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Metabolomics-Guided Discovery of Microginin Peptides from Cultures of the Cyanobacterium *Microcystis aeruginosa*

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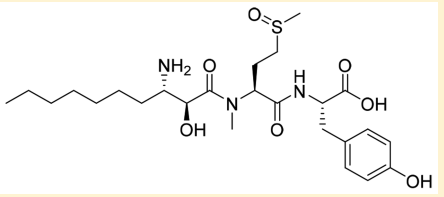
Supporting Information

ABSTRACT: We report a mass-spectrometry-based metabolomics study of a laboratory-cultured strain of *Microcystis aeruginosa* (UTEX LB2385), which has led to the discovery of five peptides (**1**−**5**) belonging to the microginin class of linear cyanopeptides. The structures and configurations of these peptides were determined by spectroscopic analyses and chemical derivitization. The microginin peptides described herein are the first reported derivatives containing *N*-methyl methionine (**1**, **5**) and *N*-methyl methionine sulfoxide (**2**−**4**). The two tripeptide microginin analogues (**4**, **5**) identified represent the smallest members of this peptide family. Their angiotensin-converting enzyme (ACE) inhibitory activity was also investigated. Microginin 527 (**4**) was the most potent of the group, with an IC_{50} of 31 μM.



Cyanobacteria are prokaryotic organisms also known as blue-green algae. Blooms of cyanobacteria are a natural phenomenon and can occur in both inland and coastal waters.[1−3] Freshwater cyanobacterial blooms are on the rise worldwide, resulting in increased attention to monitoring and regulation of the hepatotoxic microcystins often produced in these events.[4] *Microcystis* sp. is the most common bloom-forming genus of cyanobacteria[5] and, to date, has been reported on every continent except Antarctica.[6] Several classes of bioactive linear and cyclic peptides, in addition to the heptapeptide microcystins, have been isolated from *Microcystis aeruginosa* strains,[7−10] and recent genomic studies have highlighted the potential of this species in drug discovery efforts by uncovering further biosynthetic gene clusters encoding unidentified chemistry.[11,12]

Application of secondary metabolomics to guide isolation of new chemistry has been investigated in marine cyanobacteria[13,14] as well as plants,[15] nonphotosynthetic bacteria,[16] and fungi,[17] but has hardly been applied to discover new chemistry in freshwater cyanobacteria,[18] where published metabolomics studies have largely focused on untargeted analysis of primary metabolites.[19,20] The identification of strains of *M. aeruginosa* as an untapped resource for the discovery of novel chemistry coupled with its cosmopolitan distribution and implication in toxic bloom events globally make it an attractive and relevant candidate for secondary metabolomics analysis.

A rapid and robust UPLC-QTof MS metabolomics method for secondary metabolite profiling of laboratory cultures of *M. aeruginosa* (UTEX LB2385) resulted in the identification of new congeners of the microginin class of linear nonribosomal cyanopeptides. The first microginin congener was reported from *M. aeruginosa* in 1993 by Okino and co-workers, and further studies have described the isolation of numerous microginin congeners from several cyanobacteria genera including *Microcystis*, *Nostoc*, and *Oscillatoria*.[21−23] Microginin derivatives, with few exceptions, contain 4−6 residues including one or more tyrosines at the C-terminus and the characteristic N-terminal side chain, 3-amino-2-hydroxy decanoic acid (Ahda). Both mono- and dichlorinated variants of this side chain have been reported as well as some derivatives containing a modified octanoic acid moiety.[24] Congeners of this peptide class contain various amino acids between the N-terminal β-amino acid and the C-terminal tyrosine residues including Ile, Leu, Val, Ala, Pro, and Ser, some of which contain a methylated amino group. Many of the microginins reported to date exhibit angiotensin-converting enzyme (ACE) inhibition and thus may serve as lead compounds in the discovery of novel antihypertensive agents. The microginin peptides described herein include five new variants (**1**−**5**), which are the first reported microginin derivatives containing *N*-methyl methionine (**1**, **5**) and *N*-methyl methionine sulfoxide (**2**−**4**). The two tripeptide microginin analogues (**4**, **5**) identified represent the smallest members of this peptide family. The identification, isolation, and structure determination of these five microginin congeners are described as well as their ACE inhibitory activity.

RESULTS AND DISCUSSION

Metabolomics of *Microcystis aeruginosa* (UTEX LB2385). Methanolic extracts of harvested *M. aeruginosa* cells were loaded onto SPE cartridges for removal of matrix interferences, such as salts and phospholipids, prior to UPLC-QTof MS analysis. Six replicate cell extractions were processed in an identical manner in order to account for natural variance within biological systems and meet proposed minimum standards for metabolomics analysis.[25] Raw data files were imported into Progenesis QI software for processing.

