Isolation and Structure Determination of Nostocyclopeptides A1 and A2 from the Terrestrial Cyanobacterium *Nostoc* sp. ATCC53789

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The isolation and total structure determination of nostocyclopeptides A1 (**1**) and A2 (**2**) are described. These cyclic heptapeptides, which possess a unique imino linkage in the macrocyclic ring, are characteristic constituents of the cryptophycin-producing cyanobacterium *Nostoc* sp. ATCC53789. 1D TOCSY experiments proved to be very useful in identifying the seven amino acid residues in each compound, and HMBC and NOESY correlations made it possible to sequence the seven units into a total gross structure. The absolute stereochemistry was determined by directly comparing the amino acids in the acid hydrolyzate of each natural product and its peroxide oxidation and borohydride reduction products with authentic standards. Studies were carried out on the biosynthesis and initiated on the biological activity of these cyclic peptides.

The cryptophycin-producing cyanobacteria *Nostoc* sp. GSV224, isolated from a terrestrial sample collected in India,[1] and *Nostoc* sp. ATCC53789, isolated from a lichen collected in Scotland at Arron Island,[2] are significantly different both genetically and biochemically. The 16S rDNAs differ by 2.8% in homology (GenBank Accession AF062637 for GSV224 and AF062638 for ATCC53789). Although each cyanobacterium exhibits the same spectrum of >25 cryptophycins[2−6] by HPLC analysis,[7] the other secondary metabolites found in GSV224 differ from the ones found in ATCC53789. For example, GSV224 produces a class of peptolides, called nostopeptolides, which are totally absent in the ATCC53789 cyanobacterium. Furthermore, the genes that encode for the biosynthesis of the nostopeptolides in GSV224 (GenBank Accession AF204805)[8] are not found in ATCC53789. Instead ATCC53789 produces an unusual class of cyclic peptides (nostocyclopeptides)[9] that are not present in GSV224. In this paper we report the isolation and structure determination of two nostocyclopeptides found in ATCC53789: nostocyclopeptide A1 (**1**) and A2 (**2**).

Results and Discussion

Lyophilized *Nostoc* sp. ATCC53789 was extracted with acetonitrile/dichloromethane (4:1), and the concentrated extract was fractionated by reversed-phase flash chromatography using various mixtures of water in acetonitrile. Nostocyclopeptides A1 and A2 were eluted in the H_2O/CH_3CN (1:1) fraction. Reversed-phase HPLC of this fraction led to isolation of **1** and **2** in yields of 0.028 and 0.026%, respectively.

Gross Structure Determination. Unlike the nostopeptolides from GSV224, which exist as 10:3.7:1 to 2:1:1 mixtures of three conformers, the nostocyclopeptides examined to date from ATCC53789 exist as 10:1:1 mixtures of three conformers. The structure elucidations of the nostocyclopeptides were, therefore, not as complicated as the ones for the nostopeptolides.

Mass spectrometry suggested a molecular weight of 756 Da for nostocyclopeptide A1 (**1**), because the positive-ion FABMS displayed a strong MH^+ ion peak at *m*/*z* 757. Inspection of the broad band decoupled ^{13}C NMR and DEPT spectra established that **1** possessed 9 quaternary, 14 methine, 9 methylene, and 5 methyl carbons, that is, a total of 37 carbons and 47 hydrogens bonded to carbon, amounting to 500 of the 756 mass units in the molecule. Nine more protons were present and attached to heteroatoms. Eight of them were inferred by five 2°-amide signals at δ 8.51, 8.35, 7.84, 7.69, and 7.58; two 1°-amide signals at δ 7.58 and 6.81; and an alcohol OH signal at δ 5.95 in the ^1H NMR spectrum in CD_3OH. None of these ^1H signals correlated with ^{13}C signals in the HSQC spectrum. The signal for the remaining exchangeable proton was not observed; however, a phenolic OH appeared to be present. Diagnostic A_2X_2 1H signal patterns for a *p*-oxygen-substituted phenyl ring could be observed at δ 6.96 and 6.66, along with an oxygen-bearing quaternary sp^2-carbon signal at δ 157.4. Eight nitrogens and nine oxygens had to be present in **1** to account for the remaining 256 mass units. The molecular formula was, therefore, $C_{37}H_{36}N_8O_9$, and this was supported by HRFABMS.

Detailed analysis of the ^1H and ^{13}C spectral data (Table 1) for **1**, with the aid of 2D COSY, HSQC, HMBC, and NOESY and 1D TOCSY spectra,[10] suggested that glycine, glutamine, isoleucine, serine, *cis*-4-methylproline (4-MePro), and modified tyrosine and leucine units were present in the molecule. In each of the seven 1D TOCSY spectra that were obtained by irradiation of the five 2°-amide NH signals and the two H-2 methine signals at δ 4.46 and 3.68, all of the signals for the coupled protons in each amino acid unit could be observed. In the 1D TOCSY spectrum for the tyrosine unit, however, only the signals for the alanyl moiety were detected; nevertheless, HMBC couplings allowed connection of the alanyl and *p*-hydroxyphenyl moieties. Although HMBC couplings could not be observed for the 1°-amide proton signals, a NOESY correlation was visible between the *E* amide proton (δ 7.58) and the methylene protons on C-4 of the Gln unit, clearly showing that C-5 of the Gln unit was the site of the 1°-amide group. These seven amino acid units accounted for all of the nitrogens and oxygens.

