeOH gave secobatzelline A (**1**) as a dark brown amorphous solid (yield, 0.005% of wet wt) and secobatzelline B (**2**) as a red amorphous solid (yield, 0.002% of wet wt).

Secobatzelline A (1): mp >300 °C, blackened at 165−170 °C; $[\alpha]^{24}_D$ −135° (*c* 0.01, CH_3OH); UV (MeOH) λ_{max} 322 (log ϵ 4.00), 233 (4.13), 203 (4.19) nm; IR (KBr) ν_{max} 3237, 2917, 1658, 1611, 1585, 1500, 1408, 1360, 1024, 752 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.50 (1H, br s, NH-1), 9.80 (1H, br s, NH-10), 7.16 (1H, s, H-2), 6.55 (2H, br s, NH_2-6), 4.67 (1H, t, J = 6.1 Hz, H-8), 3.49 (2H, d, J = 6.1 Hz, H-9); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ 169.3 (s, C-7), 158.0 (s, C-4), 141.2 (s, C-6), 127.1 (s, C-7a), 127.1 (s, C-3), 125.8 (d, C-2), 124.4 (s, C-3a), 103.9 (s, C-5), 67.8 (d, C-8), 65.4 (t, C-9); HRFABMS (3-nitrobenzyl alcohol) *m/z* 256.055, 258.051, Δ 5 mmu for $C_{10}H_{11}ClN_3O_3$ $[M + H]^+$.

Acetylation of Secobatzelline A. A solution of secobatzelline A (10.0 mg) in pyridine (0.5 mL) and Ac_2O (0.5 mL) was stirred overnight at 50 °C. The solvents were removed in a vacuum, and the resulting gum on purification by HPLC (Si, 5 μm, 250 × 10 mm) with 2% MeOH−CH_2Cl_2 gave diacetates **3** (6.5 mg) and **4** (3.0 mg).

Secobatzelline A diacetate (3): mp 169−170 °C; $[\alpha]^{24}_D$ −24° (*c* 0.01, CH_3OH); UV (MeOH) λ_{max} 320 (log ϵ 4.11), 227 (4.32), 203 (4.36) nm; IR (neat) ν_{max} 3225, 2930, 1726, 1651, 1623, 1229, 1216, 1021, 765 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.65 (1H, br s, NH-1), 10.15 (1H, br s, NH-10), 7.27 (1H, s, H-2), 6.55 (1H, t, J = 6.7 Hz, H-8), 6.41 (2H, br s, NH_2-6), 4.30 (2H, dd, J = 11.1, 6.7 Hz, H-9), 2.06 (3H, s, OAc), 1.98 (3H, s, OAc); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ 169.8 (s, OAc), 169.5 (s, C-7), 169.4 (s, OAc), 157.2 (s, C-4), 140.2 (s, C-6), 127.0 (s, C-7a), 125.6 (d, C-2), 124.0 (s, C-3a), 120.1 (s, C-3), 104.1 (s, C-5), 67.5 (d, C-8), 64.9 (t, C-9), 20.6 (q, OAc), 20.3 (q, OAc); ^{13}C NMR (10% CD_3OD−$CDCl_3$, 125.7 MHz) δ 171.1 (s, OAc), 170.2 (s, OAc), 169.9 (s, C-7), 157.4 (s, C-4), 139.5 (s, C-6), 127.3 (s, C-7a), 125.1 (d, C-2), 124.1 (s, C-3a), 120.8 (s, C-3), 107.3(s, C-5), 68.2 (d, C-8), 64.9 (t, C-9), 20.8 (q, OAc), 20.6 (q, OAc); HRFABMS (3-nitrobenzyl alcohol) *m/z* 340.054, Δ 2 mmu and 342.047, Δ 5 mmu for $C_{14}H_{15}ClN_3O_5$ $[M + H]^+$.