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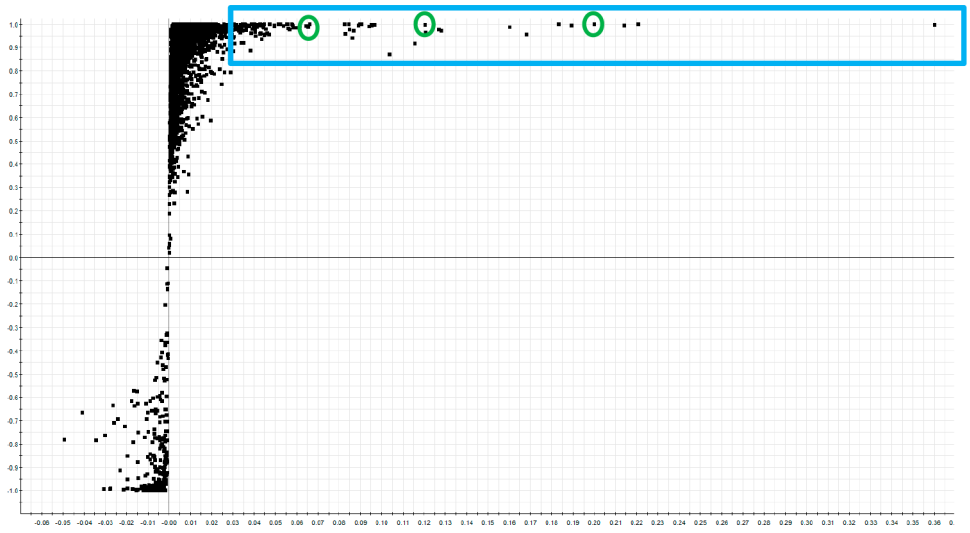

Figure 1. S-plot of *M. aeruginosa* UTEX LB2385 cell extracts (6 replicates, 3 injections of each) versus B3N media blank (1 replicate, 3 injections). The blue box indicates compound ions with loading scores of >0.03 from the cell extracts, which were selected for manual data mining and dereplication. Compound ions of microginin derivatives discovered using this approach are designated with green circles.

Compound ions of interest after application of various filters were exported to EZInfo 3.0 software for multivariate analysis, including principle component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The OPLS-DA model was used to construct an S-plot comparing compound ions from *M. aeruginosa* UTEX LB2385 cell extracts to those in the media extract (Figure 1). Compound ions with loading values of >0.03 from the cell extracts were selected for further analysis, resulting in 88 compound ions of interest. Manual data mining of the resulting compound list identified extraneous compound ions that were falsely identified by the software algorithm, most often high-intensity fragment ions from more abundant metabolites in the samples. This is a trade-off in selecting a sample concentration to maximize the number of secondary metabolites that are above the limit of detection and a caveat of natural product metabolomics data sets that contain diverse chemistry produced over a wide range of concentrations. After exclusion of compound ions of abundant fragment ions during manual data mining, a total of 41 compounds were identified in the intracellular secondary metabolome of *M. aeruginosa* (UTEX LB2385). Eight of these compounds could further be classified into three cyanopeptide classes based on characteristic fragment ions observed in the high-energy spectra.[26] The presence of three microcystins (Adda fragment at m/z 135, $[M + H - 134]^+$ neutral loss), two cyanopeptolins (Ahp-Phe substructures indicated by ions at m/z 215 and m/z 243), and three microginins (Ahda fragment at m/z 128) were identified using this method, in addition to a number of compound ions that could not be further classified by high-energy fragmentation data. This metabolite profile was consistent with genome sequencing of 10 representative *M. aeruginosa* strains, which revealed each strain contained two to nine biosynthetic gene clusters.[12] Aside from the microcystin congeners, all other metabolites identified were concluded to represent new structural variants after dereplication using both in-house and commercial databases (ChemSpider, Metlin, MassBank, Antibase, MarinLit). Compound ions not classified as cyanopeptides by high-energy fragmentation data were not able to be confidently identified using high-resolution mass (2 ppm tolerance), isotope ratio, and fragmentation scores within selected Progenesis databases (ChemSpider, MassBank, Metlin, KEGG, HMDB). Compound identification is a recognized bottleneck in metabolomics experiments due to lack of database coverage.[27] Acquisition of MS/MS data for these compound ions in order to query fragment databases such as GNPS, MoNA, and CSIFingerID is one approach to improve compound identification and is currently under investigation in our metabolomics workflow.[28]

Microginin Derivatives from *Microcystis aeruginosa* (1−5). Mass-spectrometry-guided isolation of three microginin congeners (m/z 675.3427, m/z 691.3378, m/z 528.2739; fragment ion of m/z 128.1438 in all high-energy spectra) identified by metabolomics analysis (Figure 1) using scaled-up cultures of *M. aeruginosa* UTEX LB2385 successfully resulted in the isolation of sufficient material for full structure elucidation. Futhermore, an additional two minor related compounds (m/z 512.2785 and m/z 705.3530; fragment ion of m/z 128.1438) were identified in the scaled-up material, likely not found by metabolomics due to intensities below the limit of detection in the small-scale analysis. The isolation and structure elucidation of these five related microginin congeners (**1**−**5**) is described.

Microginin 674 (**1**) was isolated as a glassy yellow solid. The HRMS and NMR data revealed that **1** had a molecular formula of $C_{34}H_{50}N_4O_8S$. The UV data (λ_{max} 224, 276 nm) were consistent with the presence of an aromatic ring, and preliminary inspection of the NMR data confirmed the presence of four ortho-coupled aromatic proton signals, an *N*-Me group (δ_H 2.65), and 16 aliphatic protons in the range δ_H 1.19−1.43 ppm. The ^{13}C spectrum contained four carbonyl resonances (δ_C 172.8, 170.7, 171.6, and 169.1) suggesting that this compound was a tetrapeptide. A doubling of many of the resonances was observed in both the ^1H and ^{13}C spectra in a ratio of ca. 3:1, consistent with the presence of an *N*-methylated amide linkage in a linear peptide.[29] The presence of two aromatic residues was confirmed by the ESIMS data, which displayed a strong fragment ion at $[M + H - 181]^+$, indicating loss of a terminal tyrosine residue, and a fragment ion at m/z 343 corresponded to a dityrosine fragment (Table 1, Figure 2). This was supported by extensive NMR analyses including

