Bohemamines from a Marine-Derived *Streptomyces* sp.

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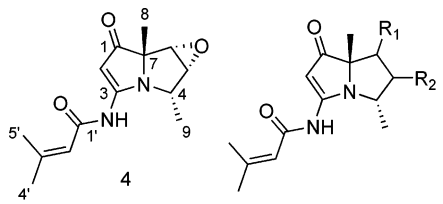
Investigation of the culture extracts of a marine-derived *Streptomyces* sp. led to the isolation of three new bohemamine-type pyrrolizidine alkaloids, bohemamine B (**1**), bohemamine C (**2**), and 5-chlorobohemamine C (**3**). The structures were elucidated using NMR methods, and the relative stereochemistry was determined using double-pulsed-field-gradient spin echo (DPFGSE) NOE studies.

Because the rate of drug discovery from traditional, soil-derived actinomycetes has diminished over the past decade, we made a considerable effort to explore marine-derived actinomycetes as a source of structurally diverse secondary metabolites and potential leads for drug discovery. The obligate marine actinomycete taxon *Salinispora*, for example, is widespread in tropical ocean sediments.[1] Initial studies of *Salinispora tropica* cultures led to the isolation of salinosporamide A,[2] a potent proteasome inhibitor that is currently in phase I clinical trials for the treatment of cancer. As part of our continued studies in this field, our attention was drawn to a chemically rich, marine-derived actinomycte, strain CNQ-583, identified by 16S rRNA gene sequence analysis as a member of the genus *Streptomyces*. In culture, this strain produced three new pyrrolizidine alkaloids, bohemamine B (**1**), bohemamine C (**2**), and 5-chlorobohemamine C (**3**), as well as two previously reported pyrrolizidine alkaloids, bohemamine (**4**) and NP25302 (**5**).[3−5] The structures for **1**−**3** were assigned on the basis of the comparison of their NMR data to those reported for bohemamine (**4**).[5] Previously, NMR data were reported for bohemamine (**4**) in $CDCl_3$.[5] However, we found the best ^1H signal dispersion was obtained in DMSO-d_6. The improved resonance band resolution was useful for selective NOE studies.



4

1 R_1 = H, R_2 = ····OH

2 R_1 = ····OH, R_2 = H

3 R_1 = ····OH, R_2 = ▬Cl

5 R_1 = H, R_2 = H

Pyrrolizidine alkaloids from numerous plant families exhibit a wide array of structural diversity and have important health implications due to their presence in food products.[6] Many pyrrolizidine alkaloids are genotoxic and mutagenic and have been shown to cause acute liver toxicity.[7] Despite the enormous number of pyrrolizidine alkaloids in the literature, the methylation pattern and presence of the amide nitrogen found in the bohemamines make this a rare pyrrolizidine subclass, which has only been isolated from actinomycete bacteria. In fact, there have only been two bohemamines, **4** and **5**, reported to date.[3−5] Additionally, a recent report revised the structures of jenamidines A, B, and C from a piperidone to a pyrrolizidine ring system, similar to that found in the bohemamines, but the jenamidines lack methyl groups on the ring.[8,9]

Bohemamine B (**1**) was isolated as a viscous oil that analyzed for the molecular formula $C_{14}H_{20}N_2O_3$, by HREIMS. The UV spectrum (see Experimental Section) was nearly identical to that reported for bohemamine (λ_{max} 248, 286, 335 nm),[3] but the molecular formula indicated that compound **1** contained two additional hydrogen atoms. Since the ^1H NMR spectrum showed additional methylene signals (see Table 1) and one additional exchangeable proton, it was clear that compound **1** was an alcohol-bearing analogue of bohemamine (**4**). COSY correlations from H-6α and H-6β to the oxygenated methine proton H-5, in addition to the COSY correlation from H-5 to H-4, confirmed the position of both the methylene and hydroxyl groups. The position was also consistent with observed correlations in the HMBC NMR spectrum.

The relative stereochemistry of bohemamine B (**1**) was determined using double-pulsed-field-gradient spin−echo (DPFGSE) 1D NOE experiments.[10,11] Selective excitation of the methyl groups at C-8 and C-9 provided a starting point for assigning the relative stereochemistry. Excitation of CH_3-9 resulted in a strong enhancement of the H-6α and H-4 signals, while excitation of CH_3-8 enhanced H-6β and H-5. The large coupling constant between H-6α and H-5 (10.1 Hz) suggested a *trans*-diaxial relationship between the two protons, which was also consistent with the assigned stereochemistry.

