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Calothrixamides A and B from the Cultured Cyanobacterium *Calothrix* sp. UIC 10520

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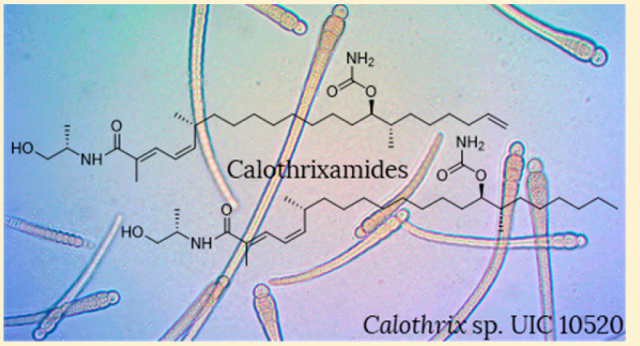
Supporting Information

ABSTRACT: Cyanobacteria are a source of chemically diverse metabolites with potential medicinal and biotechnological applications. Rapid identification of compounds is central to expedite the natural product discovery process. Mass spectrometry has been shown to be an important tool for dereplication of complex natural product samples. In addition, chromatographic separation and complementary spectroscopic analysis (e.g., UV) can enhance the confidence of the dereplication process. Here, we applied a droplet-liquid microjunction-surface sampling probe (droplet probe) coupled with UPLC-PDA-HRMS-MS/MS to identify two new natural products *in situ* from the freshwater strain *Calothrix* sp. UIC 10520. This allowed us to prioritize this strain for chemical investigation based on the presence of new metabolites very early in our discovery process, saving both time and resources. Subsequently, calothrixamides A (**1**) and B (**2**) were isolated from large-scale cultures, and the structures were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry. The absolute configurations were determined by a combination of chemical degradation reactions, derivatization methods (Mosher's, Marfey's, and phenylglycine methyl ester), and *J*-based configurational analysis. Calothrixamides showed no cytotoxic activity against the MDA-MB-435, MDA-MB-231, and OVCAR3 cancer cell lines. They represent the first functionalized long-chain fatty acid amides reported from the *Calothrix* genus and from a freshwater cyanobacterium.



Cyanobacteria continue to be a promising source of natural products with great chemical diversity.[1,2] Many cyanobacterial metabolites have been shown to have important ecological functions[3] or therapeutically relevant activities, such as antiproliferative, antimicrobial, anti-inflammatory, enzyme inhibition, and antiviral activities.[4,5] Bioassay-guided fractionation has been used traditionally to discover new bioactive compounds from various natural sources, including cyanobacteria. More recently, omics-based approaches are expanding the field of natural product discovery.[6]

Genomic and metagenomic studies constantly reveal the untapped biosynthetic potential of microorganisms. Genome mining combined with gene activation in native hosts and expression in heterologous hosts have been shown to be powerful strategies for the discovery of natural products.[7,8] Complementarily, metabolomics is used to detect the actual production of metabolites under specific conditions.[9] Biological samples represent a chemical mixture of many compounds in varying concentrations, whose analysis requires sensitive techniques with high resolving power and dynamic range. In this context, mass spectrometry (MS) has been established as an important tool in the natural product discovery workflow.[10−12] For complex mixtures, the resolving power of MS can be further increased by hyphenation with chromatographic separation. In addition, high-resolution mass spectrometry (HRMS) and tandem mass spectrometry (MS/MS) can be used to identify and dereplicate natural products.[13,14]

Dereplication is a vital step for modern natural product discovery platforms, and applying it as early as possible can greatly expedite the discovery process.[15] For instance,

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Table 1. NMR Spectroscopic Data of Calothrixamides A (1) and B (2), $CDCl_3$[a]

