Homotyrosine-Containing Cyanopeptolins 880 and 960 and Anabaenopeptins 908 and 915 from *Planktothrix agardhii* CYA 126/8

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Two homotyrosine-bearing cyanopeptolins are described from *Planktothrix agardhii* CYA 126/8. The compounds feature a common homotyrosine-containing cyclohexadepsipeptide and differ by sulfation of an exocyclically located 2-*O*-methyl-D-glyceric acid residue. In addition we describe two anabaenopeptins, which contain two homotyrosine residues, one of which is *N*-methylated. The anabaenopeptins have a common cyclopentapeptide portion and differ in the amino acid linked to it via an ureido bond, arginine and tyrosine, respectively.

We recently isolated two homotyrosine-containing microcystins, MC-HtyY and MC-HtyHty, from laboratory cultures of a unique isolate of the limnic cyanobacterium *Planktothrix rubescens* from Lake Schwarzensee in Austria.[1] We also reported a comparison of the predicted amino acid sequence of the adenylation domain of McyC of the microcystin biosynthetic gene cluster of this isolate of *P. rubescens* with the McyC adenylation domain of the cluster from *Planktothrix agardhii* CYA 126/8, a strain only known to produce microcystin-RR and a minor amount of microcystin-LR in laboratory culture. We observed that the amino acid sequences of the adenylation domains of these two species are identical with the exception of one amino acid residue.[1] This change is located in a region of the protein backbone that is part of the amino acid specificity-conferring region of the adenylation domain in the accepted model.[2,3] However, given the chemical difference between homotyrosine (Hty) and arginine (R), the difference in the McyC sequences appears to be an insufficient molecular basis for the incorporation of arginine in position 4 of the MCs in *P. agardhii* versus homotyrosine/tyrosine in the same position in *P. rubescens*.

We considered several possible explanations for the observation that the MC metabolite spectrum differed very strongly between these two species despite the high degree of genetic identity of the gene clusters. We hypothesized that *P. agardhii* CYA 126/8 might be unable to synthesize the nonproteinogenic amino acid homotyrosine in variance to this specific isolate of *P. rubescens*. Literature review in mid-2004 using Medline and SciFinder using the search term "homotyrosine" returned only three hits in conjunction with either *Planktothrix* or *Oscillatoria* sp., of which only two described defined compounds.[4,5] We therefore elected to feed synthetic homotyrosine to laboratory cultures of *P. agardhii* 126/8 in an attempt to alleviate this putative biosynthetic deficiency. Extracts of cultures of *P. agardhii* to which homotyrosine had been added were screened by LCMS for production of homotyrosine-containing microcystins. Since strain 126/8 produces MC-RR and MC-LR under standard laboratory culture conditions, one would therefore expect the production of MC-RHty or MC-LHty if McyC were capable of activating Hty in a successful precursor-directed biosynthesis experiment. However, using selected ion monitoring we did not obtain any evidence for the production of these two putative microcystins. Instead, HPLC analysis indicated increases in the concentrations of metabolites more polar than would be expected for these putative MCs. These compounds were shown on the basis of more detailed NMR-based screening to contain *para*-disubstituted phenols, as expected for homotyrosine- or tyrosine-containing compounds. In this report we describe several new natural products belonging to the cyanopeptolin and anabaenopeptin groups of cyanobacterial peptides that were isolated.

DL-Homotyrosine was prepared by a modification of a known route starting from aspartic acid.[6] The somewhat cumbersome and messy final deprotection step of the literature procedure using HBr in acetic acid was replaced by one employing iodotrimethylsilane generated *in situ* from NaI and TMS-Cl in anhydrous acetonitrile. The product was isolated as the zwitterion after ion exchange chromatography on Dowex-50.

This compound was supplied to a growing culture of *P. agardhii* over the course of two weeks, and the crude peptide fraction was isolated from the resulting cell mass by extraction with 50% aqueous MeOH followed by C18 flash chromatography and HPLC. The major noticeable difference in the HPLC chromatogram of the extract of this culture when compared to a control was a substantial increase in the intensity of one peak eluting significantly after a MC-LR standard. ^{1}H NMR analysis of this peak indicated the presence of peptidic substances containing a *para*-disubstituted aromatic ring, consistent with the presence of either homotyrosine or tyrosine. Further purification of this fraction on a different C18 stationary phase resulted in two chromatographically homogeneous, structurally closely related compounds. Structure elucidation by interpretation of high-field NMR data resulted in two new members of the cyanopeptolin group of compounds as shown below.

