Isolation and Structure Determination of Obyanamide, a Novel Cytotoxic Cyclic Depsipeptide from the Marine Cyanobacterium *Lyngbya confervoides*

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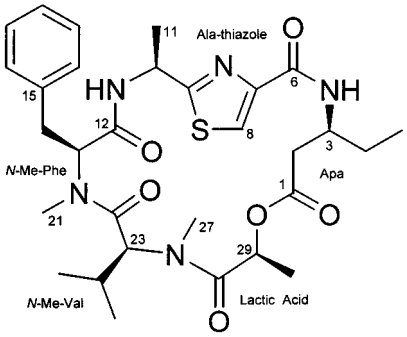
Obyanamide (**1**) was isolated from a variety of the marine cyanobacterium *Lyngbya confervoides* collected in Saipan, Commonwealth of the Northern Mariana Islands. Gross structure elucidation of this novel cyclic depsipeptide relied on extensive application of 2D NMR techniques. The absolute stereochemistry was deduced by chiral chromatography of the hydrolysis products and comparison with authentic and synthetic standards. Obyanamide (**1**) was cytotoxic against KB cells with an IC_{50} of 0.58 μg/mL.

Cyanobacteria are an ancient and diverse group of microorganisms. They are able to inhabit and thrive in an incredible variety of environments, in part because of their ability to produce a rich range of secondary metabolites. These metabolites have already been shown to display a wide spectrum of biological activities ranging from antibacterial[1] to immunosuppressive.[2] This diversity has generated considerable interest in cyanobacteria, since over half of the new drugs approved from 1983 to 1994 and 60% of the drugs currently approved for the treatment of cancer are still of natural origin.[3] One compound already in clinical trials for the treatment of cancer is based on the cryptophycins[4] isolated from terrestrial cyanobacteria. Some of the most common and accessible marine cyanobacteria in the subtropical and tropical oceans belong to the species *Lyngbya*.[5] Our ongoing investigation of cyanobacteria has led to the isolation of obyanamide (**1**) from a collection of *Lyngbya confervoides*.

Results and Discussion

The lipophilic extract of VP680 was cytotoxic and showed slight solid tumor selectivity in the Corbett assay.[6,7] Solvent partitioning of this extract followed by Si gel chromatography and repeated reversed-phase HPLC yielded **1** as a white amorphous powder. Obyanamide (**1**) exhibited moderate cytotoxicity against KB and LoVo cells with IC_{50} values of 0.58 and 3.14 μg/mL, respectively.



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Gross Structure. HRFABMS analysis established the molecular formula for **1** as $C_{30}H_{41}N_5O_6S$, indicating 13 degrees of unsaturation. From ^{1}H and ^{13}C NMR spectrum analysis, it was evident that **1** contained 14 sp^2 carbons in the form of four carbon carbon double bonds and six carbon heteroatom double bonds, which accounted for 10 degrees of the unsaturation. The remaining three degrees of unsaturation must then be in the form of rings. The IR spectrum revealed that **1** contained both amide (1628 cm^{-1}) and ester bonds (1734 cm^{-1}), indicating it was in fact a peptolide. This peptidic nature was further supported by the presence of two amide (δ 9.11, 7.90) and two *N*-methylamide proton signals (δ 3.085, 3.094) in the ^{1}H NMR spectrum. The COSY, HSQC, and HMBC data, summarized in Table 1, suggested the presence of *N*-methylvaline, *N*-methylphenylalanine, and 3-aminopentanoic acid (Apa) fragments. The low-field resonance for C-29 (δ 67.9) and the corresponding proton (δ 5.20) indicated that the acyloxy group was attached to this carbon. A COSY correlation from H-30 and a HMBC cross-peak from C-1 to H-29 along with a HMBC correlation from C-28 to H-30 expanded this particular fragment into a lactic acid moiety, which was attached to the β-amino acid (Apa) unit via an ester linkage. The downfield singlet in the ^{1}H NMR spectrum at δ 7.98 as well as the sp^2 carbon resonances at 123.2, 149.0, 160.4, and 170.1 ppm were characteristic of a 2-alkylthiazole-4-carboxylic acid unit (C-6 to C-9).[8] HMBC correlations from C-9 and C-10 to H-11 and from C-9 to H-8 connected the remaining carbons to form an Ala-thiazole fragment.