	calothrixamide A (1)				calothrixamide B (2)			
position	δ_C, mult	δ_H, mult (J in Hz)	COSY	HMBC	δ_C, mult	δH, mult (J in Hz)	COSY	HMBC
1	170.1, C				170.1, C			
2	128.9, C				128.9, C			
3	129.8, CH	7.26, ddq (11.8, 1.3, 0.9)	4, 25	1, 2, 4, 5, 25	129.8 CH	7.26, ddq (11.8, 1.3, 0.9)	4, 25	1, 2, 4, 5, 25
4	122.1, CH	6.18, ddd (11.8, 10.9, 0.9)	3, 5	2, 5, 6	122.1, CH	6.18, ddd (11.8, 10.9, 0.9)	3, 5	2, 5, 6, 25
5	145.6, CH	5.54, t (10.6)	4, 6	1, 3, 6, 7, 24	145.6, CH	5.54, t (10.6)	4, 6	3, 6, 7, 24
6	32.7, CH	2.73, m	5, 7, 24	4, 5, 7, 8	32.7, CH	2.73, m	5, 7, 24	4, 5, 7, 8
7	37.4, CH_2	1.22, m	6		37.4, CH_2	1.22, m	6	
		1.34, m				1.34, m		
8	27.5, CH_2	1.22, m			27.5, CH_2	1.22, m		
9	29.8, CH_2	1.24, m			29.8, CH_2	1.24, m		
10	29.6, CH_2	1.24, m			29.6, CH_2	1.24, m		
11	29.6, CH_2	1.24, m			29.6, CH_2	1.24, m		
12	29.7, CH_2	1.24, m			29.7, CH_2	1.24, m		
13	25.8, CH_2	1.25, m			25.8, CH_2	1.25, m		
		1.29, m				1.29, m		
14	31.6, CH_2	1.46, m	15	13, 15	31.6, CH_2	1.46, m	15	13,15
		1.51, m				1.51, m		
15	78.5, CH	4.66, dt (8.7, 4.3)	14, 16	13, 16, 17, 23, −OCONH2	78.6, CH	4.66, dt (8.7, 4.3)	14, 16	13, 16, 17, 23, $-OCONH_2$
16	36.5, CH	1.61, m	15, 17, 23	17, 23	36.5, CH	1.61, m	15, 17, 23	15, 17, 18, 23
17	32.8, CH_2	1.11, m	16	15, 16, 18, 23	32.9, CH_2	1.10, m	16	15, 16, 18, 23
		1.38, m				1.37, m		
18	26.8, CH_2	1.35, m			27.3, CH_2	1.26, m		
		1.37, m				1.32, m		
19	29.3, CH_2	1.37, m			29.7, CH_2	1.24, m		
		1.40, m						
20	33.9, CH_2	2.03, m	21	18, 19, 21,	32.0, CH_2	1.25, m		
		2.04, m		22				
21	139.2, CH	5.80, ddt (17.1, 10.2, 6.7)	20, 22	19, 20	22.8, CH_2	1.29, m	22	20, 22
22	114.4, CH_2	4.93, ddt (10.2, 2.4, 1.2)	20, 21	20, 21	14.3, CH_3	0.87, t (7.1)	21	20, 21
		4.99, ddt (17.1, 2.1, 1.6)						
23	14.5, CH_3	0.87, d (6.8)	16	15, 16, 17	14.5, CH_3	0.86, d (6.8)	16	15, 16, 17
24	21.3, CH_3	0.98, d (6.6)	6	5, 6, 7	21.3, CH_3	0.98, d (6.6)	6	5, 6, 7
25	12.9, CH_3	1.95, d (1.3)	3	1, 2, 5	12.9, CH_3	1.95, d (1.3)	3	1, 2
1′	48.3, CH	4.16, m	2′, 3′, −NH−	2′, 3′	48.3, CH	4.16, m	2′, 3′, −NH−	2′, 3′
2′	67.6, CH_2	3.58, ddd (10.9, 6.2, 4.9)	1′, −OH	1′, 3′	67.6, CH_2	3.58, ddd (10.9, 6.2, 4.8)	1′, −OH	1′, 3′
		3.72, ddd (10.9, 6.0, 3.5)				3.72, ddd (10.9, 5.8, 3.5)		
3′	17.3, CH3	1.24, d (6.9)	1′	1′, 2′	17.3, CH_3	1.24, d (6.9)	1′	1′, 2′
−OH		3.17, dd (5.9, 4.9)	2′			3.18, dd (5.7, 4.8)	2′	
−NH−		5.90, d (7.0)	1′	1, 1′		5.91, d (7.0)	1′	1, 1′
$-OCONH_2$	157.4, C	4.61, br			157.4, C	4.61, br		

[a]Frequency of 900 MHz for ^1H and 226 MHz for ^{13}C.

dereplication of microbial samples *in situ* can significantly reduce the time spent in sample preparation, with the additional benefit of providing information about the spatial distribution of metabolites. Studies have investigated the use of ambient ionization MS techniques, such as desorption electrospray ionization (DESI) and direct analysis in real time (DART), for the dereplication of microbial natural products.[16,17] With a broad use in metabolite imaging, ambient ionization MS techniques display two major disadvantages for dereplication: absence of chromatographic separation and lack of mutually supportive spectroscopic data, e.g., UV. Chromatography allows for the determination of retention time and identification of isomers, while additional spectroscopic data increase the confidence of the dereplication process. The droplet-liquid microjunction-surface sampling probe (droplet probe) combines the advantages of *in situ* analysis with the capabilities of chromatography hyphenated to analytical techniques.[18] In this study, we used a droplet probe coupled to a UPLC-UV-HRMS-MS/MS system to dereplicate cyanobacterial compounds *in situ*.

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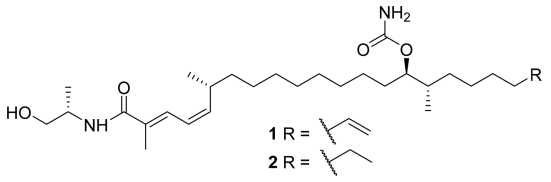
Previously used to analyze human tissue,[19] plant samples,[20] and fungal cultures,[18,21−25] the droplet probe has been shown to be a robust tool for analyzing chemistry *in situ*, and it has the advantages of requiring little, if any, sample preparation and can be applied to irregular surfaces. In short, it consists of a sampling needle that is positioned millimeters above the surface to be sampled. It dispenses a droplet of solvent that forms a microjunction with the sample surface, performing a microextraction, which is directed to the analytical system. Using this technique, two new compounds, calothrixamides A (**1**) and B (**2**), were identified in the cyanobacterial strain *Calothrix* sp. UIC 10520.



RESULTS AND DISCUSSION

Strain UIC 10520 was identified to be a *Calothrix* sp. by morphological characterization and phylogenetic analyses using a partial 16S rDNA gene sequence (see Experimental Section and Supporting Information S1 and S2). The strain was grown on solid Z medium and was analyzed by droplet probe UPLC-UV-HRMS-MS/MS. This revealed the presence of two compounds of m/z values of 515.3817 and 517.3975 ($[M + Na]^+$), consistent with the formulas $C_{29}H_{52}N_2O_4$ and $C_{29}H_{54}N_2O_4$, respectively. For both compounds, losses of $-CH_3NO_2$ and $-C_3H_9NO$ were detected in the MS^2 spectra (Supporting Information S3). Preliminary dereplication of these chemistry data, all of which were acquired *in situ*, failed to match this information with any known cyanobacterial compound, inspiring us to proceed with the isolation and characterization of these new chemical entities. Traditionally, we spend 6−8 weeks growing 8 L cultures, which are then lyophilized, extracted, and fractionated before we select strains for natural products chemistry investigations.[26] Using the *in situ* analysis afforded by the droplet probe, we can detect the presence of new compounds much earlier in the process and prioritize these for evaluation.[1,27] For this study we used 4-week-old agar plates. However, it is worth noting that droplet probe analysis of cyanobacteria can be performed after 10 to 15 days or as soon as there is microscopic growth on agar plates.