A detailed interpretation of the $^1H-^{13}C$ HMBC spectrum allowed us to sequence the seven amino acid units. Seven carbonyl signals could be seen in the δ 172−178 region of the ^{13}C NMR spectrum. The signal at lowest field (δ 177.5) had to be assigned to the C-5 carbonyl of the Gln unit,

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Table 1. NMR Data for Nostocyclopeptide A1 (**1**) in CD_3OH at 25 °C (500 MHz)

C/H no.		δ_H (*J* in Hz)	δ_C	δ_N	HMBC	NOESY
Tyr	1		176.0		H-2, H$_2$-2 Gly, NH Gly	
	2	3.68, dd (11.3, 2.2)	75.4		H-3, H-3′, H-1 Leu	H-1 Leu
	3	2.65, dd (13.4, 11.3)	40.9		H-5/9	
	3′	3.21, dd (13.4, 2.2)				
	4		129.3		H-3, H-3′, H-6/8	
	5/9	6.96, d (8.4)	132.3		H-3, H-3′, H-5/9	H-2, H-3, H-3′
	6/8	6.66, d (8.4)	116.2		H-6/8	
	7		157.4		H-5/9, H-6/8	
	OH	not observed				
	N			−70.2	H-1 Leu, H$_2$-3	
Gly	1		173.11		H$_2$-2, NH Gln	
	2	3.58, dd (17.0, 2.8)	41.7			NH
	2	4.59, dd (17.0, 10.0)				NH
	NH	8.51, dd (10.0, 2.8)		−270.8	H$_2$-2	H$_2$-2
Gln	1		175.0		H-2, H-3,H-2 Ile, NH Ile	
	2	4.36, q (7.1)	55.2		H$_2$-3, H$_2$-4	NH Ile
	3	1.99, m	26.7		H-2, H$_2$-4	NH
	4	2.28. t (7.6)	32.4		H$_2$-3	*E* CONH$_2$
	5		177.5		H$_2$-3, H$_2$-4	
	NH	8.54, d (7.1)		−260.5	H$_2$-3	H$_2$-3 Gln
	NH$_2$	6.81, br s		−273.8		
7.58, br s	H$_2$-4					
Ile	1		173.23		H-2, NH Ser, H-2 Ser	
	2	4.30, dd (8.3, 4.2)	60.1		3-Me, NH	
	3	2.10, m	37.3		H-2, H$_2$-4, 3-Me, H$_3$-5	
	3-Me	0.98, d (7.1)	16.4		H-2, H$_2$-4	
	4	1.32, m	25.5		3-Me, H$_3$-5, H-2	
	4	1.42, m				
	5	0.92, d (7.3)	12.4		H$_2$-4	
	NH	8.35, d (8.3)		−260.8	H-2	NH Ser, H-2 Gln
Ser	1		173.01		H-2, H-3, H-2 4-MePro	
	2	5.06, ddd (10.4, 9.0, 5.5)	53.3			H-5*proR* 4-MePro
	3	3.84, dt (10.4, 5.5)	64.4			OH
	3	4.17, td (10.4, 7.4)				
	OH	5.95, dd (7.4, 5.5)				H-3, NH Leu
	NH	7.69, d (9.0)		−266.9		NH Ile
4-MePro	1		174.0		H-2, H-3*proR*, H-3*proS*, NH Leu	
	2	4.46, t (8.4)	62.3		H-5 *ProR*, H-5 *ProS*	H-3*proS*
	3*proR*	1.44, ddd (12.7, 9.7, 8.4)	39.6		H-2, 4-Me, H-5 *ProR*	4-Me
	3*proS*	2.53, ddd (12.7, 8.4, 7.0)				H-2, H-4
	4	2.32, m	34.7		H-2, 4-Me, H-3*proR*, H-3*proS*, H-5*proR*, H-5*proS*	H-3*proS*, H-5*proR*
	4-Me	1.08, d (6.6)	17.6		H-5 *ProR*, H-5 *ProS*	H-3*proR*, H-5*proS*
	5*proS*	3.27, t (10.0)	56.9		H-3*proS*, 4-Me	4-Me
	5*proR*	4.25, dd (10.0, 7.2)				H-4, H-2 Ser
	N			−266.3	H-2	
Leu	1	6.87, d (2.0)	166.9		H-2, H$_2$-3, H-2 Tyr	H-2, H-2 Tyr
	2	4.43, m	51.5		H-1, H$_2$-3	H-1
	3	0.95, ddd (13.8, 8.3, 4.9)	42.0		4-Me, H$_3$-5	
	3	1.13, ddd (13.8, 9.9, 5.9)				
	4	1.32, m	26.3		4-Me, H$_3$-5	
	4-Me	0.78, d (6.9)	22.2		H$_3$-5	
	5	0.85, d (6.6)	23.2		4-Me	
	NH	7.84, d (9.0)		−267.1	H-1, H$_2$-3	OH Ser

