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NOTE

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Biosynthetic Origin of the 3-Amino-2,5,7,8-tetrahydroxy-10-methylundecanoic Acid Moiety and Absolute Configuration of Pahayokolides A and B

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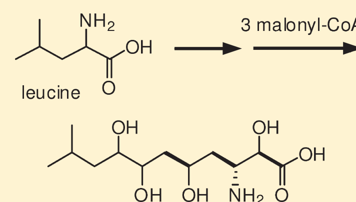
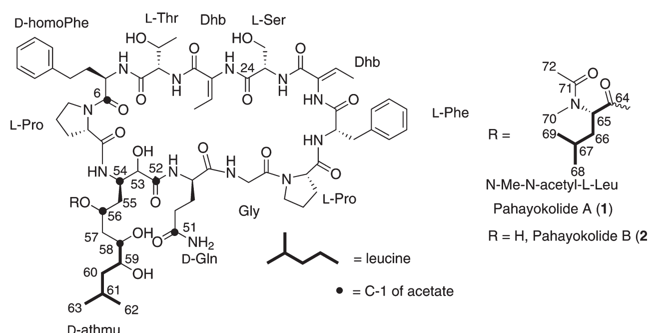
S Supporting Information

ABSTRACT: Pahayokolides A (**1**) and B (**2**) are cyclic undecapeptides that were isolated from the cyanobacterium *Lyngbya* sp. They contain the unusual α-hydroxy-β-amino acid 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid (Athmu). The absolute configurations of the amino acids of the pahayokolides, except for the four oxygen-bearing stereocenters of Athmu, have been determined by Marphy's method. Incorporation of labeled leucine and acetate precursors into the pahayokolides has established that Athmu is derived from a leucine or α-keto isocaproic acid starter unit, which is further extended with three acetate units.



Cyanobacteria have proven to be a rich source of biologically active secondary metabolites.[1,2] Natural product classes isolated from cyanobacteria include ribosomal and nonribosomal peptides, mixed nonribosomal peptides, polyketides, and alkaloids.[3,4] The Florida Everglades is an oligotrophic marsh and an abundant source of diverse species of cyanobacteria. As a potential source of secondary metabolites however, the Florida Everglades remain largely unexplored. We have previously reported studies on the planar structure,[5] isolation,[6] and cytotoxicity[7] of two related cyclic peptides, pahayokolides A (**1**) and B (**2**), which are produced by a *Lyngbya* sp., isolated from the Florida Everglades. Pahayokolides A and B contain the same cyclic undecapeptide core, and pahayokolide A contains a pendant *N*-acetyl-*N*-methylleucine moiety, which is absent in pahayokolide B. The pahayokolides are remarkably similar in structure to tychonamides A and B[8] (**4** and **5**), schizotrin A[9] (**3**), portoamides A and B[10] (**6**), and lyngbyazothrins[11] (Figure 1), cyclic peptides isolated from *Tychonema* sp., *Schizothrix* sp., and *Oscillatoria* sp., respectively. The pahayokolides, schizotrin A, and the portoamides and lyngbyazothrins[11] have an unusual β-amino acid, 3-amino-2,5,7,8-tetrahydroxy-10-methylundecanoic acid (Athmu). The tychonamides possess a similar β-amino acid, 3-amino-2,5,7-trihydroxy-8-phenyloctanoic acid (Atpoa). Like pahayokolide A, tychonamides A and B and portoamide contain a pendant *N*-acetyl-*N*-methylleucine connected via an ester linkage to the 5-hydroxy group of the β-amino acid, whereas the appendage on schizotrin A is an *N*-butyryl-*N*-methylalanine. Herein we report the absolute configuration of the amino acids of pahayokolide A (**1**) and the results of stable isotope incorporation experiments to determine the precursors of the Athmu moiety.