Cyanopeptolin 880 (**1**)[7,8] was isolated as an optically active solid, which yielded a $[M + H]^+$ pseudomolecular ion at *m/z* 881.4645 upon HRESITOFMS analysis, consistent with a molecular formula of $C_{45}H_{64}N_6O_{12}$ for **1**. A prominent fragment ion at *m/z* 863.4533 $[M + H^+ - H_2O]^+$ corroborated this molecular formula (calcd 863.4508, $\Delta = 2.5$ mmu). Analysis of ^{13}C NMR and gHSQC data suggested the presence of seven methyl, eight methylene, 16 methine, and 10 quaternary carbon atoms. The ^{1}H NMR data indicated the presence of a *para*-disubstituted and a monosubstituted phenyl ring. These structural features as well as seven carbonyl resonances accounted for 15 of the 17 degrees of unsaturation and suggested the presence of two rings.

The planar structure of **1** was established from the NMR data listed in the Experimental Section. Particularly useful was the analysis of 1D-TOCSY spectra in conjunction with ^{1}H and ^{13}C NMR data. This readily led to the identification of two isoleucine residues, one glyceric acid, a β-substituted alanine, and a γ-substituted aminobutyric acid. Analysis of gHMBC data allowed the latter two to be expanded to an *N*-methyl phenylalanine and a

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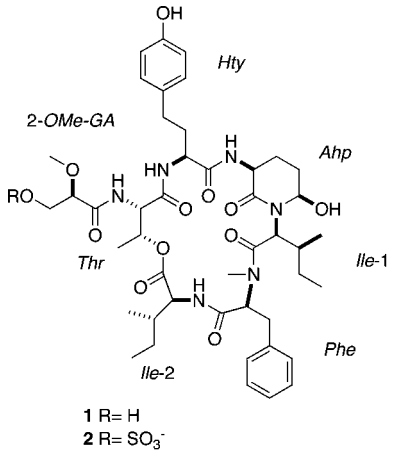

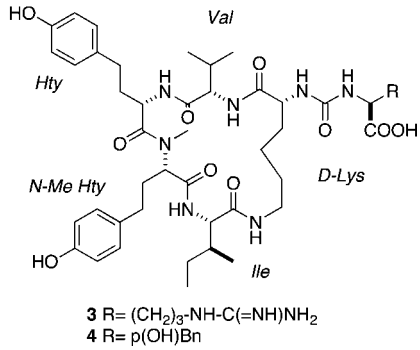

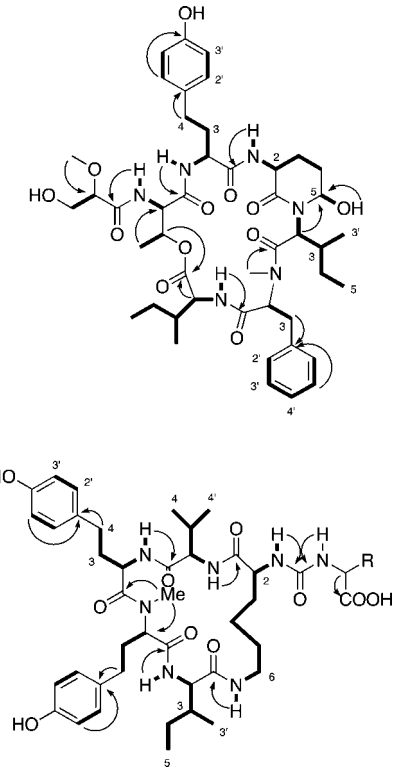

Figure 1. Individual spin systems as identified by 1D TOCSY (bold) and important gHMBC (arrows) correlations for **1**, **2**, **3**, and **4**.

homotyrosine, respectively. The most important correlations are depicted in Figure 1. It is noteworthy that the threonine residue did not yield to straightforward 1D-TOCSY analysis. This amino acid was identified on the basis of data from two 1D-TOCSY and from gHMBC experiments. Apparently, the dihedral angle between the aminomethine and the hydroxymethine protons is such that TOCSY magnetization transfer is inefficient. This explanation is borne out by coupling constant analysis, which yields $^3J_{\text{H-2/H-3}} \leq 1$ Hz. The OMe resonance at 3.54 ppm was assigned as an *O*-methyl ether function attached to C-2 of the glyceric acid residue on the basis of gHMBC data. The last residue to be identified was the aminohydroxypiperidone carboxylic acid (Ahp), which is part of the strictly conserved motif of this compound class. Here again two 1D-TOCSY experiments yielded two fragments (N-H to H-4 and OH to H-5, respectively), and gHMBC data were required to establish the atom connectivity unambiguously.

