Mozamides A and B, Cyclic Peptides from a Theonellid Sponge from Mozambique

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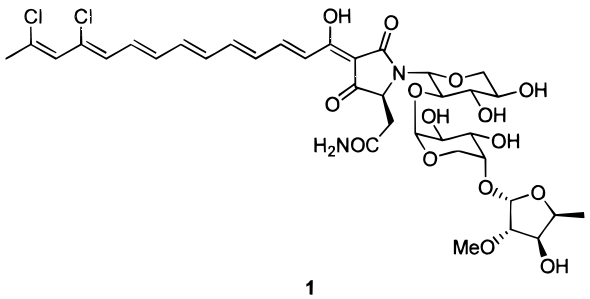
Received April 8, 1997[⊗]

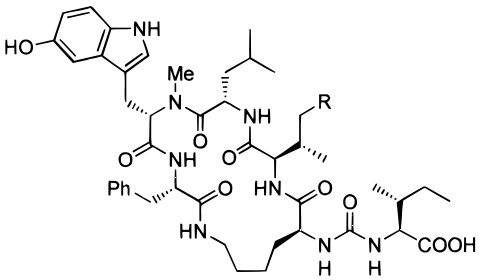
A sponge of the lithistid family Theonellidae from southern Mozambique contained the known compound aurantoside A (**1**) together with two new cyclic peptides, mozamides A (**2**) and B (**3**). The structures and absolute configurations of mozamides A and B, which contain a rare ureido group, were determined by interpretation of spectral data and chiral GC−MS analysis of the amino acids resulting from acid-catalyzed hydrolysis.

Lithistid sponges have yielded a wide variety of bioactive marine natural products that include a diverse array of cyclic peptides, which are notable for their incorporation of structurally unusual amino acids and nonproteinogenic D-amino acids.[1−3] Structural similarities between lithistid sponge metabolites and those of microorganisms led to the suggestion that the metabolites might be produced by symbiotic bacteria, or more specifically, cyanobacteria.[1,4] We have recently demonstrated that a bicyclic peptide[5] from *Theonella swinhoei* was located in and presumably produced by symbiotic filamentous bacteria, while a macrolide, swinholide A, was isolated from a mixed population of unicellular bacteria.[6] The hypothesis that cyclic peptides from lithistid sponges are produced by filamentous bacteria is of considerable interest because of the biotechnological implications. It has been demonstrated that there is a correlation between the presence of filamentous bacteria and the isolation of cyclic peptides,[7] but more research is required to determine the validity of the hypothesis.

Lithistid sponges identified as *Theonella* sp. are often superficially similar to *T. swinhoei* except that the interior tissue is colored orange or yellow, due to the presence of aurantoside A (**1**) and/or related polyenes. From one such *Theonella* sp., Fusetani and co-workers isolated both a mixture of aurantosides A and B[8] and the cyclic peptides, cyclotheonamides A and B[9] and orbiculamide.[10] Other cyclic peptides from *Theonella* spp. include keramamide A[11] and konbamide,[12] both of which incorporate a 5′-hydroxytryptophan residue and a ureido linkage joining two amino acid residues. Similar peptides have also been described from freshwater cyanobacteria, as illustrated by the ferintoic acids from *Microcystis aeruginosa*[13] and the anabaenopeptins from *Anabaena flos-aquae* and *Oscillatoria agardhii*.[14,15] We now report the isolation and identification of two new cyclic peptides, mozamides A (**2**) and B (**3**), from a theonellid sponge that also contained aurantoside A (**1**).



1



2 R = H
3 R = Me

Results and Discussion

The theonellid sponge was collected in southern Mozambique. The lyophilized sponge was sequentially extracted with EtOAc, 1:1 EtOAc−Me_2CO, and 4:1 MeCN−H_2O. Reversed-phase chromatography of the aqueous extract led directly to the isolation of aurantoside A (**1**), which was identified by comparison of its spectral data with literature values.[8] At first, aurantoside A masked the presence of the cyclic peptides, but after a multistep purification procedure, reversed-phase HPLC was successfully employed to separate mozamides A (**2**, 2.6 mg, 7×10^{-4}% yield) and B (**3**, 0.7 mg, 2×10^{-4}% yield).