because all of the protons on C-3 and C-4 showed couplings to C-5. The signal at δ 176.0 had to be attributed to the carbonyl (C-1) of the Tyr unit, because couplings could be seen from H-2 to not only C-1 of Tyr, but also C-1, a methine carbon, of the modified Leu unit (δ 166.9). The latter coupling implied that the C-1 methine of the modified Leu unit was connected to the nitrogen of the Tyr unit by an imino double bond. Both protons on C-2 of the Gly unit showed couplings to C-1 of Tyr, and, in addition to the carbonyl carbon signal at δ 173.11, the latter was ascribed to C-1 of the Gly unit. The NH of Gly therefore had to be connected to C-1 of Tyr, and this amide linkage was confirmed by HMBC. The 2°-amide NH of the Gln unit was attached to C-l of Gly as shown by a two-bond coupling in the HMBC. The signal at δ 175.0 had to be assigned to the C-1 carbonyl of the Gln unit, because H-2 of Gln and H-2 of Ile both showed couplings to this carbonyl carbon. C-1 of Gln was therefore connected to the NH of Ile, and this was confirmed by the HMBC spectrum. The signal at δ 173.23 was assigned to C-1 of Ile inasmuch as coupling was seen between this carbonyl carbon and H-2 of Ile. Coupling between the ^{1}H signal at δ 7.69 and the ^{13}C signal at δ 173.23 established the amide linkage of the Ser NH to the Ile carbonyl. The carbonyl signal at δ 173.01 was attributed to C-1 of Ser, as coupling was seen from this ^{13}C signal to the H-2 signal at δ 5.06 and the H-3 signal at δ 3.84. C-1 of Ser was attached to the nitrogen of the remaining amino acid unit because H-2 of 4-MePro (δ 4.46) showed coupling to this carbon. The carbonyl signal at δ 174.0 was assigned to C-1 of the 4-MePro unit, as the δ 4.46 signal also showed coupling to this carbon. Finally, connection of C-1 of 4-MePro to the NH of the modified Leu unit (δ 7.84) was established by HMBC. In summary, analysis of the HMBC data had generated the sequence of the seven amino acid units and the entire gross structure.

The ^{13}C NMR chemical shifts for the carbonyls of the amino acids Ser (δ 173.01), Gly (δ 173.11), and Ile (δ 173.23) were virtually identical, thus correlations to these

carbons in the HMBC spectrum were at first ambiguous due to insufficient digital resolution (22766 Hz/2048 points = 11.1 Hz/pt) in the ^{13}C dimension. Gaillet et al., however, showed that a gradient-enhanced band-selective HMBC experiment can be used to increase the digital resolution in the ^{13}C dimension by selectively exciting a small region (16 ppm) of the ^{13}C spectrum (typically 200 ppm) using shaped pulses.[11] Selective excitation prevents spectral folding, and the resulting spectrum yields highly resolved cross-peaks that are easily assigned. The experiment was applied to nostocyclopeptide A1 where the carbonyl region (ca. 16 ppm) was selectively excited with a 2.21-ms IBURP2 pulse giving a final digital resolution of 2100 Hz/2048 points (1.0 Hz/pt). The order-of-magnitude increase in digital resolution that was obtained allowed unambiguous assignment of the carbonyl carbon signals and thereby the amide linkages in **1** (see Supporting Information).

The amino-acid sequence was confirmed by ^{1}H−^{1}H NOESY data. Most importantly a strong NOESY correlation between H-2 of the Ser unit and the *pro-R* proton on C-5 of the 4-MePro unit clearly showed that C-1 of Ser was attached to the N of 4-MePro. NOESY correlations between the NH protons of Ser and Ile and between the NH of Ile and H-2 of Gln were consistent with the Gln−Ile−Ser sequence. A NOESY correlation between H-1 of the modified Leu unit and H-2 of the modified Tyr unit not only confirmed the Leu−Tyr connection, but also indicated that the geometry of the imino double bond was *E*.