As always, HMBC couplings to the *N*-methyl groups were quite informative in sequencing the peptolide. To the *N*-methyl signal at 3.085 ppm (H-21), HMBC cross-peaks from the signals for the α carbon of phenylalanine (C-13) and the carbonyl carbon of valine (C-22) were visible. This clearly indicated that the *N*-Me-Phe nitrogen was attached to the *N*-Me-Val carbonyl. Similarly the *N*-methyl signal at δ 3.094 (H-27) showed HMBC correlations from the α carbon of the valine (C-23) and the carbonyl carbon of the lactic acid fragment (C-28). Finally, a HMBC correlation between the δ 7.90 amide proton of the Ala-thiazole moiety and C-12 of the *N*-Me-Phe residue firmly established the connection of the Ala nitrogen to the *N*-Me-Phe carbonyl. Since one degree of unsaturation remained, **1** had to be cyclic with the amine of the Apa attached to the cysteine-derived carbonyl of the thiazole ring (C-6).

Absolute Stereochemistry. The absolute stereochemistry of **1** was established by analysis of the degradation products. Obyanamide was subjected to ozonolysis followed by acid hydrolysis, and the product mixture was analyzed

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Table 1. NMR Spectral Data for Obyanamide (**1**) in $CDCl_3$

C/H no.	δ_H[a] (J in Hz)	δ_C[b]	$^1H-^1H$ COSY[a]	HMBC[a,c]
		3-Aminopentanoic Acid		
1		170.4		H-2, H-29
2	2.74, dd (−11.9, 5.0)	39.1	H-3	
	2.38, dd (−11.9, 3.0)			
3	4.36, m	47.6	H-2, 3-NH, H-4	H-4, H-5
3-NH	9.11, d (10.4)		H-3	
4	1.63, m	26.2	H-3, H-5	H-2, H-5
5	1.05, t (7.4)	11.3	H-4	H-4
		Alanine-Thiazole		
6		160.4[e]		H-8[e]
7		149.0		H-8
8	7.98, s	123.2		
9		170.1		H-8, H-11, 10-NH[e]
10	5.04, dq (6.8, 5.9)	48.3	H-11	H-11, 10-NH
10-NH	7.90, d (5.9)			
11	1.42, d (6.8)	24.1	H-10	H-10
		N-Me Phenylalanine		
12		168.2		H-14, 10-NH
13	5.42, dd (8.4, 6.7)	60.9	H-14	H-14, H-21
14	3.25, dd (−13.7, 8.4)	37.2	H-13	
	2.87, dd (−13.7, 6.7)			
15		137.0		H-14
16, 20	7.17, d (7.9)	128.9	H-17	H-14
17, 19	7.21, dd (7.9, 7.2)	129.4	H-16, H-18	
18	7.08, t (7.2)	127.1	H-17	H-16
21	3.085, s	29.1		H-13
		N-Me Valine		
22		169.8		H-21,H-23, H-13
23	5.06, d (10.2)	57.9	H-24	H-26, H-25, H-27
24	2.28, m	27.6	H-23, H-25, H-26	H-23
25	0.84, d (6.6)	18.5	H-24	H-26
26	0.48, d (6.6)	18.6	H-24	H-25
27	3.094, s	29.9		H-23
		Lactic Acid		
28		173.2		H-27, H-30
29	5.20, q (6.9)	67.9	H-30	H-30
30	1.23, d (6.9)	16.0	H-29	H-29

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz. [c] Protons showing long-range correlation with indicated carbon. [d] If not indicated otherwise, correlations were observed for $^nJ_{CH} = 7$ Hz. [e] Recorded at 125 MHz.

by HPLC on a chiral column [Chirex phase 3126 (D)]. A comparison with authentic amino acid standards established the configurations of *N*-Me-Phe, *N*-Me-Val, and Ala as L. Pure L and D lactic acid standards were prepared from alanine,[9] and the *S* stereochemistry at C-29 was determined by chiral TLC and chiral HPLC. Due to the limited amount of material available, determination of the final stereocenter at C-3 necessitated the synthesis of (*R*)- and (*S*)-3-aminopentanoic acid (Figure 1). Commercially available (*R*)- and (*S*)-2-aminobutanol served as the chiral starting material for a five-step synthesis. Boc protection and tosylation of the amino alcohol followed by sodium cyanide substitution and acid hydrolysis provided the necessary standards. A comparison of the hydrolyzate mixture and these standards showed no sign of the *R*-Apa under a variety of solvent strengths and flow rates. Conversely a peak coeluting with the *S*-Apa standard was clearly evident.