Mozamide A (**2**) was isolated as a white powder. The HRFABMS spectrum indicated a molecular formula of $C_{45}H_{64}N_8O_9$. The IR spectrum appeared typical of a peptide with a broad band between 1640 and 1740 cm^{-1}. The presence in the ^{1}H-NMR spectrum of several exchangeable protons between δ 6.3 and 8.8 and six signals between 3.8 and 4.65, which may be assigned to the α-protons of amino acids, suggested that mozamide A might be a hexapeptide. A methyl signal at δ 1.85, which is unusually upfield, showed an HMQC correlation to a carbon signal at δ 27.6 and was assigned as an *N*-methyl signal. The ^{1}H-NMR spectrum also contained several other highfield signals at δ −0.46 (t, 1 H, J = 11.5 Hz), 0.22 (d, 3 H, J = 6 Hz), 0.36 (d, 3 H, J = 6.5 Hz), and 0.90 (m, 1 H) that were assigned to a leucine residue. A TOCSY experiment led to the identification of lysine, valine, leucine, and isoleucine

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[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1997.

S0163-3864(97)00195-X CCC: $14.00

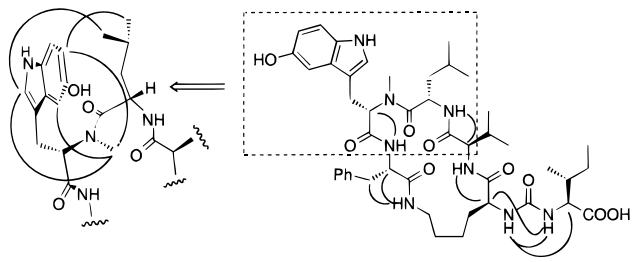

Figure 1. $^1H-^1H$ ROESY (300 MHz) correlations used to establish the structure of **1**.

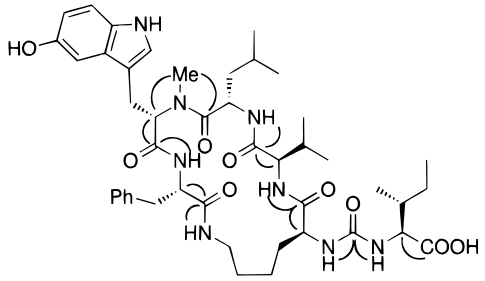

Figure 2. GHMBC (600 MHz) correlations used to establish the structure of **1**.

residues, a GCOSY experiment defined the phenylalanine residue, and the 5′-hydroxytryptophan unit was identified by analysis of the GHMQC and GHMBC experiments and by comparison of the ^1H- and ^{13}C-NMR data with literature values.[16] A ROESY experiment allowed the determination of the amino acid sequence and discrimination between the two aromatic amino acids (Figure 1). However, the ROESY experiment showed some unusual correlations for the isoleucine residue: the NH signal of isoleucine showed a strong correlation with the α-NH signal of lysine in addition to weak correlations between the α-protons of isoleucine and lysine and the NH protons on the neighboring residues. These data, together with the presence of an unassigned signal at δ 157.3 in the ^{13}C-NMR spectrum, suggested that the isoleucine residue was joined to the α-amino group of lysine through a ureido moiety. This assignment was confirmed by long-range carbon−hydrogen NMR correlations and by comparison of the NMR data with the literature values for keramamide A and konbamide.[11,12] All other spectral data, particularly those of the GHMBC experiment (Figure 2), were compatible with the structure proposed for mozamide A (**2**).

The stereochemistry of mozamide A (**2**) was determined by chiral GC−MS experiments. Acid hydrolysis of mozamide A, followed by derivatization of the resulting amino acids, gave the isopropyl ester pentafluoropropionamide derivatives that were analyzed by GC−MS using a Chirasil-Val capillary column, leading to the identification of L-leucine, L-lysine, L-phenylalanine, and D-valine. Ozonolysis of mozamide A using an oxidative workup, followed by hydrolysis and derivatization, gave *N*-methyl-L-aspartic acid,[17] indicating the presence of *N*-methyl-L-5′-hydroxytryptophan in mozamide A (**2**). The final isoleucine residue, which was attached to the urea group, was not observed under acid hydrolysis conditions, but treatment of mozamide A (**2**) with hydrazine[18] produced only one amino acid, which was derivatized and identified as L-*allo*-isoleucine.