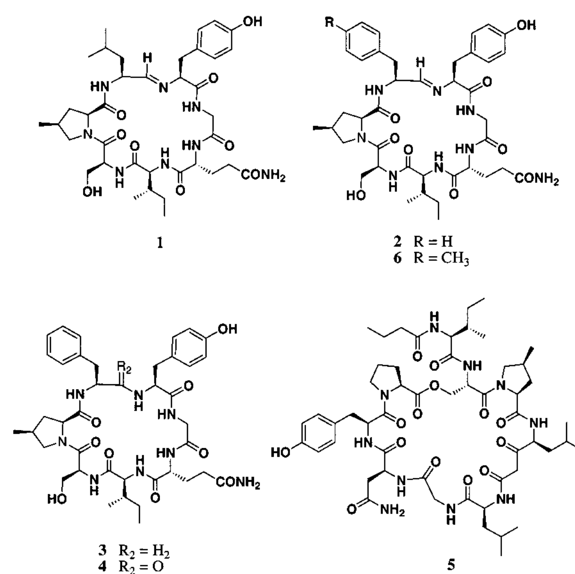
Further proof of the presence of an imine linkage and seven amide nitrogens was further substantiated by ^{1}H−^{15}N 2D NMR spectroscopy. Amide nitrogens are typically observed near δ −270 in the ^{15}N NMR spectrum, whereas imines are found at approximately δ −60 (N chemical shifts referenced externally to CH_3NO_2 in CD_3OH at δ 0.0).[12,13] Analysis of a gradient-enhanced HSQC experiment optimized for $^1J_{NH}$ = 90 Hz yielded seven NH correlations ranging from δ −260.5 to δ −273.8 (see Table 1). A gradient-enhanced HMBC experiment optimized for $^{2,3}J_{NH}$ = 7 Hz showed a single correlation from the proton at δ 6.87 (H-1 Leu) to the nitrogen at δ −70.2 (N Tyr), consistent with an imine. Optimization of the long-range coupling to $^{2,3}J_{NH}$ = 4 Hz yielded additional correlations, most noteworthy were H-3′ Tyr/N Tyr and H-2 MePro/N MePro (see Supporting Information).

A similar analysis of the spectral data for **2** ($C_{40}H_{34}N_8O_9$, 790 daltons) led to its gross structure determination. Detailed analysis of the ^{1}H and ^{13}C spectral data (Table 2) for **2**, again with the aid of 2D COSY, HSQC, HMBC, and NOESY and 1D TOCSY spectra, showed that **2** differed from **1** only in having a modified phenylalanine unit in lieu of the modified leucine unit. Again, the NMR data indicated clearly that an imine linkage connected C-1 of the modified phenylalanine to the nitrogen of the tyrosine unit.

Absolute Stereochemistry. Verification of the seven amino acid units in **1** and **2** and determination of their absolute configurations was carried out by chemical degradation. Acid hydrolysis of **1** or **2** followed by chiral HPLC analysis of the resulting hydrolyzate showed the presence of six amino acids: glycine, L-tyrosine, L-serine, L-isoleucine, D-glutamine, and (2*S*,4*S*)-4-methylproline[14] (Table 3). After sodium borohydride reduction of the imino group in either **1** or **2**, tyrosine was not detected in the acid hydrolyzate of either reduction product (eg., **3** from reduction of **2**). Peracid (*m*-chloroperbenzoic acid [*m*-CPBA] oxidation of either **1** or **2**, however, led to an oxidation product (e.g., **4** from oxidation of **2**) that now showed the presence of another amino acid in the acid hydrolyzate, L-leucine in the case of oxidized **1** and L-phenylalanine in the case of oxidized **2**.

Biosynthesis. Although the incorporation of labeled leucine into **1** was not studied, a feeding experiment with [1−^{13}C,$^{18}O_2$]acetate was carried out. The ^{13}C NMR spectrum for the enriched **1** showed strong enhancement of the carbonyl signal for the 4-MePro unit, consistent with the proposal that L-Leu is the originator of this unit in **1**. A strong enhancement of the imine carbon signal at δ 166.9 was also observed, strongly suggesting that this carbon arises from the carbonyl carbon of Leu. The carbonyl signal for the Ile unit and the C-5 carbon of the Gln unit also showed strong ^{13}C-enrichment as expected. Similar ^{13}C-enrichments were also seen in the MePro, Ile, and Gln units of **2**.

In the course of studies on the preparation of new analogues of cryptophycin by aberrant biosynthesis, it was found that L-4-methylphenylalanine was readily incorporated (resulting in **6**), in fact better than the normal L-Phe substrate, into **2** (see Experimental Section). ATCC53789 that had been fed this abnormal substrate was subjected to the same extraction and isolation procedure. The HPLC profile of the nostocyclopeptide fraction showed a new peak at a slightly longer retention time than the ones for **1** and **2**. The compound in the new peak was collected to give the analogue of **2**, which was named nostocyclopeptide A3 (**6**). The ^{1}H NMR spectrum for **6** was very similar to that of **2**. The high-field region of the spectrum matched exactly with that of nostocyclopeptide A2, except for the presence of a new methyl signal at δ 2.26 (s). In the low-field end of the spectrum the resonances for the monosubstituted phenyl group observed for **2** were missing. Instead, a pair of coupled doublets, compatible with those expected for a *p*-disubstituted phenyl group, were observed at δ 6.94 (d; 7.6) and 7.06 (d; 7.6). The NMR data indicated a structure for **6** that differed from that of **2** only in the substitution of the phenyl group by a *p*-tolyl group.