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Table 1. ESIMS Fragment Ions for Microginins 1−5

ion structure	1	2	3	4	5
$[M + Na]^+$	697	713	727	550	534
$[M + H]^+$	675	691	705	528	512
$[M + H - CH_3SOH]^+$		627	641	464	
$[M + H - Tyr]^+$	494	510			
$[M + H -$ *O*-Me Tyr$]^+$			510		
$[M + H - Tyr - CO]^+$		482			
$[M + H -$ *O*-Me Tyr $- CO]^+$			482		
$[H +$ Tyr + *O*-Me Tyr$]^+$			357		
$[H +$ Tyr + Tyr$]^+$	343	343			
[*N*-Me Met(O)]$^+$		180	180	180	
[*N*-Me Met]$^+$	164				164
$[CH_3(CH_2)_6CH{=}NH_2]^+$	128	128	128	128	128

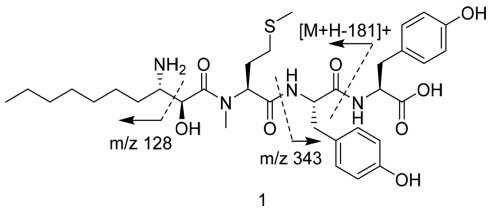
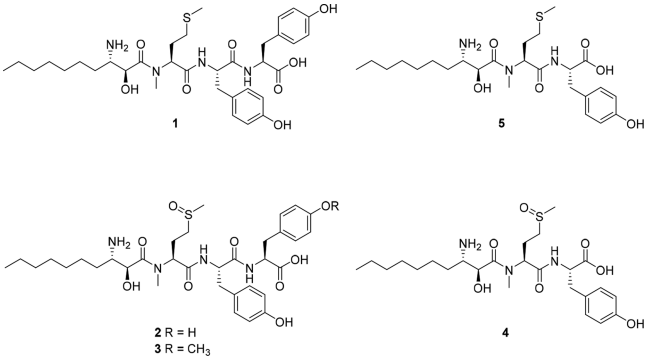


Figure 2. Schematic of ESIMS fragmentation of microginin 674 (**1**).

TOCSY, COSY, HSQC, and HMBC spectra. A characteristic fragment ion at *m/z* 128 indicated the presence of an N-terminal Ahda residue, which was supported by the ^1H and ^{13}C spectra (Table 2). The fragment at *m/z* 128 results by scission of the C-2 and C-3 bond in the Ahda moiety (Figure 2).

The presence of Ahda was further verified by $^1H-^1H$ TOCSY data, which displayed correlations from the proton on C-2 to the terminal methyl group, which was consistent with reported data for Ahda-containing microginins.[30] The NMR features of the final peptide residue contained a characteristic set of signals (δ_H 2.02 and δ_C 14.3) corresponding to an *S*-methyl group of a methionine residue. While other NMR resonances were consistent with a methionine side chain, the amide nitrogen was methylated as indicated by an *N*-methyl shift at δ_H 2.65 and δ_C 30.3. These data led to the final assignment of this residue as *N*-methyl methionine and the amino acid sequence of **1** to be Ahda-*N*-MeMet-Tyr-Tyr.



The molecular formula of microginin 690 (**2**) was determined to be $C_{34}H_{50}N_4O_9S$ based on the high-resolution MS and NMR data and denoted the presence of an additional oxygen compared with **1**. The spectroscopic data for this second compound (UV data: λ_{max} 224, 276 nm; ESIMS fragment ions at $[M + H - 181]^+$ as well as *m/z* 343) were consistent with a dityrosine C-terminus. Once again, the N-terminal Ahda was confirmed by the characteristic fragment ion at *m/z* 128 and substantiated by extensive NMR data. The NMR data easily established the presence of two tyrosine units (Table 2), but many of the resonances observed in both the ^1H and ^{13}C spectra of **2** and tentatively assigned to an *N*-methyl methionine residue appeared as doublets in a ratio of ca. 3:1. Furthermore, compared with **1**, the *S*-methyl resonances in the NMR spectra of **2** now appeared deshielded at δ_H 2.47 and δ_C 37.8 (Table 3). Additionally, ESIMS of **2** revealed a strong fragment ion of $[M + H - 64]^+$ not seen in **1**, corresponding to the loss of CH_3SOH (Table 1). Collectively these data established **2** contained an *N*-methyl methionine sulfoxide amino acid residue. The presence of the sulfoxide group in **2** as a mixture of *R* and *S* configurations resulted in the doubling of several NMR signals for this residue, making spectral interpretation more difficult. The doubling of signals due to *R* and *S* sulfoxides has been described in other methionine sulfoxide-containing cyanopeptides, and, as has been suggested for those metabolites, the sulfoxide in **2** is most likely an artifact of aerial oxidation of the *N*-methyl methionine natural product during isolation.[31,32]

The high-resolution MS and NMR data for microginin 704 (**3**) (λ_{max} 224, 276 nm) established a molecular formula of $C_{35}H_{52}N_4O_9S$, which differs from **2** by 14 Da, suggesting the presence of an additional methyl group. Indeed, the ^1H and ^{13}C NMR spectra of **3** closely resembled those of **2**, except for new signals in the NMR spectra of **3** suggesting the presence of a methoxy group at δ_H 3.60 ppm and δ_C 53 ppm (Table 2). The location of the methoxy group was determined by ESIMS fragment ions at *m/z* 510 and 357, corresponding to loss of a terminal methoxy tyrosine residue and a MeOTyr-Tyr fragment (Table 1). The presence of a methionine sulfoxide residue was supported by a strong fragment ion at $[M + H - 64]^+$ in the ESIMS of **3**, corresponding to a loss of CH_3SOH. An ESIMS fragment ion of *m/z* 128 and the 2D NMR data indicated the Ahda residue was also unchanged.