Mozamide B (**3**) was isolated as a white powder of molecular formula $C_{46}H_{66}N_8O_9$, which differs from that of mozamide A by the addition of a methylene group. Although insufficient material was available to record a ^{13}C-NMR spectrum, analysis of the ^1H-NMR data, including TOCSY, DQCOSY, and GHMQC experiments, indicated that mozamide B contained an isoleucine residue in place of the valine residue in mozamide A. Chiral GC−MS confirmed this assignment and indicated that D-valine had been replaced by D-isoleucine. Comparison of the spectral data of mozamide B (**3**) with those of mozamide A (**2**) strongly suggested that the only difference between the compounds was the replacement of D-valine by D-isoleucine but the lack of HMBC and ROESY data prevented complete confirmation.

Although mozamides A (**2**) and B (**3**) are similar in structure to keramamide A[11] and konbamide,[12] the latter compounds do not contain any D-amino acid residues. We have not been able to study the cellular localization of metabolites in this sponge but can present two relevant observations at this time. Filamentous cells are evident in the endosome (interior) of this theonellid sponge, while cyanobacteria are present only in the ectosomal layer. The orange color of the endosome appears to be due to the presence of aurantoside A (**1**). We had insufficient material to allow broad screening of mozamides A (**2**) and B (**3**), which at low concentrations showed no antimicrobial activity against Gram-positive and Gram-negative bacteria and a yeast.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol III polarimeter. IR and UV spectra were recorded on Perkin-Elmer 1600 FT-IR and Lambda 3B instruments, respectively. The ^1H, G(radient)COSY, DQCOSY, TOCSY, and ROESY spectra were recorded on a Varian Inova 300 MHz spectrometer, the ^{13}C and DEPT spectra on a Varian Gemini 400 MHz spectrometer, and the GHMQC and GHMBC spectra on a Bruker AMX-600 spectrometer. All NMR data were recorded in DMSO-d_6 solution, and those for mozamide B were recorded using H_2O-suppression sequences. TOCSY spectra were obtained using 80 msec mixing times, and ROESY spin locks were established by continuous pulsing for 300 msec. Absolute configurations were determined using a Hewlett-Packard 5890 GC−MS fitted with an Alltech Chirasil-Val capillary column. HRMS data were obtained from the UC Riverside Regional Mass Spectrometry Facility. All solvents were distilled prior to use.

Animal Material. The lithistid sponge specimen (MOZ95-032) is massive with a reddish brown exterior and a bright yellow-orange interior. The skeleton is composed of nonarticulating tetracrepid-like desmas with strongyles and acanthomicrorhabds. This sponge is comparable to *Placinolopha mirabilis*, described by de Laubenfels in 1954,[19] which is now considered to be a representative of an undescribed genus in the lithistid family Theonellidae.[20] Superficially, this sponge is remarkably similar to a co-occuring species of *Theonella*, but is easily distinguished by its unique desmas and color. A voucher specimen has been deposited in the SIO Benthic Invertebrate Collection, catalog no. P1167.

Collection, Extraction, and Purification. The sponge was collected by hand using scuba at Malangaan reef (26° 46′ S, 32° 54′ E) off southern Mozambique and was maintained frozen until extraction. The sponge

Table 1. ^{1}H-(400 MHz, DMSO-d_6) and ^{13}C-(100 MHz, DMSO-d_6) NMR Data for Mozamides A (**2**) and B (**3**)