Obyanamide (**1**) is a novel depsipeptide containing 2 *N*-methyl amino acids, an Ala-thiazole unit, and a β-amino

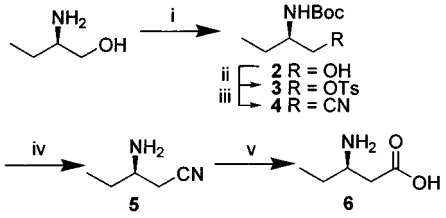

Figure 1. Synthesis of (*R*)-3-aminopentanoic acid.

acid. β-Amino acid fragments are not uncommon in marine metabolites found in cyanobacteria and certain sea hares. Structurally related units include the (2*S*,3*R*)-3-amino-2-methylpentanoic acid found in majusculamide C,[10] dolastatin 11,[11] dolastatin 12,[11] and lyngbyastatin 1.[12] Similarly, dolastatin 16[13] possesses a 3-amino-2,4-dimethylpentanoic acid fragment of unknown stereochemistry, while dolastatin D[14] has a (2*R*,3*R*)-3-amino-2-methylbutanoic acid.

Experimental Section

General Experimental Procedures. The UV spectra was determined on a Hewlett-Packard 8453 spectrophotometer, and the IR spectra were recorded on a Perkin-Elmer 1600 FTIR instrument as a film on a NaCl disk. The optical rotation was measured on a Jasco-DIP-700 polarimeter at the sodium D line (589 nm). FABMS and HRFABMS were recorded in the positive mode on a VG ZAB2SE spectrometer. The NMR spectra of **1** were recorded in $CDCl_3$ on a Varian Unity Inova 400wb at 400 MHz and a GE GN Omega 500 operating at 500 MHz using the residual solvent signal as internal reference. NMR analyses of the synthetic products were carried out at 300 and 75 MHz using a GE QE-300 spectrometer. HPLC separations were performed on a Beckman 110B apparatus coupled to a Applied Biosystems 759A absorbance detector. All synthetic reagents and amino acids were purchased from Aldrich.

Biological Material. The dark reddish-black clumps of cyanobacterium were collected at Obyan Bay in Saipan and designated VP680. A voucher is maintained in formalin at the University of Guam Marine Laboratory. The sample was identified by V. J. Paul and E. Cruz-Rivera.

Extraction and Isolation of Obyanamide (1). VP680 was extracted with 1:1 CH_2Cl_2−MeOH to yield 1.50 g of lipophilic extract. This extract was then partitioned between hexane and 80% MeOH. After drying, the aqueous methanol residue was partitioned between water and *n*-butanol to afford 154 mg of material from the organic layer. Separation on a C_{18} column with increasing amounts of MeCN in H_2O resulted in the activity concentrated primarily in the 60% MeCN in H_2O fraction (9 mg). Further purification on a RP column (50%, 60%, 70% MeCN in H_2O) gave 4.7 mg of material in the 60% MeCN fraction that after semipreparative RP HPLC (Ultracarb, 5 ODS 30, 10 × 250 mm, 65% CH_3CN in H_2O, 3 mL/min, detection at 220 nm) led to 1.6 mg of crude **1** (t_R = 16 min). Final HPLC purification with 70% MeOH (t_R = 13.8 min) yielded 1.1 mg of pure **1**.

Obyanamide (**1**) was obtained as a white powder: $[\alpha]^{27}_D$ +20° (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (5.20), 222 (4.32) nm; IR (film) ν_{max} 3315, 1734, 1628, 1551, 1521, 1458, 1279, 699 cm^{-1}; ^1H NMR, ^{13}C NMR, $^1H-^1H$ COSY, and HMBC data, see Table 1; FABMS *m/z* 622.4 $[M + Na]^+$; HRFABMS *m/z* $[M + H]^+$ 600.2873 (calcd for $C_{30}H_{42}N_5O_6S$, 600.2859).

