# Structures of Pahayokolides A and B, Cyclic Peptides from a *Lyngbya* sp.

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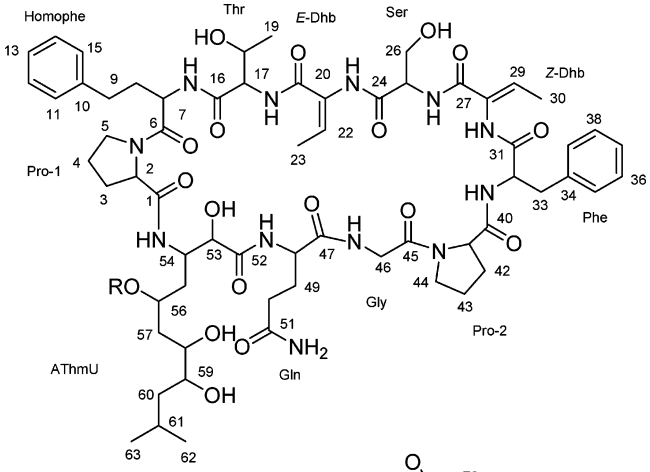
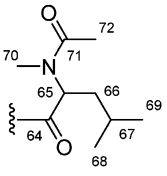
The isolation and structure elucidation of two cyclic peptides, pahayokolides A (**1**) and B (**2**), is described. Structural features determined for these compounds include a pendent *N*-acetyl-*N*-methyl leucine, both *E*- and *Z*-dehydrobutyrines, a homophenylalanine, and an unusual polyhydroxy amino acid that is most likely of mixed polyketide synthase/nonribosomal peptide synthase origin. These peptides were purified from a new species of cyanobacteria of the genus *Lyngbya*, which was isolated from a periphyton mat from the Florida Everglades.

Cyanobacteria have proven to be a rich source of biologically active secondary metabolites.[1,2] The Florida Everglades represent a relatively unexplored, yet diverse source of cyanobacteria. In this oligotrophic marsh, microbial communities are organized into either benthic or floating periphyton mats. In an effort to identify new secondary metabolites, we have recently undertaken a study of cyanobacteria from the Florida Everglades. One species in particular, identified as *Lyngbya* sp., has yielded two cytotoxic cyclic peptides. We have previously published preliminary studies on the isolation[3] and cytotoxicity[4] of one of these compounds, pahayokolide A (**1**). Herein we report the planar structures of pahayokolide A (**1**) and the related cyclic peptide pahayokolide B (**2**).




Pahayokolide A (**1**) R =



Pahayokolide B (**2**) R = H

## Results and Discussion

*Lyngbya* sp. 15-2 was isolated from a floating periphyton mat that was collected from the Florida Everglades. By using classical taxonomic features such as morphology and dimensions of the filament, the isolate was identified as *Lyngbya birgei*.[5,6] However, based on the BLAST comparison of the 16 rRNA gene sequence, the closest relationship (93%) was found to be with a number of uncultured cyanobacteria and one strain of *Leptolyngbya*. Conflicting taxonomic identification of cyanobacteria, including the genus *Lyngbya*, is well known.[7] Apparently, the existing GeneBank database does not provide adequate identification of this isolate; therefore we maintain its identification as *Lyngbya* sp.

Lyophilized biomass was extracted with MeOH−$H_2O$ (4:1), and the residue was fractionated using a $C_{18}$ SPE column followed by reversed-phase HPLC. High-resolution ESIFTMS analysis of the major fraction, pahayokolide A (**1**), indicated a $[M + Na]^+$ ion at *m*/*z* 1494.748 (average of two scans with an internal standard) and a $[M + Na_2]^{2+}$ ion at *m*/*z* 758.870 (average of two scans with an internal standard). Less accurate MALDIFTMS (external standard) gave a value of 1494.751 Da. Subsequent MALDIFTMS of the same fraction from a culture grown in the presence of $Na^{15}NO_3$ (Figure 1) showed an isotope profile and masses consistent with complete incorporation and 13 $^{15}N$ atoms. Isotopic abundances identical to the profile expected without labeling (inset) are indicative of total rather than partial isotope incorporation. While 12 and 14 nitrogen atoms would constitute an error of only 1 atom in measuring heavy atom incorporation, these values are excluded by the nitrogen rule; the number of nitrogen atoms must be odd. The isotope profile was not suggestive of the presence of any sulfur. In addition, duplicate combustion analysis of pahayokolide A (**1**) indicated that sulfur was not present and gave a C/N ratio of 5.6:1.