	mozamide A (**2**)			mozamide B (**3**)[a]		
			N-Methyl-L-5′-hydroxytryptophan			
1	169.9					
2	60.7	4.64	dd, J = 9, 2.5 Hz	61.6	4.64	dd, J = 8, 2.5 Hz
3	22.3	2.72	dd, J = 15, 9 Hz	23.0	2.78	dd, J = 15, 8 Hz
		3.04	dd, J = 15, 2.5 Hz		3.11	dd, J = 15, 2.5 Hz
NH		10.60			10.56	
2′	124.2	6.81		125.1	6.84	
3′	108.6					
4′	102.2	6.84	d, J = 2 Hz	103.0	6.82	d, J = 2 Hz
5′	150.3					
6′	111.3	6.58	dd, J = 8, 2 Hz	112.1	6.58	dd, J = 8, 2 Hz
7′	111.6	7.10	d, J = 8 Hz	112.5	7.10	d, J = 8 Hz
8′	130.4					
9′	127.9					
OH		8.50			8.50	
NMe	27.6	1.85	s, 3 H	28.2	1.96	s, 3 H
			L-Leucine			
1	172.2					
2	47.3	4.24		48.0	4.24	
3	37.0	−0.46	t, J = 11.5 Hz	37.9	−0.42	t, J = 11.5 Hz
		0.90			0.90	
4	23.1	1.39		24.0	1.30	
5	19.5	0.22	d, J = 6 Hz	20.1	0.25	d, J = 6 Hz
6	22.6	0.36	d, J = 6.5 Hz	23.4	0.38	d, J = 6.5 Hz
NH		8.42	d, J = 4 Hz		8.43	d, J = 4 Hz
		D-Valine			D-isoleucine	
1	172.3					
2	56.7	3.80	t, J = 7.5 Hz	56.8	3.98	t, J = 7.5 Hz
3	29.9	1.86		38.0	1.72	
4	18.8	0.85	d, J = 6.5 Hz	25.9	1.50	
5	18.9	0.94	d, J = 6.5 Hz	12.3	0.88	
6				16.5	0.85	
NH		6.73	d, J = 7 Hz		6.70	d, J = 7.5 Hz
			L-Lysine			
1	172.0					
2	56.7	3.84		55.2	3.86	
3	31.5	1.58		32.2	1.60	
4	20.2	1.10		21.0	1.10	
5	28.3	1.40		29.0	1.42	
6	39.0	2.85		39.1	2.88	
		3.59			3.61	
2-NH		6.44	d, J = 7 Hz		6.48	d, J = 7 Hz
6-NH		7.44	d, J = 7.5 Hz		7.48	d, J = 7.5 Hz
			L-Phenylalanine			
1	170.1					
2	54.4	4.58	ddd, J = 9, 8, 2.5 Hz	55.2	4.60	ddd, J = 9, 8, 2.5 Hz
3	39.0	2.71	dd, J = 15, 8 Hz	40.8	2.84	dd, J = 15, 8 Hz
		3.37	dd, J = 15, 2.5 Hz		3.46	dd, J = 15, 2.5 Hz
4	138.4					
5,9	128.8	7.08		129.7	7.10	
6,8	128.3	7.21		129.0	7.25	
7	126.1	7.17		129.5	7.20	
NH		8.79	d, J = 9 Hz		8.75	d, J = 9 Hz
			Urea			
1	157.3					
			L-*allo*-Isoleucine			
1	173.8					
2	57.7	4.02	dd, J = 9, 5 Hz	57.5	4.06	dd, J = 9, 5 Hz
3	37.7	1.72		37.4	1.75	
4	24.6	1.12		25.8	1.15	
		1.34			1.30	
5	11.5	0.83		12.3	0.84	
6	15.7	0.82		15.5	0.82	
NH		6.30	d, J = 9 Hz		6.34	d, J = 9 Hz

[a] ^{13}C-NMR chemical shifts were derived from the GHMQC experiment (error ± 0.3 ppm).

(364 g) was lyophilized and sequentially extracted with EtOAc (2 × 1 L), 1:1 EtOAc−Me_2CO (2 × 1 L), and 4:1 CH_3CN-H_2O (6 × 1 L). The aqueous MeCN extracts were concentrated under vacuum and chromatographed on Sephadex LH-20 using MeOH as eluent. A small portion of the aqueous MeCN extract was chromatographed on a C_{18} reversed-phase column using a CH_3CN-H_2O gradient as eluent to obtain a sample of aurantoside A (**1**) that had identical spectral data to those reported in the literature.[6] Peptide-containing fractions, which were still heavily contaminated with aurantoside A (**1**), were recombined and chromatographed on a CN-SepPak using a CH_3CN-H_2O gradient as eluent. The peptides were followed first by TLC

using cyano plates and later by ^{1}H-NMR spectroscopy. Material eluted with 30−40% CH_3CN in H_2O was purified by HPLC on a cyano column using 25% CH_3CN-H_2O as eluent followed by HPLC on a C_{18} reversed-phase column using 40% CH_3CN−0.01% aqueous TFA to obtain a mixture of mozamides A and B. Final separation was accomplished using C_{18} reversed-phase HPLC using 35% CH_3CN−0.01% aqueous TFA as eluent to obtain, after a 90-min elution time, mozamide A (**2**, 2.5 mg, 7×10^{-4}% dry wt) and mozamide B (**3**, 0.7 mg, 2×10^{-4}% dry wt).