Biological Activity. The nostocyclopeptides showed weak cytotoxicity (IC_{50} ca. 1 μM) against KB (a human nasopharyngeal carcinoma) and LoVo (a human colorectal adenocarcinoma) cell lines and were devoid of antifungal activity (eg., against *Candida albicans* at 25 μg/disk) and antibacterial activity (eg., against *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* strains 25177 and 35818

Table 2. NMR Data for Nostocyclopeptide A2 (**2**) in CD_3OH at 25 °C (500 MHz)

C/H no.		δ_H (J in Hz)	δ_C	HMBC	NOESY
Tyr	1		176.0	H-2, H_2-2 Gly, NH Gly	
	2	3.71, dd (11.2, 2.5)	74.9	H-3, H-3′, H-1 Phe	H-1 Leu
	3	2.69, dd (13.5, 11.2)	41.1	H-5/9	
	3′	3.24, dd (13.5, 2.5)			
	4		129.3	H-3, H-3′, H-6/8	
	5/9	7.04, d (8.5)	132.5	H-3, H-3′, H-5/9	H-2, H-3, H-3′
	6/8	6.73, d (8.5)	116.4	H-6/8	
	7		157.4	H-5/9, H-6/8	
	OH	not observed			
Gly	1		173.1	H_2-2, NH Gln	
	2	3.59, dd (17.0, 2.8)	41.6		NH
	2	4.58, dd (17.0, 10.0)			NH
	NH	8.47, dd (10.0, 2.8))			H_2-2
Gln	1		175.1	H-2, H_2-3, H-2 Ile, NH Ile	
	2	4.35, td (7.4, 6.1)	55.2	H_2-3, H_2-4	NH Ile
	3	1.99, m	26.7	H-2, H_2-4	NH
	4	2.28, t (7.6)	32.4	H_2-3	*E* $CONH_2$
	NH	8.56, d (6.1)			H_2-3 Gln
	$CONH_2$ *Z*	6.80, br s	177.5	H_2-4, H_2-3	
	E	7.58, br s			H_2-4
Ile	1		173.2	H-2, NH Ser	
	2	4.31, dd (8.5, 4.1)	60.0	3-Me, NH	
	3	2.11, m	37.3	3-Me, H_3-5, H-2	
	3-Me	0.97, d (6.8)	16.4	H-2	
	4	1.32, m	25.4	3-Me, H_3-5, H-2	
	4	1.42, m			
	5	0.92, d (7.3)	12.4		
	NH	8.36, d (8.5)			NH Ser, H-2 Gln
Ser	1		172.9	H-2, H-3	
	2	5.09, ddd (10.4, 9.1, 5.6)	53.3		H-5*proR* 4-MePro
	3	3.88, m	64.5		OH
	3′	4.22, m			
	OH	6.17, dd (7.3, 5.6)			H-3
	NH	7.72, d (9.0)			NH Ile
4-MePro	1		173.9	H-2, NH Phe	
	2	4.28, t (8.5)	62.2		H-3*proS*
	3*proR*	0.66, m	39.2	H-2, 4-Me, H-5*proR*	4-Me
	3*proS*	2.22, m			H-2, H-4
	4	2.15, m	34.7	4-Me, H-5*proS*	H-3*proS*, H-5*proR*
	4-Me	0.90, d (6.3)	16.9	H-5*proS*	H-3*proR*, H-5*proS*
	5*proS*	3.10, t (10.1)	56.9	H-3*proS*, 4-Me	4-Me
	5*proR*	4.17, dd (10.1, 8.8)			H-4, H-2 Ser
Phe	1	6.94, d (1.7)	165.6	H-2 Tyr	H-2, H-2 Tyr
	2	4.61, m	54.8	H-1, H_2-3	H-1, H_2-3, H-5/9
	3	2.29, dd (13.8, 8.8)	38.6	H-5/9	H-2
	3	2.61, dd (13.8, 4.5)			H-2
	4		139.1	H_2-3, H-6/8	H-2, H_2-3
	5/9	7.05, br d (7.2)	130.2	()H_2-3. H-5/9, H-7	
	6/8	7.23, t (7.2)	129.5	H-6/8	
	7	7.15, br t (7.2)	127.7	H-5/9	
	NH	7.90, d (9.5)	-		

at 25 μg/disk). None of the compounds displayed significant inhibition of protease activity against trypsin, thrombin, and plasmin (IC$_{50}$ $<$50 μM) or against chymotrypsin, elastase, and papain (IC$_{50}$ $<$25 μM).