Cell material from an 18 L culture of *Calothrix* sp. UIC 10520 was harvested, lyophilized, and extracted with CH_2Cl_2/MeOH, 1:1. The concentrated extract was fractionated using Diaion HP-20SS and a stepwise gradient of isopropyl alcohol (IPA) and H_2O, followed by automatic fractionation by RP-HPLC. The HPLC fractions containing the target compounds were analyzed by ^{1}H NMR, which further supported that the compounds were new. Compound purification by semi-preparative RP-HPLC yielded calothrixamides A (**1**) and B (**2**).

Calothrixamide A (**1**) was obtained as a pale orange oil. HRMS data in both positive and negative modes (m/z 515.3817 $[M + Na]^+$ and m/z 491.3857 $[M - H]^-$) were consistent with the formula $C_{29}H_{52}N_2O_4$, implying 5 degrees of unsaturation. The DEPTQ spectrum contained three non-protonated carbons, eight methine, 14 methylene, and four methyl carbons, accounting for all 29 carbons suggested by the molecular formula. Investigation of the ^{1}H NMR and multiplicity-edited HSQC spectra (Table 1) revealed six olefinic protons (methine δ_H 7.26, 6.18, 5.80, and 5.54; terminal methylene δ_H 4.99 and 4.93), four heteroatom-bearing protons (methine δ_H 4.66 and 4.16; methylene δ_H 3.72 and 3.58), and two aliphatic methine protons (δ_H 2.73 and 1.61). It also confirmed 12 aliphatic methylene groups and four methyl groups (δ_H 1.95, 1.24, 0.98, and 0.87), the most deshielded one consistent with a vinyl methyl group. Together, these data accounted for three out of the five degrees of unsaturation. Four protons attached to heteroatoms were also observed (1H δ_H 5.90, 2H δ_H 4.61, and 1H δ_H 3.17). Finally, two carbonyl groups (δ_C 170.1 and 157.4) accounted for the two missing degrees of unsaturation.

Interpretation of the COSY spectrum allowed the assembly of four fragments that were connected based on HMBC correlations (Figure 1). C-1′, C-2′, and C-3′ (fragment 1)

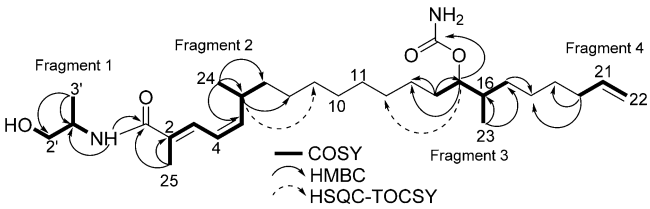


Figure 1. Key 2D NMR correlations of calothrixamide A (**1**).

composed an alaninol moiety, with COSY correlations extending to the exchangeable protons at δ_H 3.17 (−OH) and 5.90 (−NH−). HMBC correlations indicated that the −NH− group was linked to C-1 (δ_C 170.1), and C-1 was in turn connected to the methyl diene fragment by a correlation between C-1 and H_3-25. H_3-25 also showed an HBMC correlation to C-2 and a COSY correlation to C-3. The fragment from C-3 to C-7 (fragment 2) was constructed based on the interpretation of COSY correlations. The 2*E*,4*Z* configuration of the conjugated diene was assigned based on NOE correlations between H-25 and H-4, H-4 and H-5, and H-3 and H-6. This assignment was further supported by proton coupling constants ($^3J_{H\text{-}3,H\text{-}4}$ = 11.8 Hz, $^3J_{H\text{-}4,H\text{-}5}$ = 10.9 Hz). This fragment was extended based on an HMBC correlation between H-6 and C-8. Further assignment of the aliphatic chain was hampered by overlapping signals in the COSY, TOCSY, and HMBC spectra. To overcome this, we performed an ^{1}H−^{13}C HSQC-TOCSY experiment, which revealed a correlation between H-6 and H-9.

At the aliphatic terminus, fragment 4 (C-22 to C-20) was assembled based on COSY correlations and further extended to C-19 and C-18 based on HMBC. A key HMBC was observed between H-17 and C-18, allowing the connection of C-18 to fragment 3 (C-14 to C-17 and C-23). The chemical shift of C-15 (δ_C 78.5) indicated a neighboring oxygen atom. In addition, an HBMC between H-15 and the carbon signal at δ_C 157.4 was observed. This moiety was finally assigned as a carbamate ester based on the characteristic chemical shift of the carbonyl at δ_C 157.4 ppm. The carbamate ester also accounted for the broad proton signal at δ_H 4.61 integrating for two protons and with no correlations in the HSQC spectrum. The assignment of the broad proton signal as OCONH_2 was further supported by analysis of the base hydrolysis product of **1** and comparison with the intact compound in pyridine-d_5 (see Experimental Section). Fragment 3 was extended to C-13

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based on HMBC and to C-12 based on an HSQC-TOCSY correlation between H-15 and H-12. Finally, the two missing methylene groups (C-10 and C11) were assigned to the aliphatic chain between fragments 2 and 3. This structure is supported by the MS/MS data acquired *in situ* and used for preliminary dereplication. The observed losses of $-CH_3NO_2$ and $-C_3H_9NO$ are consistent with the fragmentation of the carbamate ester and the alaninol groups, respectively.