On this basis, a computerized search of possible molecular formulas for *m*/*z* 1494.748 ± 0.004, having 50−120 carbons, 50−200 hydrogens, 10−26 oxygens, 0 sulfurs, 1 sodium, and 13 nitrogens, revealed exactly one hit, namely, $C_{72}H_{105}N_{13}O_{20}Na^+$ (calculated mass of 1494.7491). This expected mass agrees to within 0.002 Da (1 ppm) of the experimental values for the unlabeled and $^{15}N_{13}$-labeled pahayokolide A (**1**) [0.001 Da (ESI), 0.002 Da (MALDI), and 0.001 Da (MALDI, $^{15}N_{13}$)].

The $^1H$ and $^{13}C$ NMR (see Table 1), MALDIMS, and ESIMS data of pahayokolide A (**1**) indicated it to be a relatively large molecule of peptide origin. Amino acid analysis of **1** revealed the presence of seven proteinogenic amino acids: glycine, serine, threonine, phenylalanine, glutamic acid or glutamine, and two prolines. Edman sequencing of a partial digest gave the sequence Gln-Gly-Pro-Phe. A MALDITOFMS of the exhaustive acetylation product gave adduct-molecule ions of *m*/*z* 1705 $[M + Na^+]$ and 1721 $[M + K^+]$, indicating the presence of five hydroxyl and/or

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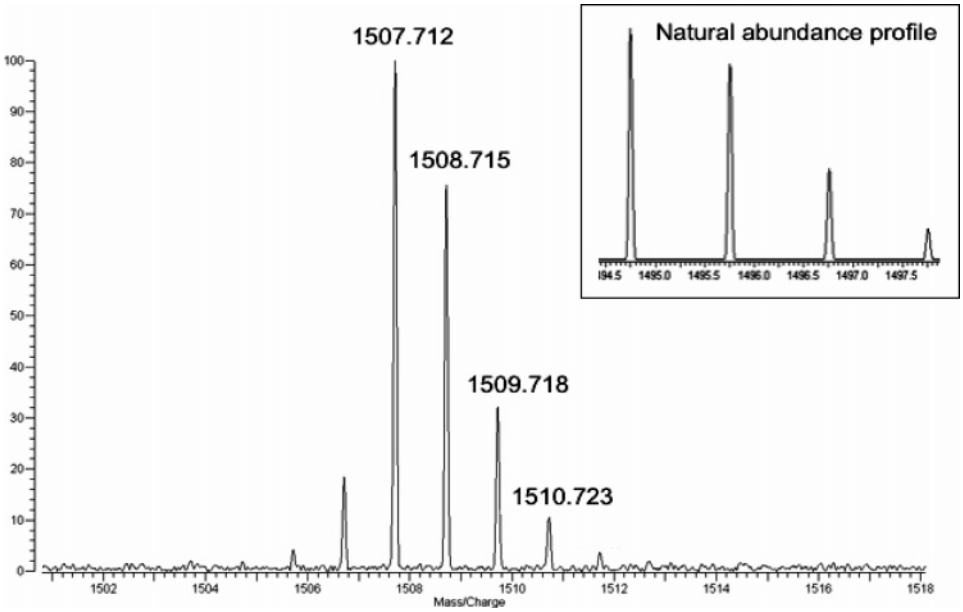


**Figure 1.** HRESIFTMS of pahayokolide A (**1**) enriched in $^{15}N$ and the corresponding isotope profile for a natural abundance sample (shown in the inset). The expected mass for the most abundant isotope for the $^{15}N_{13}$ species would be *m/z* 1507.711.

amino groups. One-dimensional (1D) proton and two-dimensional (2D) $^{1}H-^{15}N$ HSQC spectra of pahayokolide A (**1**) suggested that it exists in multiple, slowly exchanging conformations in methanol-$d_4$. In contrast, pahayokolide A (**1**) was present predominantly in a single conformation in a 3:7 mixture of DMSO-$d_6$ and $H_2O/D_2O$. The $^{1}H-^{15}N$ HSQC spectrum of pahayokolide A (**1**) obtained with $^{15}N$-labeled **1** in this solvent mixture showed nine prominent cross-peaks corresponding to backbone amide protons and two cross-peaks representing a side-chain amide group. Combined analysis of the 2D $^{1}H$ TOCSY, 2D $^{1}H$ COSY, $^{1}H-^{15}N$ HSQC, and 3D $^{1}H-^{15}N$ HSQC TOCSY NMR data confirmed the presence of all of the amino acids identified by amino acid analysis. The presence of a glutamine residue in pahayokolide A (**1**) was confirmed by the chemical shift of the $H^{\beta}$ and $H^{\gamma}$ resonances and the side-chain amide protons in the $^{1}H-^{15}N$ HSQC spectrum (see Supporting Information).