Since bohemamine C (**2**) had the same molecular formula as bohemamine B (**1**), it was most likely the regioisomer of bohemamine B (**1**). The structure of bohemamine C (**2**) was readily assigned by analysis of the 2D NMR data. More specifically, the COSY spectrum showed correlations from the H-4 methine proton to both protons of the adjacent methylene (H-5), which were coupled to an oxygenated methine proton (H-6). The elaboration of the H-4 to H-6 spin system proved that bohemamine C (**2**) was the regioisomer of bohemamine B (**1**). Bohemamine C (**2**) would presumably have identical stereochemistry to bohemamine B (**1**) since the biogenesis of both B (**2**) and C (**1**) most likely occurs by reduction of the epoxide in bohemamine (**4**). Nonetheless, the relative stereochemistry was confirmed using DPFGSE NOE experiments. Selective excitation of H-4 resulted in a stronger enhancement of H-5β (δ 2.57), thereby establishing the relative assignments for H-5β and H-5α. Subsequent irradiation of the H-6 oxygenated methine proton showed a much stronger enhancement (2:1) of proton H-5β than proton H-5α, illustrating that H-4, H-5β, and H-6 are all on the same side of the ring.

The presence of one chlorine atom in 5-chlorobohemamine C (**3**) was obvious from the isotope pattern observed in the mass spectrum. The molecular formula ($C_{14}H_{19}ClN_2O_3$) indicated that compound **3** was the chlorohydrin analogue of bohemamine (**4**). Analysis of the NMR data confirmed that compound **3** was indeed a chlorohydrin, and the COSY NMR spectrum showed a correlation

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Table 1. ^{1}H NMR Data for Compounds **1**−**4** in DMSO-d_6

position	bohemamine B (**1**)	bohemamine C (**2**)	5-chlorobohemamine C (**3**)	bohemamine (**4**)
1				
2	5.56 s	5.40 s	5.55 s	5.34 s
3				
4	3.86 dq, 6.5, 6.4	3.92 dq, 6.7, 8.7	4.07 dq, 6.8, 2.4	3.8 br q 6.7
5	4.44 dddd, 10.1, 5.9, 6.4, 3.5	α 1.75 d, 13.2 β 2.57 ddd, 13.2, 8.7, 3.9	4.21 t, 2.4	3.73 br d, 3.4
6	α 1.55 dd, 12.2, 10.1 β 1.69 dd, 12.2, 5.9	3.84 t, 3.9	3.99 ddd, 5.0, 4.4, 1.7	3.60 d, 3.4
7				
8	1.13 s	1.07 s	1.41 s	1.20 s
9	0.90 d, 6.5	1.25 d, 6.7	1.32 d, 6.8	1.35 d, 6.7
1′				
2′	5.98 dq, 1.0, 0.9	5.98 br s	5.95 dq, 1.0	5.90 dq, 1.3, 1.0
3′				
4′	1.88 d, 0.9	1.88 br s	1.89 d, 1.0	1.87 d, 1.0
5′	2.12 d, 1.0	2.12 br s	2.14 d, 1.0	2.12 d, 1.3
NH	10.24 br s	10.24 br s	10.33 br s	10.10 br s
OH	4.82 d, 3.5	4.92 d, 3.5	5.82 d, 5.0	

from the OH proton to H-6, confirming the assignment of the hydroxyl group. The relative stereochemistry of 5-chlorobohemamine C (**3**) was also based on DPFGSE NOE experiments. Selective excitation of the H-8 methyl group resulted in a strong enhancement of proton H-6 and a weak enhancement of proton H-4, while selective excitation of the H-9 methyl group resulted in a strong enhancement of H-4, H-5, and the NH protons. 5-Chlorobohemamine C (**3**) was isolated in small quantities, but larger quantities were readily obtained by stirring bohemamine (**4**) in a $CHCl_3$ emulsion with 1 N HCl. The reaction proceeded nearly to completion and resulted in only one product, as would be predicted on the basis of C-6 being more sterically hindered due to the ring junction methyl group. The product had identical characteristics to the natural product, including its HPLC retention time, indicating that the same stereoisomer had been formed. The selectivity and yield of the reaction led us to question if 5-chlorobohemamine C (**3**) could be an artifact of isolation. Consequently, we performed a time-course study to monitor the production of 5-chlorobohemamine C (**3**) over a 10-day period. Periodically, small aliquots of the culture were extracted with EtOAc, and the extract was subsequently analyzed by LCMS. Care was taken to avoid both acidic conditions and chlorinated solvents during the time-course investigation. Additionally, the pH (8.5) of the medium was not acidic, which could lead to the chlorohydrin through the presence of chloride in the culture medium. Under all cases, 5-chlorobohemamine (**3**) was produced during the time-course study, indicating that the cholorohydrin is not an artifact of isolation.