Secobatzelline B (2): mp >300 °C, blackened at 180−182 °C; $[\alpha]^{24}_D$ −18° (*c* 0.01, CH_3OH); UV (MeOH) λ_{max} 335 (log ϵ 4.32), 245 (4.00), 203 (4.32) nm; IR (KBr) ν_{max} 3456, 3347, 2918, 1667, 1623, 1580, 1549, 1509, 1408, 1351, 1290, 1060, 890, 830 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.63 (1H, br s, NH-1), 7.11 (1H, s, H-2), 7.09 (2H, br s, NH_2-6), 5.10 (1H, d, J = 5.5 Hz, OH-8), 4.87 (1H, ddd, J = 6.0, 5.5, 5.5 Hz, H-8), 4.57 (1H, t, J = 6.0 Hz, OH-9), 3.49 (1H, ddd, J = 9.0, 5.5, 6 Hz, H-9), 3.27 (1H, ddd, J = 9.0, 5.5, 6 Hz, H-9); ^1H NMR (360 MHz, 10% DMSO-d_6−CD_3COCD_3) δ 7.18 (1H, s, H-2), 6.63 (1H, br s, NH-1), 4.90 (1H, dd, J = 6.6, 4.7 Hz, H-8), 3.65 (1H, dd, J = 10.8, 4.7 Hz, H-9), 3.50 (1H, dd, J = 10.8, 6.6 Hz, H-9); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 175.8 (s, C-4), 169.5 (s, C-7), 145.9 (s, C-6), 129.0 (s, C-3), 127.5 (s, C-7a), 126.3 (d, C-2), 122.5 (s, C-3a), 104.9 (s, C-5), 67.5 (d, C-8), 66.0 (t, C-9); HRFABMS (3-nitrobenzyl alcohol) *m/z* 257.043, 259.040, Δ 1 mmu for $C_{10}H_{10}ClN_2O_4$ $[M + H]^+$.

Acetylation of Secobatzelline B. A solution of secobatzelline B (5.0 mg) in pyridine (0.3 mL) and Ac_2O (0.3 mL) was stirred overnight at 50 °C. The solvents were removed in vacuo, and the resulting gum on purification on a silica Sep-Pak with 2% MeOH−CH_2Cl_2 gave diacetate **4** (4.5 mg).

Secobatzelline B diacetate (4): mp 170−171 °C; UV (MeOH) λ_{max} 342 (log ϵ 3.73), 303 (4.15), 238 (4.27), 205 (4.29) nm; IR (neat) ν_{max} 3162, 2930, 1733, 1677, 1605, 1402, 1242, 1232, 1042 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.80 (1H, br s, NH-1), 7.28 (1H, s, H-2), 7.13 (2H, br s, NH_2-6), 6.30 (1H, dd, J = 6.3, 6.2 Hz, H-8), 4.27 (2H, m, H-9), 2.04 (3H, s, OAc), 1.98 (3H, s, OAc); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ 175.2 (s, C-4), 170.0 (2C, s, OAc), 169.6 (s, C-7), 145.9 (s, C-6), 128.0 (s, C-7a), 126.5 (d, C-2), 122.4 (s, C-3a), 121.4 (s, C-3), 105.1 (s, C-5), 66.8 (d, C-8), 64.3 (t, C-9), 20.8 (q, OAc), 20.6 (q, OAc).

Hydrolysis of Secobatzelline A. A solution of secobatzelline A (4.0 mg) in 30% aqueous EtOH (1.0 mL) was refluxed overnight on water bath. The solvent was removed in vacuo, and the resulting mixture on purification by reversed-phase HPLC (C_{18}-amino, 5 μm, 250 × 10 mm) with 35% H_2O−MeOH gave unhydrolyzed secobatzelline A (0.7 mg) and secobatzelline B (1.6 mg). The identity of the latter compound was confirmed by HPLC and NMR comparison with the natural product **2**.

Calcineurin Assay. Calcineurin activity was determined in a 96-well microtiter format using bovine calcineurin, bovine calmodulin, and *p*-nitrophenyl phosphate as substrate. Details of the methodology can be found in the preceding publication.[2]

CPP32 Assay. The stock CPP32 enzyme was kindly supplied by BASF, Worcester, MA. The details of the assay was described in the preceding publication.[2]

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