The high-resolution MS spectrum as well as NMR data revealed that microginin 527 (**4**) had a molecular formula of $C_{25}H_{41}N_3O_7S$, corresponding to seven double-bond equivalents. While the ^1H and ^{13}C NMR spectra of **4** resembled those of **2**, the difference of 163 Da and four double-bond equivalents in **4** suggested that this compound lacked a tyrosine residue. This was confirmed by the NMR data (Table 3), which displayed resonances for only one tyrosine residue. Once again the N-terminal Ahda was confirmed by a fragment ion at *m/z* 128 and by TOCSY data. The ESIMS of **4** also showed a strong fragment ion at $[M + H - 64]^+$, indicating the loss of CH_3SOH (Table 1) which, coupled with the NMR data, established the presence of an *N*-methyl methionine sulfoxide.

A molecular formula of $C_{25}H_{41}N_3O_6S$ for microginin 511 (**5**), established by high-resolution MS and NMR data, suggested that this minor component was similar to **4**, but contained an *N*-methyl methionine residue rather than the sulfoxide derivative. This residue was verified by the ^1H NMR and $^1H-^1H$ TOCSY data, which was also used to identify a single tyrosine residue and the N-terminal Ahda moiety, as indicated by the fragment ion at *m/z* 128. The MW for **5** was 163 Da less than **1**, and the similarity of the NMR data between the two compounds established that **5** was similar to **1** but contained a single C-terminal tyrosine.

While the spectrometric data for these linear peptides were relatively straightforward to interpret, as noted earlier the presence of epimeric sulfoxides in **2**−**4** and the 3:1 ratio of

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Table 2. ^{1}H and ^{13}C NMR Data for Tetrapeptide Microginins (1−3) in DMSO-d_6 (^{1}H 500 MHz, ^{13}C 125 MHz)

position		microginin 674 (1)		microginin 690 (2)		microginin 704 (3)	
		δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
Ahda	1	170.7, C		170.7, C		170.7, C	
	2	69.6, CH	4.01, br s	69.6, CH	4.01, br s	69.6, CH	4.01, br s
	3	53.2, CH	3.10, m	53.2, CH	3.10, m	53.1, CH	3.05, m
	4	28.4, CH_2	1.27, m	28.4, CH_2	1.27, m	28.4, CH_2	1.27, m; 1.43 (m)
	5	24.6, CH_2	1.19, m	24.6, CH_2	1.19, m	24.6, CH_2	1.19, m
	6	28.6, CH_2	1.22, m	28.6, CH_2	1.22, m	28.6, CH_2	1.22, m
	7	29.1, CH_2	1.21, m	29.1, CH_2	1.21, m	29.1, CH_2	1.21, m
	8	31.0, CH_2	1.23, m	31.0, CH_2	1.23, m	31.0, CH_2	1.23, m
	9	22.0, CH_2	1.24, m	22.0, CH_2	1.24, m	22.0, CH_2	1.24, m
	10	13.9, CH_3	0.85, t (6.7)	13.9, CH_3	0.85, t (6.8)	13.9, CH_3	0.85, t (6.1)
	OH		6.49, br s		6.49, br s		6.52, br s
	NH_2		7.78, br		7.79, br		7.78, br
N-Me Met/*N*-Me Met (O)	1	169.1, C		169.1, C		169.1, C	
	2	55.1, CH	5.01, m	54.9 (55.3[a]), CH	5.06 (4.99[a]), m	54.9 (55.3[a]), CH	5.06 (4.99[a]), m
	3	28.6, CH_2	2.42, m	20.9 (21.1[a]), CH_2	2.10, m	20.9 (21.1[a]), CH_2	2.09, m
	4	29.4, CH_2	2.27, t (7.8)	49.4 (49.6[a]), CH_2	2.54 (2.57[a]), m	49.4 (49.6[a]), CH_2	2.54 (2.57[a]), m
	S-Me	14.3, CH_3	2.02, s	37.8 (38.0[a]), CH_3	2.47 (2.49[a]), s	37.8 (38.0[a]), CH_3	2.45 (2.47[a]), s
	N-Me	30.3, CH_3	2.65, s	31.1 (31.2[a]), CH_3	2.63 (2.66[a]), s	32.0 (32.1[a]), CH_3	2.63 (2.66[a]), s
Tyr	1	171.6, C		171.8, C		171.8, C	
	2	50.5, CH	4.36, m	50.9, CH	4.41, m	50.9, CH	4.41, m
	3	35.8, CH_2	2.81, m	35.4, CH_2	2.89, m	35.4, CH_2	2.89, m
	4	127.0, C		127.0, C		127.0, C	
	5, 9	130.0, CH	6.99, m	130.0, CH	7.03, m	130.2, CH	7.01, m
	6, 8	115.0, CH	6.64, m	115.1, CH	6.64, m	115.1, CH	6.63, m
	7	156.0, C		156.0, C		156.0, C	
	7-OH		9.25, br		9.25, br		9.25, br
	NH		8.38, d (6.7)		8.05, d (7.5)		8.30, d (7.3)
Tyr/*O*-Me Tyr	1	172.8, C		172.8, C		172.8, C	
	2	53.8, CH	4.73, m	53.8, CH	4.72, m	53.8, CH	4.72, m
	3	35.8, CH_2	2.86, m	35.8, CH_2	2.82, m	35.8, CH_2	2.82, m
	4	127.2, C		127.6, C		127.6, C	
	5, 9	129.9, CH	7.05, m	129.9, CH	7.05, m	129.9, CH	6.99, m
	6, 8	114.9, CH	6.68, m	114.9, CH	6.68, m	114.9, CH	6.62, m
	7	155.9, C		155.9, C		155.9, C	
	7-OH		9.22, br		9.25, br		
	NH		8.18, d (7.5)		8.21, d (7.5)		8.20, d (7.4)
	7-OMe					53.0, CH_3	3.60, s