The $^{1}H$ NMR and $^{13}C$ NMR spectra of pahayokolide A (**1**) revealed an aromatic spin pattern in addition to that assigned to phenylalanine. 2D $^{1}H$ NOESY and 2D $^{13}C$ HMBC data were useful in the assignment of the other aromatic pattern to homo phenylalanine (homoPhe). 2D $^{1}H$ NOESY, 2D $^{13}C$ HMQC, and $^{13}C$ HMBC data, analyzed in conjunction, confirmed the presence of three additional nonstandard amino acid residues (in pahayokolide A, **1**), namely, *N*-acetyl-*N*-methyl leucine and two dehydrobutyrines (Dhb's). The geometry of the double bonds in the two Dhb moieties was established unambiguously on the basis of the NOE connectivity between the backbone amide proton and either the olefinic proton or the vinyl methyl group. The backbone amide proton resonance of one of the Dhb's [NH-21, at 9.38 ppm (see Supporting Information)] showed a NOE interaction with its olefinic proton (H-22, 5.91 ppm), suggesting the *E*-configuration. In a similar manner, the double bond of the other Dhb unit was assigned as the *Z*-configuration because its backbone amide proton (NH-28, 9.02 ppm) showed a strong cross-peak correlation with the allylic methyl group at 1.31 ppm.

The remainder of the molecule of pahayokolide A (**1**) was a $\beta$-amino acid unit possessing several oxygenated methines. Complete resonance assignment of the C-52 to C-63 fragment was established using the combined information content of the 2D $^{1}H$ COSY, 2D $^{13}C$ HMBC, and 2D $^{13}C$ HMQC spectra. The C-53 oxygenated methine (4.10 ppm, H-53) appeared as a doublet, indicating that it was directly coupled to only one other methine group. The 2D $^{1}H$ COSY data identified two spin patterns spanning C-53/C-58 and C-59/C-63 (Figure 2). The linkage of these two spin systems was established by 2D $^{13}C$ HMBC correlations between the H-59 methine (3.45 ppm) and C-58/C-57 (71.1 and 36.9 ppm) as well as correlations between the H-60 diastereotopic protons (1.25 and 1.19 ppm) and C-58 (71.1 ppm). Additional HMBC correlations confirmed the complete carbon−carbon linkage connecting all consecutive carbon atoms from C-52 to C-63 (Figure 2). This fragment comprised the 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid moiety (AThmU). A strong correlation was identified between the C-64 carbonyl carbon and the methine proton (H-56) in the 2D HMBC spectrum, confirming the connectivity between *N*-acetyl-*N*-methyl leucine and the oxygenated methine (C-56, Figure 2). The proton chemical shift for H-56 at 5.07 ppm supported the placement of the *N*-acetyl-*N*-methyl leucine on the C-56 hydroxyl.[8]

The 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid moiety (C-52 to C-63) is unusual and unprecedented. In addition, some spectroscopic overlap of the two isobutyl groups (AThmU and *N*-acetyl-*N*-methyl leucine) of **1** initially led to some ambiguities in the assignments. To provide additional support for this structural assignment, pahayokolide A (**1**) was treated with $NaIO_4$, in order to cleave the C-58/C-59 vicinal diol. HRFTMS analysis of the crude cleavage products revealed an ion at *m/z* 1406.660, assigned to the anticipated C-58 aldehyde. This value is within 1 ppm of the anticipated value ($C_{67}H_{93}N_{13}O_{19}Na$, *m/z* 1406.661).