Calothrixamide B (**2**) was also obtained as a pale orange oil. Its molecular formula was deduced to be $C_{29}H_{54}N_2O_4$ (*m/z* 517.3981 $[M + Na]^+$ and *m/z* 493.4015 $[M - H]^-$), with one fewer degree of unsaturation than **1**. Again, losses consistent with carbamate ester and alaninol features were observed in the MS/MS spectrum of **2**. Investigation of the DEPTQ and ^{1}H NMR spectra indicated that both compounds were closely related. The most striking difference was the lack of resonances assigned as the terminal olefin in **1** (δ_C 139.2 and 114.4, δ_H 5.80, 4.99, and 4.93). Instead, spectroscopic data of **2** showed aliphatic resonances consistent with a methylene and terminal methyl groups (δ_C 22.8 and 14.3, δ_H 1.29 and 0.87). The planar structure of **2** was elucidated by the analysis of COSY, HSQC, HMBC, and HSQC-TOCSY spectra. Coupling constants indicated the 2*E*,4*Z* configuration of the conjugated diene ($^3J_{H\text{-}3,H\text{-}4}$ = 11.8 Hz, $^3J_{H\text{-}4,H\text{-}5}$ = 10.9 Hz). Comparison of the electronic circular dichroism (ECD) spectra of **1** and **2** suggested the same absolute configuration of both compounds surrounding the conjugated diene (Supporting Information S17). Comparable coupling constants and carbon chemical shifts supported the same absolute configuration of both compounds at C-15 and C-16.

The absolute configuration of the alaninol moiety was determined to be *S* by Marfey's analysis of the acid hydrolysate of **1** and comparison to authentic standards (Supporting Information S18). To determine the absolute configuration of the stereogenic center at C-6, we subjected **2** to ozonolysis and oxidative workup followed by derivatization with (*S*)- or (*R*)-phenylglycine methyl ester (PGME).[28] Reactions were performed on calothrixamide B (**2**) due to the absence of the terminal alkene, which would create a second carboxylic acid, potentially complicating the analysis. The PGME amides **3a** [(*S*)-PGME derivative] and **3b** [(*R*)-PGME derivative] were isolated by HPLC and analyzed by ^{1}H NMR. NMR revealed the HPLC isolates to be enantiomerically enriched mixtures, containing a 3:1 ratio of the expected product and its respective enantiomer. This was due to the partial racemization of PGME under the reaction conditions (Supporting Information S19). The $\Delta\delta$ values obtained from the analysis of ^{1}H NMR spectra of **3a** and **3b** revealed the 6*R* configuration (Figure 2a). The absolute configuration of C-15 was elucidated by Mosher's ester analysis. First, **1** was subjected to base hydrolysis, yielding (2*E*,4*Z*)-15-hydroxy-2,6,16-trimethyldocosa-2,4,21-trienoic acid (**4**). Next, **4** was esterified with (*R*)- and (*S*)-MTPA-Cl at the C-15 hydroxy group to give the (*S*)- and (*R*)-MTPA esters **5a** and **5b**, respectively. $\Delta\delta$ values extracted from the comparison of ^{1}H NMR spectra of **5a** and **5b** indicated the configuration of C-15 to be 15*R* (Figure 2b). Finally, the relative configuration between C-15 and C-16 was established by *J*-based conformational analysis supported by NOE correlations.[29] The $^{2,3}J_{C,H}$ coupling constants were obtained from a phase-sensitive HMBC spectrum. The large coupling constants between C-14 and H-16 ($^3J_{C\text{-}14,H\text{-}16}$ = 7.3 Hz) and C-17 and H-15 ($^3J_{C\text{-}17,H\text{-}15}$ = 6.6 Hz) indicated both pairs to be in *anti* conformations. This allowed for only one out

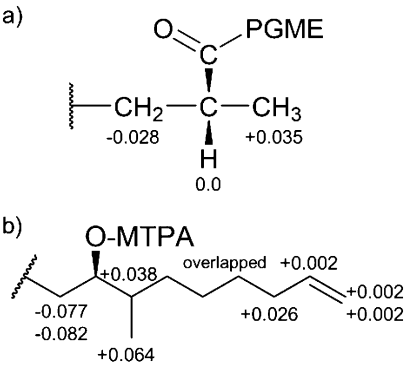

Figure 2. $\Delta\delta$ ($\delta_S - \delta_R$) values (ppm) for (a) (*S*)- and (*R*)-PGME derivatives **3a** and **3b** and (b) (*S*)- and (*R*)-MTPA derivatives **5a** and **5b**.

of the six possible relative conformations for C-15 and C-16. Additional $^{2,3}J_{C,H}$ coupling constants and strong NOE correlations between H-23/H-15 and H-15/H-16 supported the assignment of 15*R** and 16*S** (Figure 3).

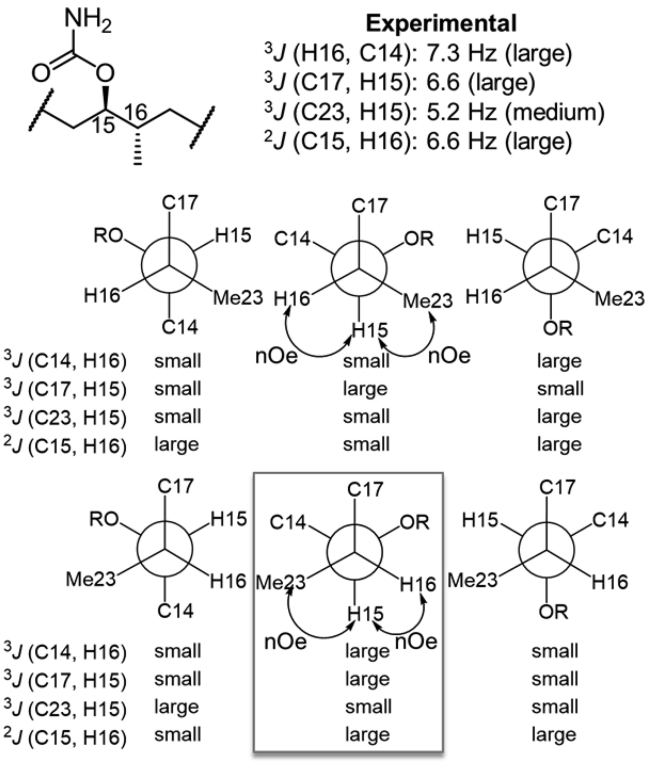

Figure 3. Newman projections for C-16/C-15. All possible conformations are presented along with predicted size of $^3J_{C,H}$ coupling constants. Double arrows indicate NOE correlations. Conformation highlighted by the box is consistent with experimental values.