The second, minor cyanopeptolin, **2**, was determined to be the sulfated version of the major one. Insufficient material was isolated for a detailed analysis of gHMBC data. However, inspection of the 1D TOCSY and the ^{1}H NMR spectra indicated that **1** and **2** are constitutionally almost identical, the chemical shift differences being smaller than 0.05 ppm (^{1}H) for all but three resonances. The only substantial chemical shift differences affect the H-3 protons as well as the methoxymethine proton H-2 of the methoxy glyceric acid residue ($\Delta\delta = 0.5$ ppm). HRESITOFMS analysis gave a pseudomolecular ion cluster $[M + H]^+$ at *m*/*z* 961.4223, suggesting a molecular formula for **2** of $C_{45}H_{64}N_6O_{15}S$. This molecular formula differs from that of **1** by the element of SO_3 and suggested **2** to be a sulfated version of **1**. Corroboration of this interpretation came from the observation of the typical sulfate stretch at 1206 cm^{-1} in the FTIR spectrum of **2**. Cyanopeptolin 960 is very closely related to micropeptin KT946,[9] differing only in the methoxyl group in the glyceric acid moiety.

The absolute configuration of the constituent amino acids of **1** was determined after acidic hydrolysis by HPLCMS analysis of the Marfey derivatives and comparison of retention times with those obtained from authentic standards.[10] The required *N*-methyl-D-phenylalanine was synthesized by an adaptation of a literature method.[11−14] The stereochemical analysis was performed on two aliquots of **1**. The first sample was oxidized with excess PCC in CH_2Cl_2 solution before hydrolysis in order to convert the aminal of the 3-amino-6-hydroxypiperid-2-one (Ahp) residue to the imide. Marfey analysis of the acid hydrolysate of the oxidized sample using LCMS in selected ion mode suggested that the configuration of the *N*-methylphenylalanine, the threonine, and the glutamic acid derived from the Ahp residue is *S*. The configuration of the isoleucine residues was determined by chromatography on chiral stationary phase (Chirex 3126, Phenomenex) using aqueous copper(II) sulfate/acetonitrile as an eluent. Under these conditions the diastereoisomers of the L-series elute approximately 5 min apart. Upon analysis of the hydrolysate of **1**, only one peak was observed, which coeluted with the L-isoleucine standard. This suggested that L-isoleucine was the amino acid present in **1** in both positions occupied by isoleucine. The homotyrosine did not give any detectable Marfey derivative, presumably as a result of oxidative degradation of the phenol during chromate oxidation. However, since the oxidation could reasonably be expected to destroy the 2-methoxy glyceric acid moiety as well, a second aliquot was hydrolyzed without prior oxidation. The Marfey derivatives of a portion of this hydrolysate were again analyzed by LCMS in negative ion mode and this time found to contain the derivative of L-homotyrosine.

The absolute configuration of the 2-methoxyglyceric acid was established by analytical HPLC of the *p*-bromophenacyl ester derivative on a chiral stationary phase (Chiracel OJ) and comparison with authentic standards. These standards were prepared from D- and L-serine by diazotization with isoamyl nitrite in methanol at elevated temperature under pressure and purified by ion exchange chromatography. Although not completely stereoselective, this method is considerably simpler than the published procedure.[15] With standards in hand, a second portion of the nonoxidized hydrolysate was derivatized as the *p*-bromophenacyl ester. The ester of 2-methoxyglyceric acid was isolated by silica chromatography and

analyzed by HPLC on chiral stationary phase. Comparison of the retention time of the derivative with that of authentic standards suggested the configuration for the 2-methoxyglyceric acid in **1** to be *R*.

^{1}H NMR-guided screening for other peptides containing *para*-disubstituted phenols that might be part of a tyrosine or homotyrosine revealed the presence of another peptide in extracts of *P. agardhii* CYA 126/8. Anabaenopeptin 908 (**3**)[7] yielded a $[M + H]^+$ pseudomolecular ion at *m/z* 909.5198 in HRESITOFMS analysis, consistent with a molecular formula for **3** of $C_{45}H_{69}N_{10}O_{10}$. The planar structure of **3** was determined on the basis of gCOSY, gHSQC, gHMBC, and 1D-TOCSY data, which are summarized in Table 1. Particularly significant was the observation of two gHMBC correlations of signals for NH protons resonating at δ_H 6.47 and 6.62 ppm, respectively, to one carbonyl resonance at δ_C 160.1, indicating the presence of a ureido function, a hallmark of the anabaenopeptin class of compounds.[16]

Analysis of 1D TOCSY and gCOSY data suggested the presence of one valine, one isoleucine, and two γ-substituted aminobutyrate residues as well as one lysine residue. The amino acid attached via the ureido function was identified as an arginine residue. Analysis of gHMBC spectra next allowed the attachment of two phenol rings to the aminobutyrate moieties to yield the homotyrosines, one of which was *N*-methylated as suggested by gHMBC data. A combination of gCOSY and gHMBC data also allowed the sequence of amino acids to be determined: the isoleucine is attached via its carboxyl group to the ε amino group of the lysine and at the *N*-terminus to the carboxyl of *N*-methyl homotyrosine. The latter is attached to a second homotyrosine residue, which is linked to a valine residue. The amino group of valine is connected to the carboxyl of the lysine residue, thereby closing a 19-membered ring. The α amino group of the lysine bears the urea carbonyl, to which the arginine side chain is connected. The most important correlations are depicted in Figure 1.