[a]Doubling of signals due to *R* and *S* sulfoxides.

conformational isomers as a result of *N*-methylation in **1**−**5** made assignment of the NMR resonances more difficult.

Configurations of the Microginins 1−5. The L-configuration of the *N*-methyl methionine and tyrosine residues in **2** was determined by oxidation of the sulfoxide to the sulfone, then acid hydrolysis followed by derivatization with Marfey's reagent and LC/MS analysis.[33] Due to lack of commercially available standards, *N*-methyl methionine sulfone required synthesis from L-methionine and D-methionine in order to determine the configuration of this amino acid. The relative stereochemistry of Ahda was determined by NMR analysis of the oxazolidinone derivative of **2**, which revealed a coupling constant of 5.4 Hz between H-2 and H-3 (numbering based on the Ahda chain prior to derivatization), which, upon comparison to previous reports for similar Ahda derivatives in other microginins,[34,35] suggests a *trans* relationship between the alcohol and amine functionalities. Consistency in the specific rotation values obtained for the five congeners suggests that the configurations of **1** and **3**−**5** are identical to those determined for **2**.

Biological Activity. Four of the microginin derivatives (**1**−**4**) reported in this study were produced in sufficient amounts to allow testing for inhibition of angiotensin-converting enzyme. The tripeptide microginin 527 (**4**) was the only congener of the four tested in this study that displayed ACE inhibition below 100 μM with an IC_{50} value of 31 μM. IC_{50} values above 100 μM were not determined due to scarcity of available material. The LC/MS assay allowed observation of the microginin peptides used for inhibition, confirming that **1** was not oxidized to **2** during the course of the assay. Despite this, there were no significant differences between **1** and **2** in inhibitory potency by this assay, indicating that the presence of the sulfoxide was not the cause for low levels of inhibition. Previous structure−activity studies on this class of ACE inhibitors indicated that the dityrosine C-terminus as well as the free amino and hydroxy groups of the Ahda side chain play a key role in ACE inhibition.[35] Early studies on snake venom peptide analogues revealed that the tripeptide residues at the C-terminus of long-chain peptides were the most important for ACE inhibition.[36] More recent studies on hydrolyzed food

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Table 3. ^{1}H and ^{13}C NMR Data for Tripeptide Microginins (4 and 5) in DMSO-d_6 (^{1}H 500 MHz, ^{13}C 125 MHz)

		microginin 527 (4)		microginin 511 (5)	
position		δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
Ahda	1	171.3, C		171.4, C	
	2	69.6, CH	4.00, br s	71.0, CH	4.00, br s
	3	53.1, CH	3.01, m	53.5, CH	3.05, m
	4	28.4, CH_2	1.27, m	28.4, CH_2	1.27, m
	5	24.6, CH_2	1.19, m	24.6, CH_2	1.19, m
	6	28.6, CH_2	1.22, m	28.6, CH_2	1.22, m
	7	29.1, CH_2	1.21, m	29.1, CH_2	1.21, m
	8	31.0, CH_2	1.23, m	31.0, CH_2	1.23, m
	9	22.0, CH_2	1.24, m	22.0, CH_2	1.24, m
	10	13.9, CH_3	0.85, t (6.6)	14.3, CH_3	0.85, t (6.5)
	OH		6.55, br s		6.51, br s
	NH_2		7.78, br		7.79, br
N-Me Met/ *N*-Me Met(O)	1	170.4, C		170.5, C	
N-Me Met (O)	2	55.9 (55.6[a]), CH	4.98 (4.91[a]), m	58.1, CH	4.95, m
	3	20.8 (20.9[a]), CH_2	2.21, m	28.8, CH_2	2.35, m
	4	49.5 (49.6[a]), CH_2	2.51 (2.59[a]), m	29.2, CH_2	2.28, m
	S-Me	37.9, CH_3	2.79 (2.82[a]), s	14.9, CH_3	2.01, s
	N-Me	31.9 (32.1[a]), CH_3	2.89 (2.92[a]), s	32.1, CH_3	2.90, s
Tyr	1	171.5, C		171.0, C	
	2	50.5, CH	4.85, m	52.0, CH	4.82, m
	3	35.9, CH_2	2.71, m	36.4, CH_2	2.71, m
	4	126.6, C		127.0, C	
	5, 9	130.2, CH	7.05, d (8.8)	131.8, CH	7.02, d (8.6)
	6, 8	115.0, CH	6.68, d (8.7)	115.0, CH	6.63, d (8.4)
	7	156.0, C		156.0, C	
	7-OH		9.25, br		9.25, br
	NH		8.11, d (7.5)		8.22, d (7.4)