Calothrixamides as pure compounds or as a 1:1 mixture of **1** and **2** were found to be inactive against MDA-MB-435 human melanoma, MDA-MB-231 breast cancer, and OVCAR3 ovarian epithelial cancer cell lines at 10 μM.

Long-chain fatty acid amides have been reported from marine cyanobacteria. They include the malyngamides,[30] pitiamides,[31,32] grenadamides,[33,34] jamaicamides,[35] semiplenamides,[36] besarhanamides,[37] credneramides,[38] kimbeamides,[39] smenamides,[40] parguerene,[41] mooreamide A,[42] columba-

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mides,[43] laucysteinamide A,[44] and others. These compounds have been associated with biological activities, such as insecticidal, cytotoxic, calcium and sodium channel modulation, and cannabinoid receptor binding. The cytotoxic nocuolin A, found in freshwater cyanobacterial species, contains structural and biosynthetic features that resemble the fatty acid amides.[45] However, it was suggested to form a new class of secondary metabolites, due to the presence of a unique oxadiazine ring. Thus, the here-reported calothrixamides are one of the first functionalized long-chain fatty acid amides isolated from freshwater cyanobacteria.

Calothrixamides also add a new chemical class to the short list of compounds reported from the *Calothrix* genus. *Calothrix* natural products include the calothrixins (alkaloids),[46] calophycin (peptide),[47] daklakapeptin (peptide),[48] brominated phenols,[49] and sesquiterpenes.[50] Distributed worldwide, *Calothrix* strains can be found in freshwater and marine environments, from temperate to tropical regions.[51] Despite genomic studies showing the potential of *Calothrix* for natural product production,[52,53] little is known about the chemical diversity of the genus.

In summary, *in situ* dereplication by droplet probe UPLC-UV-HRMS-MS/MS guided the discovery of calothrixamides A (**1**) and B (**2**) from strain *Calothrix* sp. UIC 10520. In the natural products setting, this technique has been used to analyze both plant and fungal cultures;[18,20−25] however, this is the first report on cyanobacterial cultures. We were encouraged by our ability to screen through scores of cultures with this technique, thereby selecting those most likely to biosynthesize new chemical entities for time- and resource-consuming scale up and chemical analysis. Calothrixamides are the first functionalized long-chain fatty acid amides reported from the *Calothrix* genus and from freshwater cyanobacteria. In our hand, no cytotoxicity was detected for the calothrixamides against a set of cancer cells. Ecological roles and biological activities at other targets are yet to be determined for the calothrixamides.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a PerkinElmer 241 polarimeter. UV spectra were acquired on a Varian Cary 5000 spectrophotometer. ECD spectra were obtained using a JASCO J-710 CD spectrometer. IR spectra were measured on a Thermo-Nicolet 6700 with a Smart iTR accessory. 1D and 2D NMR spectra were acquired on a Bruker 800 MHz spectrometer or on a Bruker AVII 900 MHz spectrometer, the latter equipped with a 5 mm TCI cryoprobe. NMR chemical shifts were referenced to residual solvent peaks ($CDCl_3$ δ_H 7.26 and δ_C 77.16; Pyr-d_5 δ_H 8.74 and δ_C 150.35). The majority of UPLC-UV-HRMS-MS/MS experiments were performed at UNCG using a Waters Acquity UPLC system (WatersCorp.) equipped with a photodiode array detector (PDA) and coupled to a Thermo QExactive Plus MS (Thermo-Fisher). UPLC was performed at 0.3 mL/min through a C_{18} (2.1 × 50 mm × 1.7 μm) column equilibrated at 40 °C. Mobile phases consisted of H_2O (A) and CH_3CN (B), both acidified with 0.1% formic acid (FA). A gradient program was set from 15% to 100% B for 8 min, held at 100% B for 1.5 min, and re-equilibrated to initial conditions. The PDA acquisition window was set from 200 to 500 nm and sampling rate at 5 points/s. HRMS spectra were acquired in positive and negative modes at a resolution of 70 000, scanning range from 20 to 2000 *m*/*z*, spray voltage of 3.7 kV, nitrogen sheath gas set to 25 arb, and capillary temperature of 350 °C. Automatic data-dependent fragmentation with a collision energy of 35 eV was applied to obtain the MS/MS spectra. A droplet probe system was used for *in situ* analyses, as detailed in this journal previously.[18] Sampling spots were manually selected, and a 3 μL droplet of 70:30 MeOH/H_2O was applied as the extraction solvent. This solvent system retains the droplet formation and integrity during the *in situ* analysis and is compatible with LC-MS systems.[18] The droplet was allocated to the surface and drawn back into the sampling syringe 5 times per spot before injection into the UPLC-HRMS system. Additional UPLC-UV-HRMS experiments were performed at UIC Research Resources Center on a Shimadzu LCMS-IT-TOF set as follows: C_{18} column (2.1 × 50 mm × 1.7 μm), 0.5 mL/min mobile phase flow rate, gradient of 50−100% B for 7 min with 1 min wash and 2 min re-equilibration, PDA acquiring 190−450 nm at 4.17 Hz sampling rate, HRMS acquisition in positive and negative modes from 150 to 3000 *m*/*z*.