With this structure in hand we continued screening for other members of the anabaenopeptin family and tentatively identified a compound of molecular mass 915, which also contained *para*-disubstituted phenols.

Anabaenopeptin 915 (**4**) was isolated as a colorless optically active solid. Compound **4** yielded a $[M + H]^+$ pseudomolecular ion of *m/z* 916.4828 upon HRESITOFMS analysis, consistent with a molecular formula for **4** of $C_{48}H_{65}N_7O_{11}$. Analysis of 1D-TOCSY data revealed that the amino acids constituting the ring in **4** were identical with those in **3**. The most significant difference between **3** and **4** was evident in the ^{1}H NMR spectrum, which indicated the presence of three *para*-disubstituted phenols instead of two as in **3**. Analysis of gHMBC data in conjunction with gCOSY and gHSQC data then suggested that the side chain in compound **4** was comprised of a tyrosine residue instead of the arginine present in **3**. The absolute configuration of the amino acids in **3** and **4** was determined by Marfey analysis after acid hydrolysis.

With these structures in hand, renewed literature search for cyanopeptolins and anabaenopeptins revealed that, in fact, homotyrosine-containing anabaenopeptin-type compounds had been previously discovered from *Oscillatoria agardhii*.[17a,b] The peptide in the first paper is structurally quite different,[17a] bearing an *N*-methyl alanine residue in place of the *N*-methyl homotyrosine. However, the anabaenopeptins from strain NIES 595 differ only in the amino acid *N*-terminal to the Hty residue, isoleucine in place of valine in **3** and **4**.[17b] It should be noted that the structures of the two anabaenopeptins reported in this study are closely related to anabaenopeptin T isolated from field-collected material of unreported taxonomy, the only difference being the structure of the ureido-bound exocyclic amino acid, which is isoleucine in the case of anabaenopeptin T.[16e] The failure of the keyword-based search to uncover the references for the *Oscillatoria* metabolites is puzzling and may serve as a cautionary tale; a structure-based search had no such difficulties, however.

Table 1. 500 MHz (^{1}H) and 125 MHz (^{13}C) NMR Data for **3** in CD_3OH

unit	C/H no.	δ_C	δ_H	(*J* in Hz)	HMBC
Ile	1	174.2			
	2	60.0	4.32,	m	1, 3, 4, 3′, $1_{N\text{-}Me\text{-}Hty}$
	3	37.7	2.05,	m	2, $1_{N\text{-}Me\text{-}Hty}$
	4	26.1[a]	1.36,	m	
			1.03,	m	
	5	11.8	0.82,	dd (7.6, 7.3)	4
	3′	16.6	0.86,	d (6.9)	3, 4
	NH		8.44		
N-Me-	1	171.7			
Hty	2	61.5	4.53,	dd (6.2, 8.2)	1, *N*-Me, 1_{Hty}
	3	32.1[b]	2.12,	m	
			1.78,	m	
	4	33.0	2.28		1′
	1′	133.2			
	2′/6′	130.2	6.98,	d (8.5)	1′, 4, 3′/5′, 4′
	3′/5′	116.5	6.71,	d (8.5)	2′/6′, 4′
	4′	157.0			
	N-Me	29.8	2.71,	s	1_{Hty}
Hty	1	175.2			
	2	49.5[c]	4.67,	m	1
	3	34.9	2.12,	m	
			1.78,	m	
	4	32.1[b]	2.78,	m	1′, 2, 2′/6′
			2.65,	m	
	1′	132.8			2′/6′
	2′/6′	131.1	7.07,	d (8.5)	1′, 3′/5′
	3′/5′	116.5	6.69,	d (8.5)	4′, 2′/6′
	4′	157.2			3′/5′
	NH		8.99,	d (5.2)	1_{Val}
Val	1	175.0			
	2	61.0	3.95,	dd (5.8, 8.1)	1
	3	31.5	1.99,	m	4, 4′
	4	19.9	0.98,	d (6.6)	2, 3, 4′
	4′	19.8	0.97,	d (6.5)	2, 3, 4
	NH		7.25,	d (5.8)	1_{Lys}
Lys	1	175.8			
	2	56.8	4.09,	m	1, 3, 4, C═O_{ureido}
	3	32.0[b]	1.91,	m	2
			1.78,	m	
	4	22.0	1.58,	m	
			1.36,	m	
	5	29.3	1.58,	m	
	6	40.3	3.57,	m	4, 5
			3.00,	m	
	NH		6.62,	d (5.1)	2, C═O_{ureido}
	NH_ε		7.35,	dd (6.9, 3.2)	1_{Ile}, 6
Arg	1	176.2			
	2	53.6	4.32,	m	1, 3
	3	30.8	1.91,	m	
			1.68,	m	
	4	26.3[a]	1.68,	m	
	5	42.0	3.21,	m	3, 4
	NH		6.47,	d (8.5)	6
	NH′		7.45,	t (5.3)	6
	6	158.9			
Ureido C═O		160.1			

[a] ^{13}C NMR signals are interchangeable. [b] ^{13}C NMR signals are interchangeable. [c] Overlapped with solvent signal.