The absolute configurations of the common amino acids and homophenylalanine were determined by Marfey's HPLC method[12] and the advanced Marfey's method[13] by comparison to authentic D and L standards derivatized with L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide). The assignments are as follows: L-proline (×2), L-phenylalanine, L-serine, L-threonine, D-homophenylalanine, D-glutamine, and *N*-methyl-L-leucine. In the case of *N*-methylleucine only the L isomer was commercially available. Comparison to the diastereomeric derivatives of *N*-methyl-L-leucine prepared with both L-FDLA and D-FDLA by the advanced Marfey's method showed the presence of *N*-methyl-(D/L)-leucine. However, the D/L ratio was lower under milder hydrolysis conditions, and we conclude that the D isomer arose from partial racemization of *N*-methyl-L-leucine. A similar observation was made for the tychonamides.[8] The configuration at C-54 is tentatively assigned as *R* (or D). This assignment is based on relative retention times of the diastereomeric Athmu derivatives, assuming that D-FLDA-D-Athmu (which is equivalent to L-FLDA- L-Athmu) < L-FDLA-D-Athmu.[13]



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1	Pro	Hphe	Thr	Dhb	Ser	Dhb	Phe	Pro	Gly	Gln	Athmu	[N-Ac-N-Me-Leu]
3	Pro	O-MeHty	Ser	Dhb	Ser	Val	Phe	Pro	Gly	Gln	Athmu	[N-Ac-N-Me-Leu]
4	Pro	O-MeHty	Thr	Dhb	Ser	Ile	Pro	Pro	Gly	Gln	Atpoa	[N-Ac-N-Me-Leu]
5	Pro	Hphe	Thr	Dhb	Ser	Ile	Pro	Pro	Gly	Gln	Atpoa	[N-Ac-N-Me-Leu]
6	Pro	O-MeHty	Thr	Dhb	Ser	Ile	Pro	Pro	Gly	Gln	Athmu	[N-Ac-N-Me-Tyr]

Figure 1. Alignments of the core amino acids of pahayokolide A (**1**), schizotrin A (**3**), tychonamides A and B (**4** and **5**), and portoamides (**6**). (The portoamides and the lyngbyazothrins are identical compounds isolated from different sources.)

Assignments of ^{13}C NMR signals and the results of isotope incorporations into the Athmu and *N*-methyl-*N*-acetyl leucine subunits of pahayokolides A (**1**) and B (**2**) are presented in Table 1. The first 11 carbons in Table 1 include Athmu, its attached side chain and are those that we anticipated would be enriched by one or more of the following feeding experiments. Because there is significant spectral overlap and the presence of several, slowly interconverting conformers of pahayokolide A (**1**), leading to multiple overlapping peaks in the ^{13}C NMR spectrum, we chose for comparison only those ^{13}C signals that are well resolved, always present as a single peak, and which we did not expect to be enriched. These are the last eight carbons in Table 1. The ^{13}C NMR spectrum (DMSO-d_6/D_2O) of **1** derived from [1-^{13}C]leucine fed cultures showed significant enrichment at C-58 and C-64,[14] while doubly labeled [1,2-^{13}C]leucine showed enrichment at C-58, C-59, C-64, and C-65 as well as the anticipated ^{13}C—^{13}C coupling. Because of the presence of multiple conformers of **1**, the *N*-methyl-*N*-acetyl leucine of [1-^{13}C]acetate-labeled **1** was cleaved by treatment with dilute base to provide pahayokolide B (**2**) as previously described.[5] The ^{13}C NMR spectrum (CD_3OD/D_2O) derived from [1-^{13}C]acetate-fed cultures showed enrichment at C-6, C-51, C-52, C-54, C-56, and C-58. Not shown in Table 1 are the results of feeding experiments with [1-^{13}C]valine, which failed to yield enrichment at any position.

β-Amino acids are not uncommon in cyanobacterial peptides and include Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid) in the microcystins[16] and nodularin,[17] Ahoa (3-amino-2,5-dihydroxy-8-phenyloctanoic acid) in nostophycin,[18] and Ahda (3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid) in scytonemin A.[19] The Adda moiety of the microcystins has been shown to be of mixed peptide and polyketide origin. Stable isotope feeding experiments demonstrated that the biosynthesis of the Adda side chain begins with a phenyalanine starter unit, which is extended by four rounds of condensation with malonate.[20] Thus, four PKS (polyketide synthase) modules are involved in the biosynthesis of the polyketide amino acid Adda.[21]