Biological Material. *Calothrix* sp. UIC 10520 was obtained from a sample collected in July 2015 at Dixon Page Park, Illinois (N 41°50′42″, W 89°29′54″). The unialgal strain UIC 10520 was isolated by micropipet isolation techniques.[54] For the *in situ* metabolomic analysis, UIC 10520 was grown for 4 weeks in 60 × 15 mm plates containing solid Z medium. To allow for the isolation of **1** and **2**, strain was cultured for 6 weeks in 18 L of liquid Z medium[55] (four 2.8 L Fernbach flasks, each filled with 2 L of medium, and one 13 L flask containing 10 L of medium). Cultures were kept at 22 °C, under constant sterile aeration, and illuminated by fluorescent lights at 2.6 klx following an 18/6 h light/dark cycle. Cells were harvested by centrifugation and lyophilized.

Morphological Identification. Cultured UIC 10520 was analyzed under a Zeiss Axiostar Plus light microscope equipped with a Moticam 5 camera (Supporting Information S1). Observed morphological features were as follows: heteropolar filaments with basal subspherical heterocyte and narrowed toward apex; uniseriate trichomes slightly constricted at cross-walls; barrel-shaped cells isodiametric at the base and elongated toward the end; no akinetes. Morphological identification of UIC 10520 was based on the taxonomic systems by Komarek et al.[56,57]

DNA Extraction, 16S Amplification, and Sequencing. Cyanobacterial cells from a liquid culture were concentrated by centrifugation, separated from the media, and resuspended in lysis buffer (2.5 mL, 10 mM Tris, 0.1 M EDTA, 0.5% w/v SDS) and lysozyme solution (1 mg/mL). The mixture was incubated for 30 min in a water bath at 37 °C. Proteinase K was added to the mixture to a final concentration of 100 μg/mL, and the mixture was incubated for 1 h in a water bath at 50 °C. The mixture was centrifuged to pellet the cells, and the supernatant was removed. DNA was then extracted from the cell pellet using the Nucleospin soil kit (Macherey-Nagel). Cyanobacteria-specific primers 109F and 1509R were used to amplify part of the 16S rRNA gene by PCR. The PCR reaction mixture consisted of 2 μL of DNA (50 ng/μL), 10 μL of Taq buffer, 2 μL of each primer (10 μM), 1 μL of dNTPs (10 μM), 0.5 μL of Taq DNA polymerase, and 32.5 μL of nuclease-free H_2O. PCR was executed in an Applied Biosystems 2720 thermal cycler with a program of 2 min denaturation at 95 °C, followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 49 °C, and 2 min at 72 °C, with a final 5 min extension at 72 °C. PCR products were purified using a GeneJet PCR purification kit (Thermo). Sanger sequencing was performed on the purified PCR product using 109F, 359F, and 1509R primers. The partial 16S rRNA sequence of the UIC 10520 is available in GenBank under the accession number MG984605.

Extraction and Isolation. Lyophilized cell mass of UIC 10520 was extracted three times with CH_2Cl_2/MeOH, 1:1. Remaining cell material was filtered, and the extraction solution was concentrated *in vacuo* to yield 809 mg of dry extract. Extract was fractionated by vacuum liquid chromatography using a Diaion HP-20SS column and a step gradient of IPA/H_2O (0:100, 20:80, 40:60, 70:30, 90:10, and 100:0). LC-MS analysis identified **1** and **2** in the fractions eluting at 70:30 (F4), 90:10 (F5), and 100:0 (F6) IPA/H_2O. A 1:1 mixture of F5/F6 was subjected to reversed-phase semipreparative HPLC using a Phenomenex Onyx Monolithic C_{18} column (100 × 4.6 mm), 4 mL/min flow rate, and automatic fraction collection every 30 s. The 16 min method included a gradient of 70−90% MeOH in H_2O over 10 min, a 3 min wash with 100% MeOH, and re-equilibration for 3 min.

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Fractions eluting between 4 and 6 min were shown to contain **1** and **2**. Dereplication based on ^{1}H NMR, HRMS, MS/MS, and database/literature search identified no hits, indicating these compounds to be potentially new. Compound isolation was performed by reversed-phase semipreparative HPLC using an Agilent C_{18} column (250 × 10.0 mm), 4 mL/min flow rate, and a gradient of H_2O (A) and CH_3CN (B) with the following program: 60% B for 5 min, 60−85% B for 25 min, 85−100% B for 10 min, 100% B for 10 min, 100−60% B for 5 min, 60% B for 10 min. Compounds **1** (6.5 mg) and **2** (4.5 mg) eluted at 29.5 and 34 min, respectively.

*Calothrixamide A (**1**):* pale orange oil; $[\alpha]^{25}_D$ +39 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 213 (2.97), 294 (3.01) nm; ECD (CH_3CN) λ_{max} ($\Delta\varepsilon$) 211.0 (−0.44), 261.5 (+1.33); IR (neat) ν_{max} 3351 (br), 2925, 2854, 1709, 1642, 1593, 1525, 1456, 1392, 1322, 1046, 992, 909, 781, 747 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$) Table 1; ^{1}H NMR (Pyr-d_5, 800 MHz) δ 8.10 (1H, d, *J* = 7.6 Hz, N*H*), 7.68 (1H, d, *J* = 11.8 Hz, H-3), 7.39 (2H, br, OCON*H*$_2$), 6.50 (1H, m, O*H*), 6.37 (1H, ddd, *J* = 11.8, 10.8, 0.9 Hz, H-4), 5.85 (1H, ddt, *J* = 17.0, 10.3, 6.7 Hz, H-21), 5.51 (1H, t, *J* = 10.6 Hz, H-5), 5.14 (1H, dt, *J* = 8.5, 4.1 Hz, H-15), 5.07 (1H, m, H-22a), 5.01 (1H, m, H-22b), 4.67 (1H, m, H-1′), 4.00 (1H, dt, *J* = 10.8, 4.9 Hz, H-2′a), 3.92 (1H, dt, *J* = 10.8, 5.4 Hz, H-2′b), 2.65 (1H, m, H-6), 2.14 (3H, s, H-25), 2.03 (2H, m, H-20), 1.77 (1H, m, H-16), 1.74 (1H, m, H-14a), 1.61 (1H, overlapped, H-14b), 1.60 (1H, overlapped, H-17b), 1.53 (1H, m, H-13b), 1.47 (1H, m, H-13a), 1.42 (3H, d, *J* = 6.8 Hz, H-3′), 1.37 (1H, overlapped, H-19b), 1.34 (1H, overlapped, H-19a), 1.25 (1H, overlapped, H-17a), 1.41−1.12 (14H, H-7, H-8, H-9, H-10, H-11, H-12, H-18), 1.01 (3H, d, *J* = 6.8 Hz, H-23), 0.85 (3H, d, *J* = 6.6 Hz, H-24); HRESIMS *m*/*z* 515.3817 [M + Na]$^+$ (calcd for $C_{29}H_{52}N_2O_4Na$, 515.3825).