Synthesis of 3-Aminopentanoic Acid Standards: (*R*)-*N*-Boc-2-aminobutanol (2).[15] To a 5 mL vial containing 100 μL of (*R*)-2-aminobutanol was added 50 mg of NaOH (1 molar equiv) in 1 mL of water and 750 μL of *tert*-butyl alcohol. Next 230 mg of di-*tert*-butyl carbonate was added and the cloudy solution stirred overnight at room temperature. The next day the solution was adjusted to pH 7 with 6 N HCl and partitioned between ethyl acetate and water. The organic phase was dried

over $MgSO_4$ and the solvent removed in vacuo to yield 151 mg (69%) of compound **2**: ^{1}H NMR ($CDCl_3$) δ (carbon position, *integration*, multiplicity; *J* in Hz) 0.80 (4, *3H*, t; 7.87), 1.30 (7/8/9, *9H*, s), 1.42 (3, *2H*, m), 3.38 (2, *1H*, m), 3.42 (1, *2H*, m), 5.07 (OH, *1H*, br. s); ^{13}C NMR δ 64.0 (1), 53.8 (2), 24.3 (3), 10.2 (4), 156.7 (5), 79.6 (6), 28.2 (7/8/9).

(*R*)-*N*-Boc-2-aminobutanol Tosylate (3).[16] A 151 mg sample of **2** was dissolved in 1 mL of freshly distilled pyridine, and the solution was stirred at 0 °C while 293 mg of toluenesulfonyl chloride (2 molar equiv) was slowly added. The reaction mixture was stored at −4 °C for 3 days and then poured over 30 mL of ice in a 60 mL separatory funnel and extracted with 30 mL of diethyl ether. The organic layer was subsequently washed with 6 N HCl followed by saturated aqueous $NaHCO_3$ and saturated aqueous sodium chloride. The organic layer was dried over $MgSO_4$ and evaporated to yield 263 mg (100%) of **3**: ^{1}H NMR ($CDCl_3$) δ (carbon position, *integration*, multiplicity; *J* in Hz) 0.79 (4, *3H*, t; 7.3), 1.32 (7/8/9, *9H*, s), 1.40 (3, *2H*, m), 2.36 (16, *3H*, s), 3.49 (2, *1H*, m) 3.94 (1, *2H*, m), 7.70 (12/14, *2H*, d, 7.1), 7.23 (11/15, *2H*, d; 7.8); ^{13}C NMR δ (carbon position) 71.3 (1), 65.9 (2), 24.4 (3), 10.4 (4), 155.3 (5), 79.6 (6), 28.5 (7/8/9), 145.0 (10), 128.0 (11/15), 130.0 (12/14), 158.3 (13), 21.8 (16).

(*R*)-*N*-Boc-3-aminopentanitrile (4). To 250 mg of **3** in 2 mL of wet DMSO was added 43 mg of NaCN with stirring. The reaction was left at room temperature overnight and then partitioned between diethyl ether and water. The ether layer was evaporated and the residue chromatographed on silica gel using 10:1 hexane−ethyl acetate to afford 66 mg (44%) of **4**: ^{1}H NMR ($CDCl_3$) δ (carbon position, *integration*, multiplicity; *J* in Hz) 0.90 (5, *3H*, t; 7.3), 1.39 (8/9/10, *9H*, s), 1.49 (4, *2H*, m), 2.46 (2, *1H*, dd; −16.1, 5.1), 2.68 (2, *1H*, dd; −16.1, 4.6), 3.62 (3, *1H*, m); ^{13}C NMR δ (carbon position) 117.4 (1), 40.9 (2), 49.0 (3), 23.5 (4), 10.4 (5), 155.3 (6), 79.9 (7), 28.4 (8/9/10).