Experimental Section

General Procedures. NMR spectra were recorded in $CDCl_3$, CD_3OH, or CD_3OD at 25 °C on spectrometers operating at 300, 400, and 500 MHz for ^{1}H and 75, 100, and 125 MHz for ^{13}C, using solvent signals as internal references. HSQC experiments were optimized for $^1J_{CH}$ = 140 Hz and HMBC experiments for $^nJ_{CH}$ = 7 Hz.

Isolation. Freeze-dried *Nostoc* sp. ATCC53789 (23.7 g) was extracted with CH_3CN/CH_2Cl_2 (4:1) (1 L) for 48 h. The extract was concentrated in vacuo to give a dark green solid (0.97 g), which was applied to an ODS-coated silica column and subjected to flash chromatography with CH_3CN/H_2O (25:75) (400 mL), CH_3CN/H_2O (50:50) (400 mL), CH_3CN/H_2O (65:35) (800 mL), CH_3OH (500 mL), and CH_2Cl_2 (500 mL). The fraction that eluted from the flash column with CH_3CN/H_2O (1:1) was evaporated, and the residue (38 mg) was subjected to reversed-phase HPLC (ODS YMC column, 250 mm × 10 mm, 5 μ, 3 mL/min) using two gradient elution systems, 90% 0.02N CH_3COONH_4/CH_3CN to 50% 0.02N CH_3COONH_4/CH_3CN for the first 25 min followed by 50% 0.02N CH_3COONH_4/CH_3CN to 0% 0.02N CH_3COONH_4/CH_3CN for the next 10 min. The fractions that eluted at 24.5 min and 25.3 were collected separately and evaporated to yield nostocyclopeptide A2 (**2**, 6.7 mg) and nostocyclopeptide A1 (**1**, 6.2 mg), respectively. The preceding fractions, which contained at least two other nostocyclopeptides, were not investigated.

Nostocyclopeptide A1 (1): $[\alpha]^{25}_D$ −19° (*c* 0.75, CH_3OH); UV λ_{max} (ϵ) 204 (22084), 225 (9480); IR (neat) ν_{max} 3306, 1648, 1542, 1455, 1237 cm^{-1}; ^{1}H NMR and ^{13}C NMR data shown in Table 1; FABMS *m/z* (rel int) 757 (MH$^+$, 5), 392 (5), 329 (7), 307 (33), 289 (16), 176 (15), 154 (100); HRFABMS *m/z* 757.4255 (calcd for $C_{37}H_{57}N_8O_9$, −0.6 mmu error).

Nostocyclopeptide A2 (2): $[\alpha]^{25}_D$ −57° (*c* 1.9, CH_3OH); UV λ_{max} (ϵ) 201 (17769), 223 (5443); IR (neat) ν_{max} 3306, 1647, 1542, 1455, 1237 cm^{-1}; ^{1}H NMR and ^{13}C NMR data shown in Table 2; FABMS *m/z* (rel int) 791 (MH$^+$, 10), 392 (4), 329 (3), 307 (22), 289 (12), 176 (100); HRFABMS *m/z* 791.4082 (MH$^+$, calcd for $C_{40}H_{55}N_8O_9$ −0.3 mmu error).

Table 3. Chiral HPLC Analysis of Acid Hydrolyzates of Nostocyclopeptides A1, A2, and A3

	t_R of standard (min)		amino acids found by co-elution		
amino acid	eluent A[a]	eluent B[b]	A1	A2	A3
glycine	7.5		√	√	√
L-serine	7.8		√	√	√
D-serine	8.3				
(2*S*,4*S*)-4-MePro	21.0		√	√	√
(2*S*,4*R*)-4-MePro	18.5				
(2*R*,4*R*)-4-MePro	42.0				
(2*R*,4*S*)-4-MePro	36.0				
L-glutamic acid	56.5				
D-glutamic acid	64.0		√	√	√
L-tyrosine	52.0	20.9	√	√	√
D-tyrosine	62.3	21.5			
L-leucine	44.3	21.4	√[c]		
D-leucine	57.0	23.4			
L-isoleucine	37.2		√	√	√
L-alloisoleucine	31.6				
D-isoleucine	47.9				
D-alloisoleucine	39.6				
L-phenylalanine	153.3	47.5		√[c]	
D-phenylalanine	166.3	55.2			

[a] Mobile phase: 1.9 mM $CuSO_4$ in 5:95 $MeCN/H_2O$, flow rate 1.0 mL/min. [b] Mobile phase: 1.7 mM $CuSO_4$ in 14:86 $MeCN/H_2O$, flow rate 0.8 mL/min. [c] Only seen when **3** is oxidized with *m*-CPBA in $MeOH/CH_2Cl_2$ prior to acid hydrolysis.