Inspection of the Athmu residue of the pahayokolides suggests a mixed peptide and polyketide origin as well. We anticipated that the starter unit for the biosynthesis of the Athmu side chain could be valine, leucine, or the corresponding α-keto acids: α-ketoisovaleric acid or α-ketoisocaproic acid. Leucine or α-ketoisocaproic acid seemed the more likely starter unit, as this could be further extended by three rounds of condensation with malonate units (Figure 2). Mehner et al. proposed a similar polyketide pathway for the α-hydroxy-β-amino acids Ahda, Ahoa, and Atpoa,[8] and Leao et al.[10] proposed a similar pathway for Athmu. Alternatively, Leao et al.[10] suggested that the starter unit might be acetate, which is then doubly methylated at C-2. Our data are consistent with the first hypothesis. The anticipated enrichment of C-58 upon feeding of [1-^{13}C]leucine as well as enrichment at C-58 and C-59 and ^{13}C—^{13}C coupling upon feeding of [1,2-^{13}C]leucine was observed. Enrichment at C-52, C-54, and C-56 of **2** from [1-^{13}C]acetate-fed culture demonstrates the extension of leucine with three acetate units. The enrichment of C-58 in **2** from

Table 1. Isotopic Enrichment Based on ^{13}C NMR for Pahayokolides A (1) and B (2)[a]

position	δ_C (ppm) pah A (**1**)[b]	% enrichment [1-^{13}C]leucine pah A (**1**)	% enrichment [1,2-^{13}C]leucine pah A (**1**)	δ_C (ppm) pah B (**2**)[c]	% enrichment [1-^{13}C]acetate pah B (**2**)
52	173.0	125	90	173.0	**235**
53	72.9	112	133	73.1	84
54	48.8	104	73	49.5	**193**
56	70.1	67	61	64.7	**211**
57	36.9	108	94	36.3	99
58	71.4	**332**	**410**	71.9	**197**
59	72.4	100	**260**	72.6	96
60	41.1	94	86	39.2	98
64	172.4	**436**	**311**		
65	55.4	90	**379**		
71	174.4	105	52		
2	60.9	78	118	60.7	85
6	171.6	109	95	171.4	**193**
13	126.7	111	99	126.7	116
18	67.4	112	125	67.4	126
24	171.3	103	91	170.8	103
44	47.0	88	129	46.5	141
51	176.7	136	110	176.7	**220**
70	32.8	98	97		

[a] Carbon resonances were integrated relative to the signal for C-24. Enriched signals are shown in bold. [b] DMSO-d_6/D_2O. [c] CD_3OD/D_2O. Enrichment was calculated using the integral values for carbons shown in this table and applying the method described by Sitachitta.[15] Average isotopic enrichment for carbons 2—70 above (excluding signals in bold) = 101 ± 20.

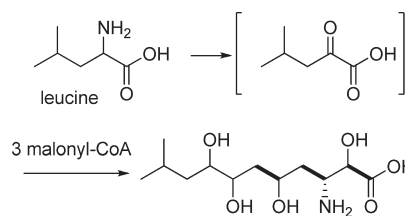

Figure 2. Proposed biogenic pathway for the β-amino acid Athmu.

[1-^{13}C]acetate-fed cultures as well as other sites of enrichment (C-51 and C-6) can be easily rationalized. The incorporation of [1-^{13}C]acetate at C-58 occurs via the condensation of acetate with α-ketoisovalerate during the biosynthesis of leucine. Enrichment at C-51 occurs via the condensation of [1-^{13}C] acetate with oxaloacetate during the biosynthesis of α-ketoglutarate to produce glutamate. Through a similar mechanism, incorporation at C-6 results from the biosynthesis of homophenylalanine from phenylalanine.[22,23] Clearly the Athmu moiety is derived from leucine and three units of acetate.

■ EXPERIMENTAL SECTION

General Experimental Procedures. One-dimensional high-resolution ^{13}C NMR data for pahayokolide A (**1**) were acquired in 30:70 DMSO-d_6/D_2O using a Bruker Avance 400 MHz spectrometer (Bruker

Biospin, Inc.) operating at 100 MHz for ^{13}C. One-dimensional high-resolution ^{13}C NMR spectra for pahayokolide B (**2**) were acquired in 50:50 CD_3OD/D_2O on a Bruker Avance II 700 MHz spectrometer equipped with a TCI cryoprobe operating at 176 MHz for ^{13}C. All data were collected at a temperature of 298 K. Chemical shifts were referenced to the deuterium CD_3 lock reference at 3.30 ppm using an absolute referencing scheme based on nuclear gyromagnetic constant ratios; this gives the center peak of the ^{13}C CD_3OD septet a chemical shift of 47.603 ppm. The NMR data were processed with Topspin software. All mass spectra were acquired on a single-quadrupole mass spectrometer (ThermoQuest Finnigan Navigator) in ESI negative ion mode. High-performance liquid chromatography (HPLC) was performed using a Thermo Finnigan Spectra System HPLC (model P4000 pump; model AS3000 autosampler; model UV6000LP PDA UV detector) with an Apollo C18 (250 × 4.6 mm i.d., 5 μm, Alltech) column. Stable isotope precursors were purchased from Cambridge Isotope Laboratories.