In conclusion, we note that all of the four peptides described in this paper contain homotyrosine. In order to establish whether these compounds are being produced only in a precursor-directed biosynthesis experiment or whether they are part of the metabolite spectrum in unsupplemented medium, we also analyzed cell mass from cultures to which exogenous homotyrosine had not been supplied. We found that three of the four compounds described in this paper are also biosynthesized by strain CYA126/8 of *P. agardhii* in the absence of added homotyrosine. The nonsulfated

cyanopeptolin 880 (**1**) was not observed when the strain was grown in nonsupplemented medium. This observation may mean that sulfation is rate limiting and that supplementation with homotyrosine results in a higher flux through the early part of the pathway. However, this observation was not pursued further.

Members of the cyanopeptolin[8] and anabaenopeptin[16] classes of compounds are known protease inhibitors. We did not observe trypsin/chymotrypsin inhibition by either **3** or **4** at concentrations up to 100 μg/mL. However, in a standard assay **4** inhibited carboxypeptidase A with an IC_{50} of 0.12 μg/mL, while **3** was inactive at concentrations up to 20 μg/mL, the highest concentration tested.

The present investigation was concerned with the peptide metabolite spectrum of strain CYA126/8 as a foundation on which a better understanding of peptide biosynthesis in cyanobacteria may be built. Last, it should be noted that **3** and **4** are obviously products of the NRPS pathway. Our current understanding of NRPS biochemistry cannot readily explain the co-occurrence of two closely related metabolites bearing two quite different amino acids, such as arginine and tyrosine in equivalent positions.

Experimental Section

General Experimental Procedures. Optical rotation data were recorded on a JASCO DIP-700 polarimeter. IR and UV spectra were recorded using a Thermo Nicolet Avatar 380 and a Beckman DU-7000 spectrometer, respectively. Nuclear magnetic resonance (NMR) spectra were recorded using Shigemi tubes on a Varian UNITY INOVA 500 instrument equipped with a 3 mm microprobe. Chemical shifts are referenced to residual protiated solvent. High-performance liquid chromatography (HPLC) was performed on a Shimadzu AS-10VP gradient system using either a 5 μm Lichrospher ODS column (4.6 × 250 mm) or a 5 μm Phenomenex Luna C-18(2) column (4.6 × 250 mm or 10 × 250 mm). HRESITOFMS spectra were recorded in the flow injection mode on an Agilent 6100 LC-MSDTOF system equipped with an Agilent 1100 LC module. HRFABMS data were recorded on a VG ZAB-70 instrument using a glycerol matrix.

Biological Material. ***Planktothrix agardhii.*** CYA126/8 was a kind gift of Kaarina Sivonen (University of Helsinki). This strain is deposited in the Norwegian Institute of Water Research culture collection and is maintained in the culture collection in the Chemistry Department at the University of Hawaii. Cultures were grown in 20 L carboys in Z+G medium.[18] Continuous illumination was by soft white fluorescent lights at 100 μE s^{-1}.

Isolation of Peptides. Cultures were concentrated by low-pressure filtration over ultrafiltration membranes. The concentrated cell suspension was pelleted by centrifugation to yield 7.4 g of cell paste. The pellet was freeze-dried (1.6 g dry wt), suspended in 50% MeOH(aq) (10 mL/g dry weight), sonicated, and extracted twice with vigorous shaking for 30 min. The combined extracts were evaporated and applied to a YMC-ODS-A flash column (4.0 g) equilibrated in 30% aqueous MeOH. The column was washed with additional 30% MeOH(aq) and then eluted with a step gradient of MeOH in H_2O (30 mL/step). Compounds **1**, **2**, and **3** eluted in the 70% fraction, whereas compound **4** eluted in the 50% fraction. Further purification was achieved by reversed-phase HPLC (Luna C-18(2), 10 × 250 mm, 3 mL/min) employing a gradient of CH_3CN(aq) containing 0.05% TFA (30% CH_3CN(aq) from 0−10 min, to 50% CH_3CN(aq) over 15 min, 50% CH_3CN(aq) for 5 min, and to 100% CH_3CN in 5 min). Compound **3** elutes after 8.6 min (4.9 mg), compound **4** after 21.5 min (3.9 mg), and compounds **1** and **2** as a mixture of anomers after 24.4 min. The cyanopeptolins are resolved by HPLC on a Lichrosphere C-18 column (4.6 × 250 mm, 1 mL/min) with a gradient starting at 45% CH_3CN(aq) ramping to 50% CH_3CN(aq) in 20 min and holding for 5 min. Cyanopeptolin (**1**) (1.5 mg) elutes after 9.5 min (anomer (1.4 mg) at 15.1 min), cyanopeptolin (**2**) (0.5 mg) after 3.4 min, and its anomer (0.8 mg) after 4.5 min, respectively.

