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A combined LC-MS/MS and molecular networking approach reveals new cyanotoxins from the 2014 cyanobacterial bloom in Green Lake, Seattle

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Abstract

Cyanotoxins obtained from a freshwater cyanobacterial collection at Green Lake, Seattle during a cyanobacterial harmful algal bloom in the summer of 2014 were studied using a new approach based on molecular networking analysis of liquid chromatography-tandem mass spectrometry (LC-MS/MS) data. This MS-networking approach is particularly well suited for the detection of new cyanotoxin variants, and resulted in the discovery of three new cyclic peptides, namely microcystin-MhtyR (**6**) which comprised about half of the total microcystin content in the bloom, and ferintoic acids C (**12**) and D (**13**). Structure elucidation of **6** was aided by a new microscale methylation procedure. Metagenomic analysis of the bloom using the 16S-ITS rRNA region identified *Microcystis aeruginosa* as the predominant cyanobacterium in the sample. Fragments of the putative biosynthetic genes for the new cyanotoxins were also identified, and their sequences correlated to the structure of the isolated cyanotoxins.

Graphical abstract

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information. Tables S1 and S2 containing BLAST matches of the amplified 16S-ITS rRNA, *mcvBA*d1, and *ferBA*d1 fragments. Figure S1 showing the structures of thiol derivatives of MCs, and Figure S2 showing their LC-MS analysis. Figures S3–S13 showing HR-MS/MS spectra of **1–8** and **11–13**.

INTRODUCTION

Cyanobacteria have a long history of ecological and health impacts. This unique class of photosynthetic microorganisms can generally survive in nearly all phototrophic aquatic environments, including recreational water bodies, fisheries, and reservoirs. In the last two decades, worldwide attention has been given to the ecological effects of cyanobacteria and to their production of secondary metabolites. They represent a not yet fully explored source of novel lead compounds for drug discovery, such as the dolastatins,¹ cryptophycins,² and curacins,³ that have in turn inspired the development of synthetic analogues with improved bioactivity and pharmacokinetics. Some of these have successfully reached the phase II and phase III of clinical trials, and one is a FDA clinically approved drug.⁴ In addition, it has been shown that symbiotic cyanobacteria are often the real producers of secondary metabolites that were originally isolated and thought to be produced by invertebrates.^{5,6,7} Interestingly, smenothiazole B,⁸ recently isolated from the sponge *Smenospongia aurea* during our anticancer screening program, closely resembles that of the neurotoxin jamaicamide B,⁹ a hybrid peptide/polyketide obtained from the cyanobacterium *Moorea producens* (formerly known as *Lyngbya majuscula*). The two molecules share a similar vinyl chloride moiety and alkynyl terminus.

On the other hand, cyanobacteria growing in freshwater and marine recreational areas may have a strongly negative impact on human health. Some species can form extensive blooms, and there is evidence that these are increasing during recent decades due to nutrient enrichment, especially phosphorus.¹⁰ Many cyanobacteria produce toxic secondary metabolites (cyanotoxins) with differing effects on health, ranging from mild skin irritations to severe illness.¹¹ Economic losses from freshwater and marine cyanobacterial blooms in the United States account for millions of dollars per year, and include monitoring programs, closure of recreational areas and fisheries, loss of livestock, water treatment and population wellness related costs.¹²

Because cyanobacteria are an attractive research topic from both the risks as well as potential remedies they represent, we have initiated a worldwide program to study their chemistry and biochemistry. Here we report our analysis of a sample of the cyanobacterial scum collected at Green Lake, Seattle (WA, USA) during the 2014 summer bloom. This study involved a new approach that combined liquid chromatography (LC)-high resolution mass spectrometry (HRMS) with automated data analysis using the molecular networking

technique,¹³ and led to the rapid identification of one new microcystin (MC) (**6**) and two new ferintoic acid congeners (**12** and **13**). In addition, metagenomic analysis of the sample allowed us to identify the bloom-forming cyanobacteria as a strain of *Microcystis aeruginosa*, and to detect a fragment of the *mcyBAd1* gene from the MC biosynthetic cluster. The sequence and predicted function of this gene nicely correlate with the structure of the MCs detected in the sample.

MATERIAL AND METHODS

Collection and Extraction

A sample of cyanobacterial scum was collected at Green Lake, Seattle (WA, USA) on September 5, 2014 during a cyanobacterial harmful algal bloom. A voucher sample is stored at Dipartimento di Farmacia, Università degli Studi di Napoli “Federico II” with the reference number SEAGL14. An aliquot (2 mL) of the cyanobacterial suspension was sonicated for 5 min and extracted with BuOH. The organic and aqueous layers were separated, filtered (Whatman, 0.2 µm, PTFE) and dried, yielding 1 mg of dry organic extract; the extract was resuspended in H₂O at a concentration of 5 mg/mL and subsequently analyzed by LC-HRMS.