Mozamide A (2): white powder; $[\alpha]_D$ −66° (*c* 0.024, MeOH); IR (film) 2940, 2920, 1740−1640 (br), 1550, 1260, 1205, 1120, 1080, 1030 cm^{-1}; UV (MeOH) λ_{max} 208 (ϵ 19 600), 268 (ϵ 2600) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; HRFABMS (NBA matrix) *m*/*z* 861.4729 [M + H]$^+$, calcd for $C_{45}H_{65}N_8O_9$, 861.4875.

Mozamide B (3): white powder; $[\alpha]_D$ −33° (*c* 0.018, MeOH); IR (film) 2955, 2925, 1740−1640 (br), 1555, 1260, 1200, 1125, 1075, 1035 cm^{-1}; UV (MeOH) λ_{max} 208 (ϵ 19 600), 268 (ϵ 2600) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; HRFABMS (NBA matrix) *m*/*z* 875.4854 [M + H]$^+$, calcd for $C_{46}H_{67}N_8O_9$, 875.5031.

Hydrolysis and Derivatization of Mozamides A (2) and B (3) for Chiral GC−MS Analysis. Mozamides A and B (150 μg of each) were placed in 1-mL conical vials containing 6 N HCl (0.5 mL), and the sealed vials were heated at 110 °C for 15 h. After evaporation of the solvent under nitrogen, isopropyl alcohol (0.4 mL) and acetyl chloride (0.1 mL) were added, and the sealed vials were again heated at 110 °C for 1 h. After evaporation of the reagents under nitrogen, pentafluoropropionic anhydride (400 μL) in CH_2Cl_2 (0.4 mL) was added, and the sealed vials were again heated at 110 °C for 15 min. The derivatives were again dried under nitrogen and redissolved in EtOAc (100 μL). Aliquots (3 μL) were injected onto a Chirasil-Val capillary column, and the temperature was ramped from 50 to 210 °C over a period of 45 min. Elution times were measured by GC−MS and compared with those of standards that had been derivatized in the same manner. In this manner, L-leucine, L-lysine, L-phenylalanine, and D-valine were identified.

Ozonolysis of Mozamide A (2) and Chiral GC−MS Analysis of Hydrolysis Products. Ozone in oxygen was bubbled through a cooled solution of mozamide A (200 μg) in MeOH (4 mL) at −78 °C for 30 min. The reaction was quenched with 50% H_2O_2 (10 drops) and allowed to warm to room temperature. After 1 h, the solvent was removed under nitrogen, and the ozonolysis product was transferred to a 1-mL conical vial and treated exactly as in the hydrolysis experiment above. Chiral GC−MS analysis revealed the presence of an additional peak that was identified as *N*-methyl-L-aspartic acid. The isopropyl ester of racemic *N*-methylaspartic acid was synthesized as follows. A solution of aspartic acid (10 mg) in isopropyl alcohol (1 mL) containing acetyl chloride (300 μL) was heated to 110 °C for 1 h. The product was dried under nitrogen then redissolved in MeOH (2 mL), to which was added iodomethane (12 mg) and K_2CO_3 (12 mg), and the solution was stirred overnight at 25 °C.

Hydrazinolysis of Mozamide A (2). A solution of mozamide A (100 μg) in hydrazine (0.5 mL) was heated in a sealed conical vial at 110 °C for 18 h. The hydrazine was removed under high vacuum, and the sample was relyophilized from H_2O to remove the last traces of hydrazine. After the resulting mixture had been derivatized as described above, a single peak was identified by chiral GC−MS as L-*allo*-isoleucine.

Acknowledgements. The sponge was collected by Dr. Brad Carté, SmithKline Beecham, and Dr. Philip Coetzee, University of Port Elizabeth, and was provided to us by Dr. Mike Davies-Coleman, Rhodes University. We thank Dr. M. Kelly-Borges for generously sharing her ideas concerning the classification of lithistid sponges. This research was supported by grants from the National Science Foundation (CHE95-27064) and the California Sea Grant College Program (NOAA NA36RG0537, project R/MP 60). The Varian Inova 300 MHz NMR spectrometer was purchased using a grant from the National Science Foundation (CHE95-23507).

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NP970195X