*Calothrixamide B (**2**):* pale orange oil; $[\alpha]^{25}_D$ +50 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 210 (2.74), 297 (2.79) nm; ECD (CH_3CN) λ_{max} ($\Delta\varepsilon$) 208.5 (−0.52), 268.0 (+1.57); IR (neat) ν_{max} 3340 (br), 2924, 2854, 1708, 1646, 1593, 1526, 1456, 1392, 1320, 1046, 990, 780, 746 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$) Table 1; HRESIMS *m*/*z* 517.3975 [M + Na]$^+$ (calcd for $C_{29}H_{54}N_2O_4Na$, 517.3981).

Acid Hydrolysis and Marfey's Analysis. Approximately 0.3 mg of **1** was hydrolyzed with 1 mL of 6 N HCl in a high-pressure tube kept at 110 °C for 18 h. The hydrolysate was sequentially dried and resuspended with 0.5 mL of H_2O twice to remove the residual HCl. The hydrolysate of **1** and (*S*)-alaninol and (*R*)-alaninol standards were derivatized with Advanced Marfey's reagent (FDLA) according to the following protocol. Approximately 0.2 mg of the hydrolysate or standards was dissolved in 110 μL of acetone, 50 μL of H_2O, and 20 μL of 1 N $NaHCO_3$. Next, 20 μL of L-FDLA solution (10 mg/mL in acetone) was added, and the mixtures were stirred for 1 h at 40 °C. Once at room temperature (rt), reactions were quenched with 20 μL of 1 N HCl. Reaction mixtures were dried under vacuum and resuspended in CH_3CN for LC-MS analysis. LC-MS analyses were carried out using a Phenomenex Kinetex C_{18} column (50 × 2.1 mm, 1.7 μm) and a gradient of H_2O (A) and CH_3CN (B) both with 0.1% formic at 0.5 mL/min flow rate. A gradient program was set from 25% to 65% B for 9 min. Extracted ion chromatograms for *m*/*z* 370.17 revealed the following retention times: 3.4 min for (*S*)-alaninol derivative, 4.2 min for (*R*)-alaninol derivative, 3.4 min for the derivatized hydrolysate of **1** (Supporting Information S18). The absolute configuration of the alaninol moiety was assigned as *S*.

Ozonolysis and PGME Derivatization. A solution of **2** (3.5 mg, 0.007 mmol) in MeOH (140 μL) was cooled to −78 °C, and ozone was bubbled through the solution until a pale blue color persisted. The reaction vessel was opened to the atmosphere, and the mixture was slowly warmed to rt. The resulting mixture was concentrated *in vacuo*. Formic acid (95% in H_2O, 70 μL) and H_2O_2 (35% in H_2O, 70 μL) were added to the resulting residue and stirred for 17 h at rt. The reaction mixture was concentrated *in vacuo*. Toluene (3 × 2 mL) was then successively added to the mixture and removed under vacuum to facilitate the removal of any volatile aqueous reagents. The resulting residue containing 11-(carbamoyloxy)-2,12-dimethyloctadecanoic acid (HRESIMS *m*/*z* 394.2920 [M + Na]$^+$, calcd for $C_{21}H_{41}NO_4Na$ 329.2933) was concentrated to dryness and separated into two equal portions for derivatization with PGME. Each portion was mixed with *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1 mg, 2.6 μmol), 1-hydroxybenzotriazole (HOBt) (0.5 mg, 3.7 μmol), (*S*)- or (*R*)-PGME (0.5 mg, 2.5 μmol), dimethylformamide (DMF) (25 μL), triethylamine (25 μL), and one drop of CH_2Cl_2 to ensure solubility. Reaction mixtures were stirred for 5 h at rt. After 5 h, 1 mL of EtOAc was added to the mixtures, forming organic layers, which were each washed with a saturated aqueous solution of NH_4Cl (3 × 1.0 mL). The organic layers were concentrated and purified by HPLC to yield enantioenriched mixtures of (*S*)-PGME (**3a**) or (*R*)-PGME (**3b**) amide derivatives of 11-(carbamoyloxy)-2,12-dimethyloctadecanoic acid [Agilent Microsorb Dynamax C_{18} column (250 mm × 10 mm), 4 mL/min flow rate, gradient of H_2O (A) and CH_3CN (B) set from 60% to 85% B over 20 min, from 85% to 100% B for 10 min, and held at 100% for 10 min before re-equilibration].

3a: ^{1}H NMR ($CDCl_3$, 800 MHz) δ 7.36−7.31 (PGME's aromatic protons), 6.51 (1H, d, *J* = 7.0), 5.60 (1H, d, *J* = 7.2), 4.68 (1H, m), 4.53 (2H, br), 3.73 (3H, s), 2.26 (1H, m), 1.63 (1H, m), 1.61 (1H, m), 1.53 (overlapped), 1.46 (1H, m), 1.37 (1H, m), 1.35−1.18 (overlapped), 1.15 (3H, d, *J* = 6.9), 0.88 (3H, t, *J* = 7.0), 0.87 (3H, d, *J* = 6.8); HRESIMS *m*/*z* 541.3650 [M + Na]$^+$ (calcd for $C_{30}H_{50}N_2O_5Na$, 541.3617).