Sequential resonance assignments of the polypeptide portion were accomplished based on the $^{\alpha}H-^{\alpha}H$ NOE connectivity observed in the 3D $^{1}H-^{15}N$ HSQC NOESY data collected using a doubly ($^{15}N$, $^{13}C$) labeled **1** (see Supporting Information). The spectroscopic resolution obtained from the 3D data helped in resolving some ambiguities in the resonances assigned using 2D NMR data. Analysis of the sequential NOE connectivity revealed the sequence of AThmU-Gln-Gly and Phe-(*Z*)Dhb-Ser-(*E*)Dhb-Thr-homoPhe. Combined with the partial peptide sequence obtained by automated Edman sequencing, the sequence of 10 amino acids was determined as AThmU-Gln-Gly-Pro(2)-Phe-(*Z*)Dhb-Ser-(*E*)Dhb-Thr-homoPhe. The HMBC relationship between the *N*-methine proton at $\delta$ 4.07 (H-54) and the carbonyl carbon of proline-1 at 173.0 ppm (C-1) suggested the connectivity of these two amino acid units. Several lines of evidence suggested a cyclic structure for the polypeptide chain: (i) **1** was amenable to Edman sequencing only after acid hydrolysis; (ii) the absence of significant fragmentation in the mass spectra; (iii) the degree of unsaturation calculated from the

**Table 1.** NMR Spectroscopic Data for Pahayokolides A (**1**) and B (**2**) in DMSO-$d_6$/$D_2O$ (3:7)

| | pahayokolide A (**1**) | | pahayokolide B (**2**) | |
|---|---|---|---|---|
| unit | $\delta_H$ (*J* in Hz)[a] | $\delta_C$ (or $\delta_N$) | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| Pro-1 | | | | |
| 1 | | 173.0, s | | 170.4, s |
| 2 | 4.18, m | 60.9, d | 4.21, m | 60.9, d |
| 3 | 2.19, m, 1.71, m | 29.6, t | 2.11, m, 1.65, m | 29.5, t |
| 4 | 1.85, m | 24.6, t | 1.81, m | 24.4, t |
| 5 | 3.45, m, 3.25, m | 47.6, t | 3.48, m, 3.23, m | 47.6, t |
| homophe | | | | |
| 6 | | 171.5, s | | 171.9, s |
| 7 | 4.37, m | 51.2, d | 4.35, t (3.5) | 51.5 d |
| 8 | 1.98, m | 32.0, t | 1.95, m | 31.6, t |
| 9 | 2.50, m | 31.2, t | 2.47, m | 31.2, t |
| | 2.65, dd (14.2, 7.2) | | 2.63 dd (14.2, 7.2) | |
| 10 | | 136.1, s | | 136.4, s |
| 11/15 | 7.19, d (7.8) | 128.9, d | 7.18, d (7.8) | 128.9, d |
| 12/14 | 7.32, t (7.8) | 129.2, d | 7.32, t (7.8) | 129.2, d |
| 13 | 7.22, t (7.8) | 126.7, d | 7.21, t (7.8) | 126.7, d |
| NH-7 | 8.09, s | 121.7[b] | | |
| Thr | | | | |
| 16 | | 171.9, s | | 171.8, s |
| 17 | 4.29, d (3.6) | 59.8, d | 4.28, d (2.4) | 59.3, d |
| 18 | 4.25, m | 67.2, d | 4.22, m | 67.5, d |
| 19 | 1.18, d (6.4) | 19.5, q | 1.14, d (6.4) | 19.5, q |
| NH-17 | 7.73, s | 113.7[b] | | |
| *E*-Dhb | | | | |
| 20 | | 166.4, s | | 166.4, s |
| 21 | | 128.6, s | | 129.2, s |
| 22 | 5.91, q (7.3) | 130.3, d | 5.91, q (7.3) | 131.4, d |
| 23 | 1.89, d (7.3) | 13.6, q | 1.89, d (7.3) | 13.7, q |
| NH-21 | 9.38, s | 129.1[b] | | |
| Ser | | | | |
| 24 | | 171.2, s | | 171.4, s |
| 25 | 4.32, t (4.2) | 56.9, d | 4.30, t (4.8) | 56.8, d |
| 26 | 3.93, dd (12.0, 4.2) | 61.5, t | 3.88, dd (12.0, 4.2) | 61.4, t |
| | 3.87, dd (12.0, 4.2) | | 3.83, dd (12.0, 4.2) | |
| NH-25 | 8.05, s | 112.7[b] | | |
| *Z*-Dhb | | | | |
| 27 | | 166.3, s | | 166.3, s |
| 28 | | 128.1, s | | 128.1, s |
| 29 | 6.61, q (7.1) | 134.9, d | 6.58, q (7.2) | 134.9, d |
| 30 | 1.31, d (7.1) | 13.1, q | 1.35, d (7.2) | 13.1, q |
| NH-28 | 9.02, s | 122.8[b] | | |
| Phe | | | | |
| 31 | | 172.4, s | | 172.6, s |
| 32 | 4.54, t (8.4) | 56.2, d | 4.52, t (8.4) | 56.1, d |
| 33 | 3.01, dd (14.0, 8.0) | 36.4, t | 3.12, d (8.4) | 36.4, t |
| | 3.05, dd (14.0, 8.0) | | | |
| 34 | | 140.8, s | | 140.8, s |
| 35/39 | 7.28, d (7.8) | 129.5, d | 7.29, d (7.8) | 129.5, d |
| 36/38 | 7.34, t (7.8) | 129.1, d | 7.33, t (7.8) | 129.0, d |
| 37 | 7.26, t (7.8) | 127.6, d | 7.28, t (7.8) | 127.6, d |
| NH-32 | 8.60, s | 121.5[b] | | |
| Pro-2 | | | | |
| 40 | | 174.3, s | | 174.3, s |
| 41 | 4.39, m | 60.4, d | 4.33, m | 60.4, d |
| 42 | 2.05, m, 1.82, m | 29.4, t | 2.06, m, 1.78, m | 29.4, t |
| 43 | 1.85, m | 24.5, t | 1.81, m | 24.3, t |
| 44 | 3.52, m | 47.0, t | 3.50, m | 47.0, t |
| Gly | | | | |
| 45 | | 169.1, s | | 169.1, s |
| 46 | 4.01, d, (15.4) | 41.9, t | 4.02, d, (15.2) | 42.0, t |
| | 3.96, d (15.4) | | 3.91, d (15.2) | |
| NH-46 | 8.13, s | 108.3[b] | | |