The BuOH extract was subjected to preparative reversed-phase HPLC separation on a Agilent 1260 Infinity Quaternary LC apparatus equipped with a Diode-Array Detector (DAD) [column 150 × 4.6 mm, 5 µm, Kinetex C18; eluent A: H₂O; eluent B: ACN; gradient: 20→99% B, over 22 min, flow rate 0.2 mL/min], which afforded two fractions (fraction A, *t_R* = 15 min, and fraction B, *t_R* = 16 min) containing, respectively, MC-HtyrR (**5**) and MC-MhtyR (**6**) (Figure 2) as the sole microcystins, as determined by LC-HRMS analysis.

LC-HRMS and LC-HRMS/MS

Experiments were performed using a Thermo LTQ Orbitrap XL high-resolution ESI mass spectrometer coupled to an Agilent model 1100 LC system, which included a solvent reservoir, in-line degasser, binary pump, and refrigerated autosampler. A 5 µm Kinetex C18 column (50 × 2.1 mm), maintained at 25 °C, was operated using a gradient elution of H₂O and ACN running at 200 µL/min. The gradient program was as follows: 10% ACN for 5 min, 10%–99% ACN over 17 min, 99% ACN for 3 min. All the mass spectra were recorded in the positive-ion mode. MS parameters were a spray voltage of 5 kV, a capillary temperature of 230 °C, a sheath gas rate of 15 units N₂ (ca. 150 mL/min), and an auxiliary gas rate of 5 units N₂ (ca. 50 mL/min). Data were collected in the data-dependent acquisition (DDA) mode, in which the first and second most intense ions of a full-scan mass spectrum were subjected to tandem mass spectrometry (MS/MS) analysis. MS/MS scans were obtained for selected ions with CID fragmentation, isolation width 2.0, normalized collision energy 36, Activation Q 0.250, and activation time 30 ms. Mass data were analyzed using the Thermo Xcalibur software.

Methylation of microcystins

A mixture containing MC-LR and MC-YR (Sigma) (1.75 µg each) in 350 µL of MeOH was treated with (trimethylsilyl)diazomethane (2M solution in Et₂O, 100 µL).¹⁴ The reaction mixture was kept at room temperature overnight, then dried and redissolved in H₂O/MeOH (9:1) (100 µL) for subsequent LC-HRMS analysis. Similarly, fraction A (0.2 mg) and fraction B (0.5 mg) from the HPLC separation described above were each dissolved in 400 µL of MeOH and methylated with (trimethylsilyl)diazomethane (50 µL, 2M solution in Et₂O) as described above.

Molecular Networking

The mass spectral data were converted to mzXML format using the program msconvert.¹⁵ A molecular network was obtained using the online workflow at the GNPS website.¹⁶ The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.1 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.6 and more than 10 matched peaks. Furthermore, edges between two nodes were kept in the network only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS's spectral libraries. The data were then imported into Cytoscape 2.8.3¹⁷ and displayed as a network of nodes and edges. The network was organized with the FM3 layout plug-in.¹⁸ Dereplication and search for analogues were performed separately after the creation of the network. All matches within the library spectra were required to have a score above 0.7 and at least 10 matched peaks with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. Analogue search was enabled against the library with a maximum mass shift of 200 Da.

DNA isolation, PCR amplification, and clone library construction

Metagenomic DNA from the Green Lake cyanobacteria was extracted from a pellet (about 40 mg) obtained by centrifugation of the cyanobacterial scum. Primers used in the PCR experiments were the 16S-ITS rRNA cyanobacterial specific primers CYA359F (5'-GGGGAATYTTCCGCAATGGG-3')¹⁹ and 373R (5'-CTAACCACCTGAGCTAAT-3'),²⁰ the specific *mcyB* primers FAA (5'-CTATGTTATTTATACATCAGG-3') and RAA (5'-CTCAGCTTAACTTGATTATC-3'),²¹ and the degenerate primers MTF (5'-GCNGGYGGYGCNTAYGTNCC-3') and MTR (5'-CCNCGDATYTTNACYTG-3').²¹ PCR products were subcloned with the TOPO TA cloning_R kit (Invitrogen) and sequenced. Sequences were analyzed by the RDP Seq Match tool,²² BLASTn²³ and BLASTx. Full experimental details are reported in the Supporting Information.

Partial sequences of 16S-ITS rDNA from *Microcystis aeruginosa* SEAGL14, *mcyB_Ad1*, and *ferB_Ad* were deposited into GenBank under the accession numbers KT359577-KT359579.