Feeding Experiment. DL-Homotyrosine (100 mg) was suspended in 14 mL of distilled water, and the minimum amount of diluted HCl was added to effect dissolution. The resulting solution was dispensed through a sterile filter in equal 1 mL portions every day over two weeks starting 10 days after inoculation. Fermentation was continued for another 10 days after the last addition of compound.

Cyanopeptolin 880 (1): white powder; $[\alpha]_D$ −38 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 279 (2.95) nm; IR (film) ν_{max} 3435, 1645, 1456, 1384 cm^{-1}; ^{1}H NMR (500 MHz, MeOH-d_3) δ *Ahp*: 7.69 (1H, d, J = 9.0 Hz, N-H), 6.34 (1H, brd, O-H), 5.04 (1H, d, J = 1.7 Hz, H-5), 4.56 (1H, m, H-2), 2.78 (1H, m, H-3a), 1.88 (3H, m, H-3b, H-4); *Hty*: 8.41 (1H, d, J = 7.5 Hz, N-H), 6.97 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.66 (2H, d, J = 8.5 Hz, H-3′, H-5′), 4.32 (1H, m, H-2), 2.66 (1H, m, H-4a), 2.51 (1H, m, H-4b), 2.51 (1H, m, H-3a), 1.88 (1H, m, H-3b); *Ile-1*: 4.50 (1H, d, J = 10.7 Hz, H-2), 1.88 (1H, m, H-3), 1.12 (1H, m, H-4a), 0.70 (1H, m, H-4b), 0.66 (3H, dd, J = 6.8, 5.0 Hz, H-5), −0.13 (3H, d, J = 6.5 Hz, H-3′); *Ile-2*: 8.13 (1H, d, J = 9.0 Hz, N-H), 4.42 (1H, t, J = 8.6 Hz, H-2), 1.88 (1H, m, H-3), 1.45 (1H, m, H-4a), 1.21 (1H, m, H-4b), 0.96 (3H, d, J = 6.9 Hz, H-3′), 0.88 (3H, t, J = 7.4 Hz, H-5); *N-Me-Phe*: 7.29 (2H, d, J = 7.4 Hz, H-2, H-6), 7.24 (2H, t, J = 7.4 Hz, H-3, H-5), 7.16 (1H, t, J = 7.4 Hz, H-4), 5.24 (1H, dd, J = 11.6, 2.8 Hz, H-2), 3.52 (1H, dd, J = −14.5, 2.8 Hz, H-3a), 2.83 (3H, s, N-Me), 2.80 (1H, dd, J= −14.5, 11.6 Hz, H-3b); *Thr*: 8.08 (1H, d, J = 9.0 Hz, N-H), 5.60 (1H, q, J = 6.6 Hz, H-3), 4.83 (1H, d, J = 9.0 Hz, H-2), 1.39 (3H, d, J = 6.6 Hz, H-4); *2-OMe-GA*: 3.96 (1H, dd, J = 6.0, 3.7 Hz, H-2), 3.85 (1H, dd, J = −11.7, 3.7 Hz, H-3a), 3.77 (1H, dd, J = −11.7, 6.0 Hz, H-3b), 3.54 (3H, s, O-Me); ^{13}C NMR (125 MHz, MeOH-d_3) δ *Ahp*: 171.1, 76.2, 51.2, 31.4, 22.3; *Hty*: 174.3, 156.8, 132.8, 130.6, 116.3, 54.8, 33.7, 32.1; *Ile-1*: 172.8, 56.6, 34.6, 25.1, 15.0, 10.8; *Ile-2*: 175.5, 58.2, 38.0, 26.6, 16.2, 10.2; N-Me-Phe: 172.0, 139.0, 130.9, 130.0, 128.0, 63.0, 35.4, 31.5; *Thr*: 171.3, 73.4, 56.8, 18.9; *2-OMe-GA*: 173.9, 84.5, 63.9, 58.9; HRESITOFMS *m/z* $[M + H]^+$ 881.4645 (calcd for $C_{45}H_{65}N_6O_{12}$, 881.4660, 1.5 ppm error).