An attempt to determine the absolute stereochemistry using *R*- and *S*-MTPA esters was undertaken. Both the *R*- and *S*-MTPA esters of 5-chlorobohemamine (**3**) were synthesized and analyzed by ^{1}H NMR methods. However, the ^{1}H signals at positions 4, 5, 6, and 9 were shifted ($\delta S - \delta R > 0$), while H-2 and H-8 had the same chemical shift in each of the two esters. Attempts to make the MTPA ester of bohemamine B (**1**) resulted in formation of a high molecular weight species (*m*/*z* 697). Although the product was not fully characterized by 2D NMR methods, the mass spectrum indicates that a bis-MTPA ester was formed. We hypothesize that enolization of the ketone could lead to a bis-MTPA ester. This hypothesis was further supported by the observation that proton H-2 exchanges slowly in 1:1 $CDCl_3/CD_3OD$. Full exchange was observed after 16 h, by NMR analysis, and is similar to the rate of exchange observed by Snider et al.[8] After equilibrating bohemamine B (**1**) in $CHCl_3/CH_3OH$, proton H-2 was once again observed in the ^{1}H NMR spectrum.

Compounds **1**−**4** were tested for inhibition of the HCT-116 colon carcinoma cell line and antimicrobial activity, but were found to be essentially inactive. Although NP25301 and bohemamine were shown to be LFA-1/ICAM-1 adhesion inhibitors, we had no access to this bioassay system.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Rudolph Research Autopol III polarimeter. UV spectra were obtained with a Beckman Coulter DU640 spectrophotometer. IR spectra were obtained with a Perkin-Elmer 1600 series FTIR spectrophotometer. All ^{1}H and ^{13}C spectra were obtained at 500 and 125 MHz in the indicated solvent at 25 °C on a Varian INOVA spectrometer. Proton shifts are reported in parts per million relative to the reference solvent signals of DMSO-d_6 at δ 2.49 ppm for ^{1}H and δ 39.5 ppm for ^{13}C. High-resolution mass spectrometric analyses were performed at UC Riverside (compounds **1** and **4**) using a VG 7070 and at The Scripps Research Institute (compounds **2** and **3**) using a Micromass Q-Tof micro.

Biological Material. Strain CNQ-583 was cultured from a marine sediment sample collected using a surface-deployed sediment grab (Kahlsico, El Cajon, CA, model #214WA110) at a depth of 82 m off the island of Guam on January 26, 2002. The sediment was air-dried overnight in a laminar flow hood and plated by dilution stamping onto a medium consisting of 500 mg of soluble seaweed SSE (*Ascophyllum nodosum*, USA of America, Hudson, FL), 100 mg of casamino acids, cyclohexamide (100 μg /mL), nystatin (50 μg /mL), 8 g of Noble (purified) agar, and 1 L of seawater. Once obtained in pure culture, the strain was identified as a member of the genus *Streptomyces* on the basis of 98.3% 16S rRNA gene sequence identity with the *S. luteosporus* type strain (NCBI accession number AB184607). CNQ-583 also shares between 99.6% and 99.7% sequence identity with two marine-derived *Streptomyces* strains (CNR-876 and CNR-926) previously isolated from sediments collected off the Republic of Palau, Micronesia (NCBI accession numbers DQ448784 and DQ448729, respectively). These two strains also produce new secondary metabolites and are the subject of a separate study.

Fermentation, Extraction, and Isolation. A seed culture of isolate CNQ-583 was grown for 2 days in 25 mL of medium TCG (3 g of tryptone, 5 g of casitone, 4 g of glucose, 1 L of seawater) while shaking at 230 rpm and 27 °C. Production fermentation was performed in 2.8 L Fernbach flasks (20 × 1 L) in medium A1BFe + C (10 g of starch, 4 g of yeast extract, 2 g of peptone, 1 g of $CaCO_3$, 40 mg of $Fe_2(SO_4)_3 \cdot 4H_20$, 100 mg of KBr, 1 L of seawater) while shaking at 230 rpm and 27 °C. After 7 days of cultivation, Amberlite XAD-7 resin (20 g/L) was added to adsorb extracellular secondary metabolites. The culture and resin were shaken at 215 rpm for an additional 2 h. The resin and cell mass were then collected by filtration through cheesecloth and washed with DI water to remove salts. The resin, cell mass, and cheesecloth were then extracted with 4 × 1.25 L of acetone, and the solvent was removed under vacuum to yield 58 g of crude extract.