molecular formula requires an additional double-bond equivalent that cannot be accounted for by a linear structure. A NOESY cross-peak between the methylene protons at 2.50 and 2.65 ppm ($H_2$-9) and the proline-1 protons at 3.45 ppm ($H_2$-5) indicates that proline-1 should be connected with homoPhe, which led to the macrocyclic gross structure of **1**.

Additional evidence for the proposed structure was obtained from low-resolution (ESI ion trap) MS/MS taken from the protonated molecules. While sodium adduct ions were readily observed and used in the exact mass measurements, these ions often provide less structural specificity than protonated molecules. Deliberate acidification of the solutions was thus used to increase the contribution from the protonated molecules sufficiently for MS/MS product ion studies (see Supporting Information). The major ions from collision-induced dissociation of the protonated molecules can be explained by losses of combinations of three small molecules. The small molecules are water (18 Da), ammonia (17 Da), and an ion corresponding to a fragment from the proposed *N*-acetyl-*N*-methyl leucine side chain at C-56 ($C_9H_{15}NO_2$ or 169.1 Da). The specific losses were 18, 36 (18+18), 53 (18+18+17), 54 (18+18+18), 169,

**Table 1.** Continued

| | pahayokolide A (1) | | pahayokolide B (2) | |
|---|---|---|---|---|
| unit | $\delta_H$ (*J* in Hz)[a] | $\delta_C$ (or $\delta_N$) | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| Gln | | | | |
| 47 | | 173.3, s | | 173.7, s |
| 48 | 4.20, m | 53.2, d | 4.25, m | 53.0, d |
| 49 | 2.04, m, 1.91, m | 27.7, t | 2.06, m, 1.78, m | 27.7, t |
| 50 | 2.28, t (7.2) | 31.5, t | 2.26, t (7.8) | 31.2, t |
| 51 | | 177.4, s | | 177.4, s |
| NH-48 | 8.02, s | 120.0[b] | | |
| $NH_2$-51 | 7.50, s, 6.70, s | 111.8[b] | | |
| AThmU | | | | |
| 52 | | 172.8, s | | 173.2, s |
| 53 | 4.10, d (3.2) | 72.1, d | 4.14, d (3.6) | 73.0, d |
| 54 | 4.07, m | 48.8, d | 4.29, m | 49.4, d |
| 55 | 1.93, m, 1.71, m | 35.0, t | 1.89, m, 1.68, m | 32.1, t |
| 56 | 5.09, m | 69.8, d | 3.64, m | 64.6, d |
| 57 | 1.77, m, 1.52, m | 36.9, t | 1.50, m, 1.33, m | 38.4, t |
| 58 | 3.24, m | 71.1, d | 3.61, m | 71.4, d |
| 59 | 3.45, m | 72.6, d | 3.51, m | 72.6, d |
| 60 | 1.25, m, 1.19, m | 41.1, t | 1.18, m | 40.8, t |
| 61 | 1.64, m | 24.3, d | 1.66, m | 24.2, d |
| 62 | 0.84, d (6.6) | 23.4, q | 0.82, d (6.6) | 23.6, q |
| 63 | 0.89, d (6.6) | 21.6, q | 0.87, d (6.6) | 21.4, q |
| NH-54 | 7.81, s | 117.7[b] | | |
| *N*-met Leu | | | | |
| 64 | | 172.6, s | | |
| 65 | 5.03, dd (10.4, 4.8) | 55.4, d | | |