Cyanopeptolin 960 (2): white powder; $[\alpha]_D$ −39 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 279 (2.94) nm; IR (film) ν_{max} 3440, 1645, 1455, 1361, 1206 cm^{-1}; ^{1}H NMR (500 MHz, MeOH-d_3) δ *Ahp*: 7.69 (1H, d, J = 9.0 Hz, N-H), 6.34 (1H, brd, O-H), 5.04 (1H, d, J = 5.4 Hz, H-5), 4.56 (1H, m, H-2), 2.78 (1H, m, H-3a), 1.88 (3H, m, H-3b, H-4); *Hty*: 8.41 (1H, d, J = 7.5 Hz, N-H), 6.97 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.66 (2H, d, J = 8.5 Hz, H-3′, H-5′), 4.32 (1H, m, H-2), 2.66 (1H, m, H-4a), 2.51 (1H, m, H-4b), 2.38 (1H, m, H-2a), 1.88 (1H, m, H-2b); *Ile-1*: 4.50 (1H, d, J = 10.7 Hz, H-2), 1.88 (1H, m, H-3), 1.12 (1H, m, H-4), 0.70 (1H, m, H-4), 0.66 (3H, dd, J = 6.8, 5.0 Hz, H-5), −0.13 (3H, d, J = 6.5 Hz, H-3′); *Ile-2*: 8.13 (1H, d, J = 9.0 Hz, N-H), 4.42 (1H, dd, J = 8.6, 8.6 Hz, H-2), 1.88 (1H, m, H-3), 1.45(1H, m, H-4a), 1.21 (1H, m, H-4b), 0.96 (3H, d, J = 6.9 Hz, H-3′), 0.88 (3H, t, J = 7.4 Hz, H-5); *N-Me-Phe*: 7.29 (2H, m, H-2′, H-6′), 7.24 (2H, m, H-3′, H-5′), 7.16 (1H, m, H-4′), 5.24 (1H, dd, J = 11.6, 2.8 Hz, H-2), 3.52 (1H, dd, J = −14.5, 2.8 Hz, H-3a), 2.80 (1H, dd, J= −14.5, 11.6 Hz, H-3b); *2-OMe-GA sulfate*: 4.49 (1H, dd, J = 4.7, 2.7 Hz, H-2), 4.35 (1H, dd, J= −10.4, 2.7 Hz, H-3a), 4.28 (1H, dd, J = −10.4, 4.7 Hz, H-3b), 3.54 (3H, s, O-Me); *Thr*: 8.08 (1H, d, J = 7.5 Hz, N-H), 5.60 (1H, q, J = 6.6 Hz, H-3), 1.39 (3H, d, J = 6.6 Hz, H-4); ^{13}C NMR (125 MHz, MeOH-d_3) δ *Ahp*: 171.1, 76.2, 51.2, 31.4, 22.3; *Hty*: 174.3, 156.8, 132.8, 130.6, 116.3, 54.8, 33.7, 32.1; *Ile-1*: 172.8, 56.6, 34.6, 25.1, 15.0, 10.8; *Ile-2*: 175.5, 58.2, 38.0, 26.6, 16.2, 10.6; *N-Me-Phe*: 172.0, 139.0, 130.9, 130.0, 128.0, 63.0, 35.4, 31.5; *2-OMe-GA sulfate*: 173.9, 84.5, 63.9, 58.9; *Thr*: 171.3, 73.4, 56.8, 18.9; HRESITOFMS *m/z* $[M + H]^+$ 961.4223 (calcd for $C_{45}H_{65}N_6O_{15}S$, 961.4229, 0.6 ppm error).

Anabaenopeptin 908 (3): $[\alpha]_D$ −29 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 280 (3.39) nm; IR (film) ν_{max} 1652 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESITOFMS *m/z* $[M + H]^+$ 909.5198 (calcd for $C_{45}H_{69}N_{10}O_{10}$, 909.5204, 0.7 ppm error).