The crude extract was partitioned using 25:34:20 CH_2Cl_2/MeOH/H_2O. The extract was suspended in 500 mL of the upper aqueous layer and extracted with 4 × 500 mL of the lower organic layer to yield 1.64 g of organic partition. The organic partition was separated into

Table 2. ^{13}C NMR Data for Compounds **1**−**4** in DMSO-d_6[a]

position	1	2	3	4
1	204.6	200.8	200.8	199.0
2	93.1	93.3	94.9	91.7
3	167.7	166.1	166.8	168.6
4	57.9	53.8	63.8	55.5
5	72.2	43.1	70.9	63.8
6	35.6	72.4	78.9	56.0
7	69.0	78.8	76.9	72.8
8	26.1	23.5	24.4	18.8
9	9.8	19.8	17.0	13.9
1′	164.0	163.8	163.8	163.8
2′	117.6	117.7	117.5	117.5
3′	156.4	156.0	156.6	156.7
4′	27.3	27.2	27.3	27.3
5′	20.0	19.9	20.0	20.0

[a] Carbon assignments by HSQC and DEPT NMR methods.

two portions using Sephadex LH-20 (3.5 cm × 23 cm) using 1:1 CH_2Cl_2/MeOH. The bohemamines were concentrated in the fourth fraction eluting between 190 and 440 mL. Final purification was performed using preparative C-18 HPLC using isocratic conditions (90% H_2O/10% CH_3CN) for 5 min followed by a linear gradient to 50% CH_3CN over 55 min followed by a gradient to 100% CH_3CN over 5 min. Bohemamine B (**1**) eluted at 27.2 min; bohemamine C (**2**) eluted at 28.2 min; bohemamine (**4**) eluted at 30.6 min; NP25302 (**5**) eluted at 38.2 min; and 5-chlorobohemamine C (**3**) eluted at 39.2 min.

Bohemamine B (1): $[\alpha]^{25}_D$ −6.8 (*c* 1, MeOH); UV (MeOH) λ_{max} (log ϵ) 250 (4.3), 284 (4.0), 332 (3.9) nm; IR (NaCl disk) ν_{max} 3280, 3185, 2970, 2925, 1715, 1640, 1620, 1580 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* $[M]^+$ 264.1474 (calc for $C_{14}H_{20}N_2O_3$, 264.1473).

Bohemamine C (2): $[\alpha]^{25}_D$ −12 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ϵ) 250 (4.2), 281 (3.7), 325 (3.4) nm; IR (NaCl disk) ν_{max} 3280, 3185, 2970, 2925, 1715, 1640, 1620, 1580 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* $[M + H]^+$ 265.1552 (calc for $C_{14}H_{21}N_2O_3$, 265.1543), $[M + Na]^+$ 287.1366 (calc for $C_{14}H_{20}N_2O_3Na$, 287.1372).

5-Chlorobohemamine C (3): $[\alpha]^{25}_D$ −14.5 (*c* 0.4, MeOH); UV (MeOH) UV (MeOH) λ_{max} (log ϵ) 250 (4.1), 281 (4.0), 330 (3.8) nm; IR (NaCl disk) ν_{max} 3285, 3185, 2970, 2925, 1710, 1640, 1625, 1570 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* $[M + H]^+$ 299.1169 (calc for $C_{14}H_{20}{}^{35}ClN_2O_3$, 299.1162), $[M + H]^+$ 301.1135 (calc for $C_{14}H_{20}{}^{37}ClN_2O_3$, 301.1132), $[M + Na]^+$ 321.0982 (calc for $C_{14}H_{19}{}^{35}ClN_2O_3Na$, 321.0982), $[M + Na]^+$ 323.0952 (calc for $C_{14}H_{19}{}^{37}ClN_2O_3Na$, 323.0952).

Bohemamine (4): $[\alpha]^{25}_D$ +16 (*c* 2, MeOH); UV (MeOH) λ_{max} (log ϵ) 248 (4.2), 286 (4.0), 335 (3.7) nm; IR (NaCl disk) ν_{max} 3285, 3185, 2975, 2925, 1715, 1645, 1625, 1570 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* $[M^+]$ 262.1317 (calc for $C_{14}H_{18}N_2O_3$, 262.1317).

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Supporting Information Available: Copies of ^1H spectra for compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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