(*R*)-3-Aminopentanitrile (5).[9] A solution of 66 mg (0.3 mmol) of **4** in 1 mL of concentrated trifluoroacetic acid was stirred for 10 min. Evaporation of the solvent under N_2 and partitioning of the residue between EtOAc and water yielded 30 mg (92%) of **5** from the aqueous phase: ^{1}H NMR (MeOH-d_4) δ (carbon position, *integration*, multiplicity; *J* in Hz) 1.09 (5, *3H*, t; 7.6), 1.85 (4, *2H*, m), 2.98 (2, *2H*, d; 5.6), 3.51 (3, *1H*, m); ^{13}C NMR δ (carbon position) 115.8 (1), 25.4 (2), 49.3 (3), 20.3 (4), 8.5 (5).

(*R*)-3-Aminopentanoic Acid Hydrochloride (6). A solution of 30 mg of **5** in 1 mL of 6 N HCl was refluxed overnight. Purification over DOWEX 50 resin eluting with 1 M HCl resulted in 40 mg (87%) of **6**: IR (Nujol) ν_{max} 3415 (s), 3300 (s), 3100 (br), 1712 (s); H NMR (MeOH) δ (carbon position, *integration*, multiplicity; *J* in Hz) 0.98 (5, *3H*, t; 7.8), 1.72 (4, *2H*, m), 2.69 (2, *1H*, dd; −17.3, 4.7), 2.84 (2, *1H*, dd; −17.3, 8.3), 3.6 (3, *1H*, m), 7.01,7.19, 7.36 (NH_3).

(*S*)-3-Aminopentanoic Acid Hydrochloride. The *S* enantiomer was synthesized in the same manner as described above starting with (*S*)-2-aminobutanol.

L and D-Lactic Acid.[9] A solution of L-Ala (100 mg) in 1 mL of 4 N HCl at 4 °C was treated with excess sodium nitrite (500 mg in 1 mL of water) and left to stir overnight. The mixture was repeatedly extracted with ethyl ether and evaporated to dryness to give L-lactic acid. D-Lactic acid was prepared in the same manner from the enantiomer.

Chiral TLC analysis. The acid hydrolyzate of **1** was subjected to TLC analysis on Chiralplate (Macherey-Nagel) using 1:9 MeOH−CH_2Cl_2 as the developing solvent. With V_2O_5 spray reagent[17] the lactic acid was visualized as an intense blue spot. Authentic L-lactic acid and D-lactic acid showed R_f values of 0.63 and 0.60, respectively. The L-lactic acid in the hydrolyzate had an R_f of 0.63.

Ozonolysis and Acid Hydrolysis of Obyanamide (1). For analysis, 200 μg of **1** was dissolved in 1 mL of CH_2Cl_2 and ozonized for 15 min. The residue was dissolve in 400 μL of 6 N HCl and refluxed at 118 °C for 16 h. After evaporation of the solvent the sample was passed over a C_{18} column (100 mg) with 10% CH_3CN.

Absolute Stereochemistry of the Amino Acid Derived Units. After hydrolysis the mixture was analyzed by Chiral HPLC, comparing the retention times of the components of the hydrolyzate with those of authentic standards [Column Chirex Phase 3126 (D) (4.6 × 250 mm), Phenomenex; solvent 2 mM $CuSO_4$ for Ala and *N*-Me-Val, $CuSO_4$−MeCN (95:5) for lactic acid and 3-aminopentanoic acid and $CuSO_4$−MeCN (85:15) for *N*-Me-Phe, flow rate 1 mL/min, except alanine 0.8 mL/min; detection at 254 nm]. The retention times (t_R, min) of the standards were L-Ala (12.1), D-Ala (16.8), *N*-Me-L-Val (20.5), *N*-Me-D-Val (26.5), L-lactic acid (18.5), D-lactic acid (31.5), *N*-Me-L-Phe (28.3), *N*-Me-D-Phe (29.9), (*S*)-3-aminopentanoic acid (8.2), and (*R*)-3-aminopentanoic acid (13.0). The retention times of the amino acid components of the hydrolyzate were (*S*)-3-aminopentanoic acid (8.0), L-Ala (11.8), L-lactic acid (18.5), *N*-Me-L-Val (20.5), and *N*-Me-L-Phe (28.3). The identities of the peaks were also confirmed by co-injection.

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