Culture of *Lyngbya* sp. *Lyngbya* sp. strain 15-2 was maintained in 2 L cultures in BG11 medium, buffered with 2-(*N*-morpholino)-ethanesulfonic acid buffer (2.6 mM) at pH 7.2. Cultures were supplemented with 150 mg of ^{13}C-labeled L-leucine (50 mg each on days 30, 34, and 38) or 1.3 g of ^{13}C-labeled sodium acetate (430 mg each on days 30, 34, and 38). Cultures were harvested after six weeks of growth. Pahayokolides A (**1**) and B (**2**) were isolated from the biomass as previously described.[5]

Preparation of FDLA Derivatives. To 40 μL of a 2 μM solution of each amino acid standard was added 20 μL of 1 M sodium bicarbonate and 80 μL of 1% (w/v) L- or D-FDLA in acetone as previously described.[12,13] After incubating at 40 °C for 60 min the reactions were quenched by the addition of 10 μL of 2 M HCl and stored at 4 °C. As *N*-Me-D-Leu was not available, the D-FDLA derivative of *N*-Me-L-Leu was used as a standard in lieu of its enantiomer, the L-FDLA-*N*-Me-D-Leu derivative. A 100 μg amount of pahayokolide A was hydrolyzed at 110 °C for 14 h with 500 μL of 4 M HCl. This solution was divided into two portions and dried under a stream of N_2. Each portion was derivatized with either L- or D-FDLA as described above and stored at 4 °C.

HPLC/PDA Conditions. The mobile phase used for the separation of the L- and D,L-FDLA derivatives of pahayokolide A was CH_3CN/0.01 M TFA, step gradient [4:6 for 24 min; ramp to 1:1 from 24 to 34 min; 7:3 after 34 min] at a flow rate of 0.4 mL/min. The FDLA derivatives were detected with a PDA UV detector at 340 nm. The retention times for the L-FDLA derivatives of *N*-Me-L-Leu and L-homoPhe were very close, at 47.3 and 47.7 min, respectively. In separate experiments, the L-FDLA-derivatized pahayokolide A hydrolysate was spiked with the L-FDLA derivatives of *N*-Me-L-Leu or L-homoPhe, confirming the presence of *N*-Me-L-Leu and the absence of L-homoPhe. Similarly, the retention times for the L-FDLA derivatives of D-Phe and *N*-Me-D-Leu were very close, at 50.0 and 50.2 min, respectively. In separate experiments, the L-FDLA-derivatized pahayokolide A hydrolysate was spiked with the L-FDLA-D-Phe derivative and the D-FDLA-*N*-Me-L-Leu derivative, confirming the presence of *N*-Me-D-Leu and the absence of D-Phe. When pahayokolide A was hydrolyzed in 6 M HCl, the L:D ratio of *N*-Me-Leu was 2.75:1. However, hydrolysis of pahayokolide A in 4 M HCl resulted in a L:D ratio of 5.5:1. We conclude that the *N*-Me-D-Leu arose from epimerization of *N*-Me-L-Leu during hydrolysis. A similar observation was made for the tychonamides.[8] The FDLA derivative of Athmu was observed only when the hydrolysis was performed in 4 M HCl. When the hydrolysis was carried out in 6 M HCl, a derivative having a molecular ion at m/z 668 was observed, corresponding to a loss of water from the FDLA-Athmu derivative. Either one of the alcohols is dehydrated to an alkene or the lactone is formed under these conditions.

ESI LC/MS Conditions. The sample probe was set at 400 °C and 4 kV with an entrance cone voltage of 10 V. Chromatographic conditions were as described for the HPLC-PDA analysis with a flow rate of 0.5 mL/min.

■ ASSOCIATED CONTENT

Supporting Information. Marfey's analysis of pahayokolide A and ^{13}C NMR spectra for natural abundance pahayokolides A and B, ^{13}C-enriched pahayokolide A [1-^{13}C]leucine, [1,2-^{13}C]leucine, and ^{13}C-enriched pahayokolide B, [1-^{13}C]acetate. This material is available free of charge via the Internet at http://pubs.acs.org.

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