Acid Hydrolysis of Nostocyclopeptides. Each nostocyclopeptide (0.5−1 mg) in 0.5−1 mL of 6 N HCl was heated in a sealed tube at 109 °C for 12 h. The solution was evaporated under a nitrogen stream, and the dried residue was passed through a small C_{18} column with 10% MeOH in H_2O. The solvent of the eluate was removed in vacuo, and the residual acid hydrolyzate was dissolved in 0.2−0.5 mL of H_2O. Aliquots of this solution were analyzed by HPLC on a Phenomenex Chirex d-pencillamine column using one of the following mobile-phase solvent systems: (a) 1.9 mM $CuSO_4$ in CH_3CN/H_2O (5:95), flow rate 1.0 mL/min (eluent A) or (b) 1.7 mM $CuSO_4$ in CH_3CN/H_2O (14:86), flow rate 0.8 mL/min (eluent B); UV detection at 245 nM. The nature of the amino acid residues in the nostocyclopeptides and the retention times for the various standards are summarized in Table 3. Glycine, L-serine, (2*S*,4*S*)-4-methylproline,[14] D-glutamic acid, L-tyrosine, and L-isoleucine were identified in the acid hydrolyzates of **1** and **2**.

Peracid Oxidation of Nostocyclopeptides. Each nostocyclopeptide (0.5 mg) was oxidized with *m*-CPBA (0.25 mg) in 1 mL of $EtOH/CH_2Cl_2$ (1:1) for 4 h. Excess Me_2S was added, and after 0.5 h the solvent was evaporated and the oxidized nostocyclopeptide subjected to acid hydrolysis and HPLC analysis (see Table 3). In addition to the six amino acids found by acid hydrolysis of unoxidized **1** and **2**, L-leucine and L-phenylalanine were identified in the acid hydrolyzates of oxidized **1** and **2**, respectively.

Borohydride Reduction of Nostocyclopeptides. To a solution of **2** in 0.5 mL of MeOH was added $NaBH_4$ (0.3 mg). After 3 h of stirring at room temperature, 4 drops of a saturated sodium sulfate solution were added followed by a few drops of water. The solution was concentrated and the concentrate passed through a small reversed-phase ODS column. The column was eluted with H_2O (2 mL) and H_2O/CH_3OH (1:1) (3 mL). The latter fraction was evaporated, and the residue was purified by reversed-phase HPLC [YMC pack ODS AQ, 250 × 10 mm, 5 μ, 3 mL/min, 0.02 N TFA in H_2O/CH_3CN (65:35)] to obtain the reduction product **3** (1.1 mg): ^1H NMR ($CDCl_3$) δ *Tyr* 3.13 (H-2, br d; J = 12.4 Hz), 2.61−2.68 (3-H_2, m), 7.13 (H5/9, d; J = 8.2 Hz), 6.79 (H-6/8, d; J = 8.2 Hz); *Gly* 3.64 (H-2, d; J = 16.5 Hz), 4.47 (H-2, dd; J = 16.5, 9.1 Hz), 8.34 (NH, dd; J = 9.4, 1.7 Hz); *Gln* 4.34 (H-2, m), 1.92−2.02 (3-H_2, m), 2.22−2.29 (4-H_2, m), 8.54 (NH, d; J = 6.3 Hz); *Ile* 4.23 (H-2, dd; J = 8.2, 4.1 Hz), 2.03−2.09 (H-3, m), 0.98 (3-CH_3, d; J = 6.9 Hz), 1.28−1.35 (H-4, m), 1.42−1.47 (H-4, m), 0.94 (H-5, t; J = 7.4 Hz), 8.28 (NH, d; J = 8.2 Hz); *Ser* 5.03 (H-2, m), 3.89 (H-3, dd; J = 10.2, 5.5 Hz), 4.06 (H-3, t; J = 10.2 Hz), 7.57 (NH, br d; J = 9.6 Hz); *MePro* 4.27 (H-2, t; J = 8.5 Hz), 0.86−0.91 (H-3, m), 2.27−2.33 (H-3, m), 2.16−2.25 (H-4, m), 0.95(4-CH_3, d; J = 6.3 Hz), 3.08 (H-5, t; J = 9.9 Hz), 4.10 (H-5, dd; J = 9.9, 7.2 Hz); *Phe* 2.22−2.29 (H-1, m), 2.61−2.68 (H-1, m), 4.04−4.09 (H-2, m), 2.41−2.48 (H-3, m), 2.50 (H-3, dd; J = −13.5, 9.9 Hz), 6.85 (H-5/9, d; J = 7.4 Hz), 7.12−7.20 (H-6/-7/-8, m), 7.60 (NH, d; J = 8.5 Hz); FABMS *m*/*z* (rel int) 815 (MNa^+), 793 (MH^+); HRFABMS *m*/*z* 815.4048 (MNa^+, calcd for $C_{40}H_{56}N_8O_9$, 2.0 mmu error).

A similar reduction could be carried out with **1**. Glycine, L-serine, (2*S*,4*S*)-4-methylproline,[11] d-glutamic acid, and L-isoleucine were identified in the acid hydrolyzates of borohydride-reduced nostocyclopeptides; L-tyrosine was not seen in either hydrolyzate.