[a]Doubling of signals due to *R* and *S* sulfoxide.

proteins have shown that ACE inhibition is enhanced in peptides containing aromatic and branched chain amino acids at the three C-terminal positions.[37,38] This substrate preference could explain the vast differences in inhibitory activity between microginin 527 and the tripeptide degradation product of microginin Ahda-Ala-Val, which display IC_{50} values of 31 and 460 μM respectively.[35]

Conclusion. The UPLC-QTof MS method for obtaining secondary metabolite profiles of laboratory-cultured cyanobacteria described here allows rapid access to the cyanobacterial secondary metabolome using minimal biomass. This method successfully captured a large number of cyanopeptide metabolites and is amenable to high-throughput processing. The discovery of five congeners of the microginin class of linear cyanopeptides using this method adds to the mounting evidence for freshwater cyanobacteria as a source of new chemistry. A large proportion of *Microcystis* spp. have yet to be analyzed for secondary metabolite production, and genomic analysis on a small number of *Microcystis* strains has revealed the biosynthetic machinery for more unidentified products. Consequently, metabolomics evaluation of this genus will undoubtedly provide new chemistry as well as new insight into the biological functions of cyanobacterial secondary metabolites. This preliminary metabolomics study serves as a baseline of the secondary metabolome of *M. aeruginosa* (UTEX LB2385) under standard culture conditions, and further studies using changes in culture parameters or addition of chemical elicitors may facilitate the expression of cryptic biosynthetic clusters, allowing access to additional chemistry. Furthermore, as the occurrence of freshwater cyanobacterial blooms persists globally, an in-depth understanding of the complex metabolome of *Microcystis* spp. is essential to rapidly and accurately assess the true threat of bloom events on human health.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol III automatic polarimeter. UV spectra were recorded on an HP1100 diode array detector. ^{1}H and 2D homonuclear (TOCSY, COSY) NMR spectra were measured on an Avance 500 MHz NMR spectrometer (Bruker) using 5.0 mm TXI and 1 mm TXI probe heads. ^{13}C and 2D heteronuclear (HSQC, HMBC) NMR spectra were also measured on an Avance 500 MHz NMR spectrometer using a 5 mm BBO probe head. All NMR spectra were acquired in DMSO-d_6, and the chemical shifts were referenced to DMSO-d_6 (δ_H 2.50 for proton and δ_C 39.5 for carbon). Low-resolution ESI mass spectra were measured on a Micromass ZQ with electrospray ionization (capillary voltage 3.1 kV; cone voltages 30 V/50 V; source temperature 80 °C; desolvation temperature 150 °C; cone gas flow 50 L/h; desolvation gas flow 300 L/h) (Waters Corporation) in positive ion mode with a mass range of 100−1400 Da and a scan time of 0.5 s acquired using MassLynx v 4.1 software. High-resolution data for accurate molecular weight, MS/MS fragmentation, and metabolomics were measured using a Xevo G2-XS QTof spectrometer controlled by MassLynx v 4.1 software (capillary voltage 2.5 kV; sampling cone 80 V; source offset 80 V; source temperature 120 °C; desolvation temperature 550 °C; cone gas flow 100 L/h; desolvation gas flow 800 L/h) (Waters Corporation) coupled with an Acquity iClass UPLC system (Waters Corporation) with flow-through needle. HPLC separations were performed using a Waters HPLC system (model 515 pumps and a model 680 automated gradient controller) equipped with a Waters 2487 dual λ UV detector. All MS solvents and modifiers were Fisher Optima MS grade, and all HPLC solvents were BDH HiPerSolv Chromanorm HPLC grade.

Biological Material. *Microcystis aeruginosa* (UTEX LB2385) was obtained from the University of Texas Culture Collection and cultured in our laboratory in Bold 3N (B3N) media and a 14/10 h light−dark cycle.[39]

Metabolomics Sample Preparation. Cells from 1.0 L of *M. aeruginosa* (UTEX LB2385) were harvested by vacuum filtration and stored on glass fiber filters at −20 °C prior to extraction. Six replicate harvests were processed individually. Cells were extracted with 80:20 MeOH/H_2O (2×), sonicating for 15 min prior to filtration. Combined and filtered extracts were taken to dryness *in vacuo* and resuspended in 10 mL of 95:5 H_2O/MeOH for processing. An aliquot (1 mL) of the extract was loaded onto an SPE column (Waters Oasis Prime HLB 6 cm^3/200 mg) and washed with 1 column volume (CV) of 95:5 H_2O/MeOH in order to remove matrix interferences. The column was then eluted with 1 CV of 90:10 MeCN/MeOH collected into a scintillation vial. An aliquot (10%) of this fraction was filtered using a 0.2 μm 13 mm PTFE syringe filter (Pall Acrodisc) directly into an MS certified autosampler vial with preslit cap (Waters TruView). B3N media (100 mL) was also processed using this protocol. A quality control (QC) sample containing equal parts (50 μL) of each of the six replicate cell extracts and media control was prepared from the filtered samples.