| 66 | 1.67, m | 36.8, t | | |
| 67 | 1.40,m | 24.8, d | | |
| 68 | 0.86, d (6.6) | 22.9, q | | |
| 69 | 0.90, d (6.6) | 21.2, q | | |
| 70 | 2.89, s | 32.8, q | | |
| Acyl | | | | |
| 71 | | 174.3, s | | |
| 72 | 2.1, s | 21.7, q | | |

[a] The NH NMR data were acquired in DMSO-$d_6$−$H_2O$ (3:7). [b] The chemical shift for N, acquired from 2D $^{15}N$,$^1H$-HSQC.

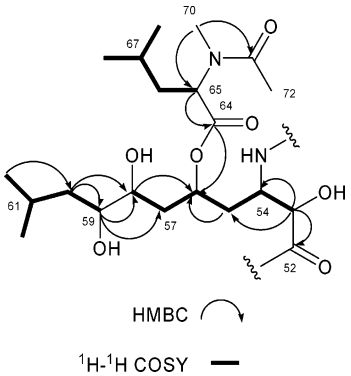


**Figure 2.** Key connectivities identified for the AThmU moiety by 2D $^{13}C$ HMBC and 2D $^1H$ COSY.

187 (169+18), 205 (169+36), 222 (169+53), and 223 (169+54) Da. Thus, loss of 169.1 Da was observed with and without accompanying losses of 18, 36, 53, and 54 Da. These MS/MS data are consistent with the cyclic nature of the structure, the proposed identity of the C-56 substituent, and the presence of at least three labile OH substituents and one $NH_2$ group.

Pahayokolide B (**2**) was obtained as a minor component of the mixture and was the most polar compound collected from the HPLC effluent. The amino acid analysis of pahayokolide B (**2**) was identical to that of **1**. Treatment of pahayokolide A (**1**) with mild base yielded pahayokolide B (**2**). This suggested that pahayokolide B may be derived from **1** by cleavage of an ester bond. HRESIFT-MS revealed a $[M + Na]^+$ ion at *m*/*z* 1325.640 ($z = 1$, average of two scans with internal standard), or 169.109 Da less than the calculated mass of pahayokolide A (**1**). This corresponds to a difference of $C_9H_{15}NO_2$. Hydrolysis of the *N*-acetyl-*N*-methyl leucine unit is consistent with these data. The molecular formula of pahayokolide B was assigned as $C_{63}H_{90}N_{12}O_{18}$. Comparison of the $^1H$ and $^{13}C$ NMR data of **1** and **2** indicated that both compounds share the same polypeptide−polyketide skeleton. The two compounds differ in that the signals of two carbonyls, one *N*-methyl and *N*-methine, one acetyl methyl, one methylene, and one isopropyl group that are present in pahayokolide A (**1**) are absent in pahayokolide B (**2**). This again suggested the loss of the *N*-acetyl-*N*-methyl leucine unit. In comparison with pahayokolide A (**1**), it was observed that the C-56 methine resonances in pahayokolide B (**2**) showed significant upfield shifts in both the $^1H$ and $^{13}C$ NMR spectra. The prominent upfield shifts supported the location of the *N*-acetyl-*N*-methyl leucine moiety on the C-56 hydroxyl of pahayokolide A (**1**). The proposed structure of pahayokolide B (**2**) was consistent with HMBC and COSY spectra (see Supporting Information).