3b: ^{1}H NMR ($CDCl_3$, 800 MHz) δ 7.36−7.31 (PGME's aromatic protons), 6.39 (1H, d, *J* = 7.0), 5.58 (1H, d, *J* = 7.2), 4.68 (1H, m), 4.50 (2H, br), 3.73 (3H, s), 2.26 (1H, m), 1.64 (1H, m), 1.61 (1H, m), 1.52 (overlapped), 1.47 (1H, m), 1.37 (1H, m), 1.35−1.18 (overlapped), 1.12 (3H, d, *J* = 6.9), 0.88 (3H, t, *J* = 7.0), 0.87 (3H, d, *J* = 6.8); HRESIMS *m*/*z* 541.3647 [M + Na]$^+$ (calcd for $C_{30}H_{50}N_2O_5Na$, 541.3617)

Base Hydrolysis and Mosher's Ester Analysis. Approximately 1 mg of **1** was dissolved in 0.5 mL of MeOH and reacted with 0.5 mL of 0.8 N NaOH in a high-pressure tube at 110 °C for 18 h. The hydrolysate was dried, resuspended in H_2O, and neutralized with HCl to pH 7. The reaction mixture was extracted with EtOAc three times. The combined organic layers were dried to afford (2*E*,4*Z*)-15-hydroxy-2,6,16-trimethyldocosa-2,4,21-trienoic acid (**4**).

4: ^{1}H NMR (Pyr-d_5, 800 MHz) δ 8.13 (1H, d, *J* = 12.0 Hz), 6.52 (1H, dd, *J* = 12.0, 11.0 Hz), 5.87 (1H, ddt, *J* = 16.8, 10.3, 6.8 Hz), 5.48 (1H, t, *J* = 10.4 Hz), 5.08 (1H, m), 5.02 (1H, m), 3.79 (1H, dt, *J* = 8.3, 3.7 Hz), 2.82 (1H, m), 2.38 (3H, s), 2.06 (2H, m), 1.75 (4H, m), 1.66 (1H, m), 1.61 (1H, m), 1.52 (1H, m), 1.44 (1H, m), 1.43−1.18 (overlapped), 1.12 (3H, d, *J* = 6.8 Hz), 0.95 (3H, d, *J* = 6.6 Hz); HRESIMS *m*/*z* 415.3196 [M + Na]$^+$ (calcd for $C_{25}H_{44}O_3Na$, 415.3188).

For the Mosher's ester reaction, two equal portions of **4** were separated in oven-dried 4 mL vials and dried under vacuum. To each vial were added four activated molecular sieves, a stir bar, and 200 μL of pyridine-d_5. Vials were stirred for 10 min at rt, after which 1.8 μL of *R*- or *S*-MTPA-Cl was added. The reaction mixtures were stirred for 16 h at rt to allow formation of the *S*-MTPA ester of **4** (**5a**) and *R*-MTPA ester of **4** (**5b**), respectively. Direct ^{1}H NMR analysis of the reaction mixtures was hindered due to overlapping reagent and impurity signals, and derivatives were isolated by RP-HPLC [Agilent Microsorb Dynamax C_{18} column (250 mm × 10 mm), 4 mL/min flow rate, gradient of H_2O (A) and CH_3CN acidified with 0.1% FA (B) from 80% to 100% B over 20 min, 100% B for 15 min, followed by re-equilibration].

5a: partial ^{1}H NMR (Pyr-d_5, 800 MHz) δ 8.07 (1H, d, *J* = 10.6 Hz), 6.47 (1H, m), 5.87 (1H, ddt, *J* = 16.8, 10.7, 6.9), 5.64 (1H, m), 5.31 (1H, m), 5.10 (1H, m), 5.04 (1H, m), 2.84 (1H, m), 2.23 (3H, br), 2.05 (1H, m), 1.79 (1H, m), 1.70 (1H, m), 1.57 (1H, m), 1.00 (3H, d, *J* = 6.6), 0.96 (3H, d, *J* = 6.8); HRESIMS *m*/*z* 631.3563 [M + Na]$^+$ (calcd for $C_{35}H_{51}F_3O_5Na$, 631.3586).

5b: partial ^{1}H NMR (Pyr-d_5, 800 MHz) δ 8.07 (1H, d, *J* = 10.5 Hz), 6.47 (1H, m), 5.87 (1H, ddt, *J* = 16.8, 10.7, 6.9), 5.65 (1H, m), 5.31 (1H, m), 5.10 (1H, m), 5.04 (1H, m), 2.84 (1H, m), 2.23 (3H, br), 2.03 (1H, m), 1.78 (1H, m), 1.75 (1H, m), 1.64 (1H, m), 0.99

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(3H, d, J = 6.5), 0.90 (3H, d, J = 6.8); HRESIMS m/z 631.3552 [M + Na]$^+$ (calcd for $C_{35}H_{51}F_3O_5Na$, 631.3586).

Antiproliferative Assay. Cytotoxicity assays against MDA-MB-435 human melanoma cancer cells, MDA-MB-231 human breast cancer cells, and OVCAR3 human ovarian cancer cells were performed following established protocols.[58] Taxol was used as positive control (IC_{50} values of 3.1, 2.6, and 7.5 nM against MDA-MB-435, MDA-MB-231, and OVCAR3, respectively).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00432.

Photomicrograph and phylogenetic analysis of *Calothrix* sp. UIC 10520, results from the droplet probe UPLC-UV-HRMS-MS/MS analysis of *Calothrix* sp. UIC 10520, 1D and 2D NMR and ECD spectra of **1** and **2**, results of Marfey's analysis (PDF)

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Notes

The authors declare no competing financial interest.

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