Metabolomics Data Acquisition. Chromatographic separation for metabolomics analysis was achieved using a Waters Acquity BEH C_{18} 2.1 × 100 mm 1.7 μm column with gradient elution (mobile phase

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A: H_2O + 0.1% formic acid (FA); mobile phase B: MeCN + 0.1% FA; held at 5% B for 1 min, 5−65% B over 8 min, 65−100% B over 1 min, hold at 100% B for 1.5 min, re-equilibrate at 5% B for 4.5 min; flow rate: 0.5 mL/min). Samples were injected in triplicate (1 μL), and QC samples were analyzed at the beginning, middle, and end of the data acquisition in order to aid the software in alignment of the chromatograms during processing. Continuum data were acquired in resolution mode using the MS^e function (mass range 50−1800 Da; scan time 0.1 scans/s; function 1 CE off; function 2 CE ramp 45−50 V; lockmass data acquired but not applied). Leucine enkephalin (200 pg/μL, infused at 20 μL/min) was used as the lockmass solution.

Metabolomics Data Processing Parameters. Continuum data were uploaded into Progenesis QI software (Nonlinear Dynamics) for alignment, lock mass calibration, and peak picking. QC samples were chosen for alignment reference, and peak picking parameters were set to default settings (automatic sensitivity threshold; no minimum chromatographic peak width), with the exception of retention time, which was restricted to 1−10 min. Progenesis identified 6287 compound ions under the selected threshold values. This data set was filtered to remove compounds with ANOVA p-values of >0.05 as well as compound ions with the highest mean intensity in the solvent blank sample, resulting in 3724 compound ions, which were then subjected to statistical analysis using an OPLS-DA model to construct an S-plot comparing cell extracts to the media extract using EZInfo 3.0 software (UMetrics) (Figure 1).

Extraction and Isolation Procedure for Microginins. The 80% aqueous MeOH extract of cells collected on filter papers by vacuum filtration (from 256 L of culture, extracted in 32 L batches) was washed with hexane. The aqueous layer was dried *in vacuo* and applied to a reversed-phase column eluted with a MeOH gradient (Phenomenex Strata C_{18} 20g/60 mL; step gradient 20−100% MeOH), yielding fractions containing the masses of interest. Further purification of these fractions by gel permeation chromatography (LH-20), followed by C_{18} reversed-phase HPLC (Waters SunFire RPC$_{18}$ 19 × 250 mm; 40−100% MeOH + 0.05% trifluoroacetic acid (TFA) over 40 min; flow rate 10 mL/min, UV detection at 280 nm), provided five new microginins (**1**−**5**). Final purification of these peptides was accomplished by Amide C_{16} reversed-phase HPLC (Supelco Discovery Amide C_{16} 10 × 250 mm; 25−45% MeCN + 0.05% TFA over 20 min; flow rate 2.5 mL/min, UV detection 280 nm) to yield **1** (18.2 min, 14.0 mg), **2** (12.1 min, 23.0 mg), **3** (13.5 min, 6.5 mg), **4** (9.7 min, 9.0 mg), and **5** (15.0 min, 2.2 mg).

*Microginin 674 (**1**):* $[\alpha]_D^{26}$ −41 (c 0.28, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.19), 276 (3.39); ^{1}H and ^{13}C NMR, Table 2; positive ESIMS m/z (relative intensity) 675 (53.7), 494 (100), 343 (36.2), 164 (25), 128 (18.7); HRMS m/z 675.3427 $[M + H]^+$ (calcd for $C_{34}H_{51}N_4O_8S$, 675.3428).

*Microginin 690 (**2**):* $[\alpha]_D^{26}$ −21 (c 0.175, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.19), 276 (3.39); ^{1}H and ^{13}C NMR, Table 2; positive ESIMS m/z (relative intensity) 691 (100), 627 (7.6), 510 (54.1), 343 (49.3), 180 (29.3), 128 (8.0); HRMS m/z 691.3378 $[M + H]^+$ (calcd for $C_{34}H_{51}N_4O_9S$, 691.3377).

*Microginin 704 (**3**):* $[\alpha]_D^{26}$ −16 (c 0.40, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.19), 276 (3.39); ^{1}H and ^{13}C NMR, Table 2; positive ESIMS m/z (relative intensity) 705 (100), 641 (6.2), 510 (68.3), 357 (40.6), 180 (31.9), 128 (8.5); HRMS m/z 705.3530 $[M + H]^+$ (calcd for $C_{35}H_{53}N_4O_9S$, 705.3533).

*Microginin 527 (**4**):* $[\alpha]_D^{26}$ −38 (c 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.18), 276 (3.34); ^{1}H and ^{13}C NMR, Table 3; positive ESIMS m/z (relative intensity) 528 (37.4), 464 (3.3), 180 (100), 128 (29.6); HRMS m/z 528.2739 $[M + H]^+$ (calcd for $C_{25}H_{42}N_3O_7S$, 528.2743).

*Microginin 511 (**5**):* $[\alpha]_D^{26}$ −20 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.18), 276 (3.34); ^{1}H and ^{13}C NMR, Table 3; positive ESIMS m/z (relative intensity) 512 (100), 164 (6.8), 128 (17.5); HRMS m/z 512.2785 $[M + H]^+$ (calcd for $C_{25}H_{42}N_3O_6S$, 512.2794).