The isolation of the cytotoxic pahayokolides from *Lyngbya* sp. 15-2 provides another example of the potential of cyanobacteria, particularly those of the genus *Lyngbya* or related genera, to yield novel secondary metabolites. This work also demonstrates the potential of cyanobacteria from the Florida Everglades to yield new and useful cyanobacteria. To the best of our knowledge, this is the first example of any secondary metabolite derived from an Everglades cyanobacterial isolate. Pahayokolides A (**1**) and B (**2**) may indeed be the largest cyclic peptides isolated from any cyanobacteria. Additionally, these compounds exhibit unusual structural features, including the pendent *N*-acetyl-*N*-methyl leucine moiety, not found in any cyanobacterial metabolite, and the unprecedented 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid moiety. The pahayokolides also share some features with other cyanobacterial metabolites. Dehydrobutyrines have been identified in several microcystin variants,[9−11] in nodularin,[12] and in nostocyclin.[13] Homophenylalanine has been previously identified in a microcystin variant,[14] and *N*-methylhomophenylalanine has been

previously identified in the cyanobacterial metabolite antillatoxin B.[15] *N*-Methylleucine is present in many secondary metabolites from bacteria and sponges, but only one from a cyanobacterium: the linear peptide microginin FR1, from *Microcystis* sp.[16] Investigations on the biosynthesis of the pahayokolides may be expected to unravel novel biosynthetic pathways.

## Experimental Section

**General Experimental Procedures.** Amino acid analysis and amino acid sequencing (Edman degradation) was performed at the Molecular Structure Facility at the University of California at Davis. Mass spectrometry experiments were preformed using either (1) an Ion Spec 9.4 T FTMS with MALDI (MALDI high-resolution FT-MS) or ESI (HRESIFTMS) ionization; (2) a Bruker Reflex III MALDI TOF (MALDI MS); or (3) a Bruker Esquire ion trap (ESI HPLC/MS, ESI flow injection (FIA) MS or ESI/MS/MS). In MALDI and high-resolution MALDI experiments either SA or HCCA was used as the MALDI matrix and data were acquired in the reflectron mode. In the LC/ESI experiments samples were analyzed by flow injection analysis and/or using a standard reversed-phase $C_{18}$ column (typically 2.1 mm $\times$ 50 mm) with a standard acetonitrile/water gradient system.

The measurement of exact mass values was done by ESI FIA directly into the 9.4 T FTMS. For the MS/MS experiments using FIA, ESI, and the ITMS, the samples were acidified with 0.1% FA rather than being mixed with the HP tune mix.

NMR spectroscopic data were acquired on a Bruker DMX-500 spectrometer equipped with a triple-resonance cryoprobe and triple-axis pulsed-field gradients or Bruker 400 or 600 MHz AVANCE spectrometers. The NMR data were processed with Topspin/XWIN-NMR and analyzed using Sparky software.[17] Proton frequencies were referenced directly to internal DSS at 0.00 ppm, while the heteronuclear dimensions were referenced indirectly on the basis of the gyromagnetic ratios.[18] Two-dimensional homonuclear experiments [$^1$H-TOCSY (mixing time 75 ms),[19] DQF-COSY,[20] water-gated NOESY (mixing time 300 ms)[21]] were acquired on unlabeled pahayokolide samples dissolved in 30% DMSO-$d_6$ + 70% $H_2O$. 2D $^1$H$-^{15}$N HSQC, $^{15}$N HSQC TOCSY (55 ms mixing time),[22] and $^{15}$N HSQC NOESY (150 ms mixing time) spectra were collected in 30% DMSO-$d_6$ + 70% $H_2O$. 2D $^1$H$-^{13}$C HMQC[23] and 2D $^1$H$-^{13}$C HMBC[24] were acquired in 30% DMSO-$d_6$ + 70% $D_2O$. 2D and 3D heteronuclear NMR experiments were acquired using double ($^{15}$N/$^{13}$C) labeled pahayokolide samples (>1 mM). $^{15}$N or $^{13}$C decoupling was achieved using appropriate decoupling pulse schemes.