Incorporation of Sodium [1−^{13}C,$^{18}O_2$]Acetate into 1. A mixture of labeled acetate (400 mg) and unlabeled acetate (400 mg) was fed to each of two 20-L cultures of *Nostoc* sp. ATCC53789 in eight equal portions every other day beginning on day 10 after inoculation. The cultures were harvested on day 21 and the lyophilized cyanobacterium (24.3 g) processed to give 3.9 mg of labeled **1**. The ^{13}C NMR spectrum of the labeled **1** showed ^{13}C enriched singlets (1.3%) at δ 166.9 (Leu C-1), 173.1 (Ile C-1), 174.0 (4-MePro C-1), and 177.4 (Gln C-5). No ^{18}O-label could be seen on the three carbonyl carbons.

Aberrant Biosynthesis of Nostocyclopeptide A3 (6). To a carboy (20 L) of *Nostoc* sp. ATCC53789 growing under standard conditions was added L-4-methylphenylalanine (60 mg) in eight equal doses on every alternate day starting from day 10 after inoculation. After harvesting, the freeze-dried alga (9.8 g) was extracted with 0.5 L of CH_3CN/CH_2Cl_2 (4:1) for 48 h, and the extract was concentrated in vacuo to give a dark green solid (0.48 g). The crude extract was applied to an ODS-coated silica column and subjected to flash chromatography with CH_3CN/H_2O (25:75) (400 mL), CH_3CN/H_2O (50:50) (400 mL), CH_3CN/H_2O (65:35) (800 mL), CH_3OH (500 mL), and CH_2Cl_2 (500 mL). The fraction eluted from the flash column with CH_3CN/H_2O (1:1) was evaporated, and the residue (34 mg) was subjected to reversed-phase HPLC (ODS YMC column, 250 mm × 10 mm, 5 μ, 3 mL/min) using a gradient elution 90% 0.02N CH_3COONH_4/CH_3CN to 50% 0.02N CH_3COONH_4/CH_3CN in the first 25 min, followed by elution at 50% 0.02N CH_3COONH_4/CH_3CN to 0% 0.02N CH_3COONH_4/CH_3CN in the next 10 min. The fractions eluted at 21.3 min and 22.2 were collected separately and evaporated to yield **2** (2.6 mg) and **1** (3.1 mg), respectively. The peak collected at 24.3 min was evaporated to obtain nostocyclopeptide A3 (**6**, 5.8 mg).

Nostocyclopeptide A3 (6): ^1H NMR ($CDCl_3$) δ *Tyr* 3.70 (H-2, dd; J = 11.3, 2.4 Hz), 2.70 (H-3, dd; J = 13.4, 11.3 Hz), 3.24 (H-3, dd; J = 13.4, 2.4 Hz), 7.05 (H-5/-9, d; J = 8.5 Hz), 6.73 (H-6/-8, d; J = 8.5 Hz); *Gly* 3.58 (H-2, d; J = 17.1 Hz), 4.57 (H-2, d; J = 17.1 Hz); *Gln* 4.36 (H-2, t; J = 7.6 Hz), 1.92−2.03 (H-3, H_2, m), 2.27 (H-4, t; J = 7.6 Hz); *Ile* 4.31 (H-2, d; J = 4.0 Hz), 2.08−2.15 (H-3, m), 0.99 (3-CH_3, d; J = 7.0 Hz), 1.27−1.37 (H-4, m), 1.38−1.48 (H-4, m), 0.94 (H-5, t; J = 7.9 Hz); *Ser* 5.09 (H-2, dd; J = 10.5, 5.5 Hz), 3.87 (H-3, dd; J = 10.5, 5.5 Hz), 4.22 (H-3, t; J = 10.5 Hz); *MePro* 4.27 (H-2, t; J = 8.5 Hz), 0.64 (H-3, m), 2.10−2.28 (H-3, m), 2.10−2.28 (H-4, m), 0.92(4-CH_3, d; J = 6.7 Hz), 3.10 (H-5, t; J = 10.1 Hz), 4.18 (H-5, dd; J = 10.1, 8.4 Hz); *MePhe* 6.95 (H-1, d; J = 2.1 Hz), 4.54−4.61 (H-2, m), 2.28 (H-3, dd; J = 13.7, 11.2 Hz), 2.60 (H-3, dd; J = 13.7, 4.6 Hz), 7.06 (H-5/-9, d; J = 7.6 Hz), 6.94 (H-6/-8, d; J = 7.6 Hz), 2.26 (7-CH_3); FABMS *m*/*z* 827 (MNa^+), 805 (MH^+); HRFABMS *m*/*z* 805.4258 (MH^+, calcd for $C_{41}H_{57}N_8O_9$ −0.9 mmu error).

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Supporting Information Available: Copies of 500 MHz ^{1}H NMR spectra of **1** and **2**, the $^{1}H-^{13}C$ HMBC spectrum of **1** in the carbonyl carbon region, and the $^{1}H-^{15}N$ HMBC spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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