Determination of the Configuration of Microginin 690 (2). Following reported methods,[40] to a solution of *N*-Boc-L-methionine (100 mg) in MeOH (2.5 mL) was added oxone (500 mg), and the solution was stirred at room temperature for 2 h. The mixture was diluted with H_2O and extracted with CH_2Cl_2 to afford *N*-Boc-L-methionine sulfone (m/z 282 $[M + H]^+$). The sulfone was suspended in dry THF (2 mL). NaH (50 mg) and MeI (330 μL) were added, and the mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with H_2O and extracted with EtOAc. The aqueous layer was then acidified with 20% citric acid and extracted with EtOAc. The combined organic layers were concentrated to afford *N*-Boc-*N*-methyl-L-methionine sulfone (m/z 296 $[M + H]^+$), which was dissolved in equal volumes of 1 M HCl (0.5 mL) and EtOAc (0.5 mL) and stirred at room temperature for 2 h. The solution was concentrated to give *N*-methyl-L-methionine sulfone (m/z 196 $[M + H]^+$). This material was used for the Marfey's analysis. *N*-Methyl-D-methionine sulfone was synthesized from *N*-Boc-D-methionine in the same manner. To a solution of microginin 690 (**2**, 0.5 mg) in MeOH (0.5 mL) was added oxone (5 mg in 100 μL of H_2O), and the mixture was stirred at room temperature for 2 h. The solution was diluted with H_2O and applied to a small SPE column (Waters Oasis Maxx 6 cm^3), washed with 10% MeOH, and eluted with 100% MeOH to afford microginin 690 sulfone (m/z 707 $[M + H]^+$). Microginin 690 sulfone (0.5 mg) was dissolved in 6 N HCl (0.5 mL) and heated at 110 °C for 5 h. To the acid hydrolysate of a 500 μg portion of microginin 690 sulfone were added 100 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) (10 mg/mL) and 1 M $NaHCO_3$ (100 μL), and the reaction mixture heated at 40 °C for 1 h. After cooling to room temperature, the reaction was quenched with 6 N HCl (5 μL) and concentrated under a stream of nitrogen. D- and L-Amino acid standards were treated with L-FDAA in the same manner. The L-FDAA derivatives were resuspended in MeOH (100 μL) and filtered (0.2 μm 13 mm GHP syringe filter) for analysis by reversed-phase UPLC-MS (Waters Acquity BEH C_{18}, 2.1 × 100 mm 1.7 μm column; 5−40% MeCN + 0.1% FA over 9 min; flow rate 0.65 mL/min). Retention times (t_R) of the standard amino acids: L-tyrosine (5.97 min), D-tyrosine (6.70 min), *N*-Me-L-methionine sulfone (4.97 min), *N*-Me-D-methionine sulfone (5.05 min). The absolute configuration of the amino acids in **2** were determined as L-tyrosine (t_R 5.97 min) and *N*-Me-L-methionine sulfone (t_R 4.97 min).

Formation of the Oxazolidinone Derivative. A vial containing microginin 690 (**2**, 2.1 mg) was desiccated under vacuum prior to addition of carbonyl diimidiazole (2 mg) and dry THF (700 μL) under a blanket of nitrogen prior to sealing. The reaction was subjected to magnetic stirring at room temperature for 24 h, after which the sample was dried under a stream of nitrogen. The sample was purified by HPLC (Waters XTerra MS C_{18} 4.6 × 150 mm; 20−45% MeCN + 0.05% TFA over 25 min; flow rate 1.0 mL/min) and analyzed by NMR.

Angiotensin-Converting Enzyme Inhibitory Assay. ACE inhibitory activity was determined by a modified method of Cushman and Cheung.[41] The reaction mixture (100 μL) contained potassium phosphate buffer (50 mM; pH 8.3), NaCl (300 mM), $ZnCl_2$ (10 μM), hippuryl-L-histidyl-L-leucine (5 mM) (Sigma), and ACE (5 mU) (Sigma). Test compounds were dissolved in DMSO for testing, keeping the concentration of the DMSO in the reaction mixture at 5%. At this concentration DMSO did not significantly affect enzyme activity. Test compounds were preincubated with the substrate for 30 min at 37 °C, after which the ACE was added and the tubes were again incubated at 37 °C for 30 min. At the end of the incubation, tubes were placed on ice, and the reaction was terminated by the addition of 1 N HCl (100 μL). The hippuric acid (HA) was then extracted with EtOAc (1 mL). An aliquot of the EtOAc layer (200 μL) was transferred to Target DP vials (National Scientific) and evaporated using a SpeedVac (Thermo). The HA was then dissolved in MeOH (1 mL), and the solution was analyzed by LC/MS (Waters XTerra MS C_{18}, 2.1 × 30 mm; 10−100% MeCN + 0.1% acetic acid over 6.1 min; flow rate 0.5 mL/min). The LC/MS method was validated using pure HA (Sigma). A standard curve of HA (1−250 μM) was generated and found to be linear with the HA produced due to the hydrolysis of the substrate falling within this range. Typically, about 1% of the substrate was hydrolyzed with 5 mU of ACE, corresponding to about 60 μM HA. To further validate this method, the effect of captopril and EDTA, known inhibitors of ACE activity, was determined. The IC_{50} values

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were 25 nM and 14 μM for captopril and EDTA, respectively, and are in agreement with published values.[42]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00829.

Spectra of microginins **1**–**5**, including NMR and MS (PDF)

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Notes

The authors declare no competing financial interest.

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