**Microbial Material.** *Lyngbya* sp. strain 15-2 was isolated from the floating periphyton mat in the Florida Everglades as previously described.[25] The organism has straight, unbranched trichomes, 20 $\mu$m in diameter, with rounded apexes enclosed in a yellowish-brown sheath.

**Purification of Pahayokolides A (1) and B (2).** Pahayokolide A (**1**) was purified as detailed by Berry et al.[3]

**Pahayokolide A (1):** white, amorphous powder; $[\alpha]^{25}_D$ −18 (*c* 0.0015, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 201 (1.78) nm; $^1$H and $^{13}$C NMR data, see Table 1; HRESIFTMS *m/z* $[M + Na]^+$ 1494.748 (calcd for $C_{72}H_{105}N_{13}O_{20}Na$, 1494.7507); *anal.* 56.56% C, 7.18% H, 11.77% N, 0% S, calcd for $C_{72}H_{105}N_{13}O_{20}\cdot 2H_2O$, 56.44% C, 6.91% H, 11.89% N.

**Pahayokolide B (2):** white, amorphous powder; $[\alpha]^{25}_D$ −20 (*c* 0.001, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 201 (1.79) nm; $^1$H and $^{13}$C NMR data, see Table 1; HRESIFTMS *m/z* $[M + Na]^+$ 1325.640 (calcd for $C_{63}H_{90}N_{12}O_{18}Na$, 1325.639)

**Preparation of $^{13}$C-Labeled Pahayokolides.** *Lyngbya* sp. strain 15-2 was cultured as described above using $Na_2{}^{13}CO_3$. From 2.77 g of dried biomass, 12.5 mg (0.45%) of pahayokolide A (**1**) was isolated.

**Preparation of $^{15}$N-Labeled Pahayokolides.** *Lyngbya* sp. strain 15-2 was cultured as described above using $Na^{15}NO_3$. From 2.05 g of dried biomass, 9.6 mg (0.47%) of pahayokolide A (**1**) was isolated.

**Preparation of $^{13}$C and $^{15}$N Doubly Labeled Pahayokolides.** *Lyngbya* sp. strain 15-2 was cultured as described above using $Na_2{}^{13}CO_3$ and $Na^{15}NO_3$ (75% $^{15}$N). From 2.20 g of dried biomass, 11.7 mg (0.53%) of pahayokolide A (**1**) was isolated.

**Preparation of Pahayokolide B (2) from Pahayokolide A (1).** Pahayokolide A (**1**) (1 mg) was dissolved in MeOH (1 mg/mL) and added to pH 10.0 buffer (1 mL). The mixture was left overnight at room temperature and monitored by HPLC (20 mM $NH_4OAc-CH_3CN$, 70:30). Pahayokolide B (**2**) was purified by reversed-phase HPLC as described above and was obtained in nearly quantitative yield.

**Exhaustive Acetylation of Pahayokolide A (1).** Pahayokolide A (**1**) (300 $\mu$g) and acetic anhydride (45 $\mu$L) were dissolved in $CH_2Cl_2$ at 0 °C. DMAP (50 $\mu$g) and triethylamine (65 $\mu$L) were added sequentially. The mixture was stirred overnight and quenched with sodium bicarbonate solution, extracted three times with ethyl acetate, washed with brine, dried over $Na_2SO_4$, filtered, and concentrated.

**Periodate Oxidation of Pahayokolide A (1).** Sodium periodate-saturated water (1 mL) was added to a solution of pahayokolide A (**1**) (500 $\mu$g) in MeOH (0.5 mL) at 0 °C. The solution was stirred at 0 °C for 1 h and quenched with excess ethylene glycol. The mixture was poured into brine and extracted with EtOAc. The extract was dried by evaporation. The residue was dissolved in MeOH and applied to a 1 g $C_{18}$ SPE cartridge. The cartridge was washed with water and eluted with MeOH.

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**Supporting Information Available:** 1D $^1$H and $^{13}$C NMR, 2D $^1$H COSY, and 2D $^{13}$C$-^1$H HMQC and HMBC spectra of pahayokolides A (**1**) and B (**2**) and 2D $^{13}$C$-^1$H TOCSY and NOESY, $^1$H$-^{15}$N HSQC, 3D $^1$H$-^{15}$N HSQC NOESY, $^1$H$-^{15}$N HSQC-TOCSY, and ESIMS/MS for pahayokolide A (**1**) are available free of charge via the Internet at http://pubs.acs.org.

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