Anabaenopeptin 915 (4): white powder; $[\alpha]_D$ −21 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 280 (3.46) nm; IR (film) ν_{max} 1652 cm^{-1}; ^{1}H NMR (500 MHz, MeOH-d_3) δ *Ile*: 8.42 (1H, d, J = 9.6 Hz, N−H), 4.30 (1H, dd, J = 9.6, 6.2 Hz, H-2), 2.03 (1H, m, H-3), 1.38 (1H, m, H-4a), 1.10 (1H, m, H-4b), 0.85 (3H, d, J = 6.9 Hz, H-3′), 0.79 (3H, t, J = 7.4 Hz, H-5); *N-Me-Hty*: 6.97 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.68 (2H, d, J = 8.5 Hz, H-3′, H-5′), 4.52 (1H, dd, J = 6.5, 7.2 Hz, H-2), 2.70 (3H, s, N-Me), 2.32 (1H, dt, J = −12.4, 5.0 Hz, H-4a), 2.26 (1H, dt, J = −12.4, 4.6 Hz, H-4b), 2.12 (1H, m, H-3a), 1.80 (1H, m, H-3b); Hty: 8.96 (1H, d, J = 4.7 Hz, N-H), 7.06 (2H, d, J = 8.3 Hz, H-2′, H-6′), 6.68 (2H, d, J = 8.3 Hz, H-3′, H-5′), 4.66 (1H, m, H-2), 2.78 (1H, m, H-4a), 2.64 (1H, m, H-4b), 2.12 (1H, m, H-3a), 1.80 (1H, m, H-3b); *Val*: 7.28 (1H, d, J = 4.7 Hz, N-H), 3.92 (1H, dd, J = 8.0, 5.9 Hz, H-2), 1.97 (1H, m, H-3), 0.98 (6H, d, J = 6.8 Hz, H-4, H-4′); *Lys*: 7.31 (1H, dd, J = 7.0, 3.0 Hz, N-H_ε), 6.64 (1H, d, J = 4.7 Hz, N-H), 4.06 (1H, m, H-2), 3.54 (2H, m, H-6), 1.91 (1H, m,

H-3a), 1.76 (1H, m, H-3b), 1.56 (1H, m position, H-5a), 1.53 (1H, m, H-4a), 1.31 (2H, m, H-4b, H-5b); *Tyr*: 7.02 (2H, d, $J = 8.3$ Hz, H-2′, H-6′), 6.68 (2H, d, $J = 8.3$ Hz, H-3′, H-5′), 6.06 (1H, d, $J = 8.0$ Hz, N−H), 4.45 (1H, m, H-2), 3.03 (1H, m, H-3a), 2.91 (1H, m, H-3b); ^{13}C NMR (125 MHz, MeOH-d_3) δ *Ile*: 174.2, 60.0, 37.7, 26.1, 16.6, 11.7; *N-Me-Hty*: 171.7, 156.9, 133.1, 130.2, 116.3, 61.4, 34.9, 33.0, 29.8; *Hty*: 175.2, 157.5, 132.8, 131.1, 116.5, 51.3, 34.9, 31.9; *Val*: 175.0, 61.2, 31.5, 20.1, 19.8; *Lys*: 175.9, 56.5, 40.4, 32.0, 29.2, 21.9; *Tyr*: 177.0, 157.2, 131.5, 129.1, 116.5, 56.6, 38.4; *ureido*: 159.8; HRESITOFMS *m/z* $[M + H]^+$ 916.4828 (calcd for $C_{48}H_{65}N_7O_{11}$, 916.4821, 0.7 ppm error).

Carboxypeptidase A Assay. In an Eppendorf tube, 100 μL of 25 mM Tris, 0.5 M NaCl, pH 7.5, 10 μL of carboxypeptidase A [6 U/mL, Sigma Aldrich], and 10 μL of anabaenopeptin solution of varying concentrations (dissolved in 50% MeOH(aq)) were combined and incubated for 30 min at 37 °C. The mixture was cooled to room temperature, and the reaction was initiated by addition of 380 μL of hippuryl-L-phenylalanine (1 mM in 25 mM Tris, 0.5 M NaCl, pH 7.5). After 5 min a 100 μL aliquot of the reaction was quenched with 1 μL of 50% trichloroacetic acid(aq). The amount of phenylalanine liberated was determined by HPLC using the OPA derivatization reaction.[19]

Briefly, the OPA derivatization buffer was prepared as follows, 1 mL of *o*-phthalaldehyde [0.25 mg per 50 mL of MeOH], 4 mL of borate buffer [0.1 M boric acid, 0.1 M NaOH, pH 9.3], and 162 μL of 3-mercaptopropionic acid were added in that order and mixed thoroughly, and the pH was adjusted to 9.3 with NaOH. The derivatization buffer was stored at 4 °C until use (buffer was prepared at least 90 min before use and used within three days).

To analyze the amount of phenylalanine liberated, 25 μL of the quenched reaction mixture was added to 50 μL of OPA derivatization buffer. The mixture was allowed to incubate at room temperature for 20 min, at which time it was subjected to HPLC analysis.

HPLC was performed on a 5 μm LUNA C18(2) column (4.6 × 250 mm) pre-equilibrated with an isocratic solvent system of 48% 40 mM sodium phosphate buffer, pH 7.8/52% CH_3CN/MeOH/H_2O (45:45:10). The peaks were observed at 338 nm and recorded on an HP 3995 integrator. At a flow rate of 1 mL/min the phenylalanine derivative eluted at 5.6 min.

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Supporting Information Available: Procedures and analytical data for the synthesis of standards, ^{1}H NMR spectra for compounds **1**−**4**, and ^{13}C NMR spectra for **1**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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