RESULTS

Molecular Networking

A cyanobacterial bloom sample collected from Green Lake (Seattle, WA, USA) during the bloom occurring in the summer 2014 was extracted and analyzed by LC HRMS and LC-HRMS/MS. Data obtained were used to generate a molecular network using the Global Natural Product Social Molecular Network (GNPS)¹⁶ (See Experimental section for details). The mass spectral molecular networking results in a fast identification of known metabolites from natural extracts (dereplication)¹³ as well as new analogues.²⁴ Molecular networking provides a visual representation of structural relationships as revealed by MS/MS data. A single chemical species is represented as a node and the relatedness between compounds is represented by an edge.

Libraries containing a large number of MS/MS spectra of known natural products are available at the GNPS website for the purpose of dereplication (additionally, users can provide their own libraries). During the process of generation of the network, the spectra from one or more LC MS/MS runs are compared pairwise, and each spectrum is also compared with spectra in the libraries. This strategy allows the identification not only of known metabolites, but also of their structural analogues (either new or known compounds which are not present in the library) in a fast and automatic way, and directly from raw LC-MS/MS data. To date, this new approach has not yet been used in the analysis of a cyanobacterial bloom.

Because the data obtained in this study were high-resolution MS/MS spectra, we reasoned that smaller values for the MS/MS fragment ion tolerance than the default 0.5 Da would improve the quality of the network and minimize false clusters between structurally unrelated compounds. After some trials, we empirically determined an optimal value of 0.1 Da for this parameter. Moreover, because peptides are known to contain a large number of fragment ions, we selected a threshold of 10 matched peaks (compared to the default of 6 peaks) for two nodes to be connected. The Green Lake two-dimensional network obtained using these parameters, and visualized using the Cytoscape software,¹⁷ is displayed in Figure 1. The network contains 13 clusters ranging from 2 to 10 nodes.

Dereplication and search for analogues were performed separately after the creation of the network, in part because not all of the available libraries of natural products contained high-resolution spectra. Therefore, the default MS/MS fragment ion tolerance of 0.5 Da was restored, and the specific dereplication workflow available at the GNPS website was used. This resulted in the identification of three known cyanobacterial compounds, namely microcystins LR (**1**),²⁵ ferintoic acid A (**11**),²⁶ and aerucyclamide A,²⁷ along with several analogues of these metabolites. The corresponding nodes of Figure 1 are color-coded accordingly.

While the molecular networking algorithm allows a fast and automated identification of analogs of known compounds, it does not directly provide a chemical structure for these analogues. Therefore, the structures of the analogues of microcystin and ferintoic acids were

established from an in-depth analysis of the high resolution MS/MS spectra combined with nanogram-scale chemical derivatization.

Microcystins

MCs are monocyclic heptapeptides biosynthesized via the nonribosomal peptide synthetase (NRPS) pathway as encoded by the *mcy* gene cluster.²⁸ MCs contain a common set of five amino acids (including the unique β -amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, or Adda) and two variable L-amino acids at positions 2 and 4. MCs are named after their variable L-amino acids. The most common MC, which contains leucine (L) at position 2 and arginine (R) at position 4, is therefore called MC-LR (**1**, Figure 2). Substitutions at position 2 and 4 give rise to more than 20 primary MC analogues, with alterations in other constituent amino acids resulting in more than 100 reported MCs to date.^{29,30} MCs are hepatotoxins; they are strong inhibitors of type 1 and 2A serine protein phosphatases (PP1 and PP2A).³¹ These enzymes are vital to various cellular processes such as cell growth and tumor suppression and therefore these toxins are potent potential cancer promoters.³²

The MC cluster contained six nodes, indicating the presence of five compounds closely related to MC-LR (**1**, m/z 995.5535). The relative amounts of the six MCs were estimated from the areas of the relevant peaks in the extracted-ion chromatograms from the LC-MS run (Figure 2). The molecular weight of **2** (m/z 1029.5361) and **3** (m/z 1045.5320) suggested they were the relatively common MC variants, MC-FR and MC-YR, respectively. This was confirmed by comparison of their retention times and MS/MS spectra with those of authentic standards. The high-resolution MS/MS fragmentation patterns of **1–3** were studied in detail (Figure 3). Most fragment ions derived from the cleavage of two amide bonds, which is the typical fragmentation mode of cyclic peptides.³³ In all of these fragment ions, the positive charge was retained on the fragment containing arginine. Fragments deriving from the loss of part of the Adda residue (ions *a*, *l*, and *m* in Figure 3) were also observed. These analyses completed and confirmed previous interpretations of low- resolution³⁴ and high-resolution³⁵ spectra of MCs, and provided a basis for the subsequent analysis of the MS/MS spectra of MCs **4–6**.

The molecular formula of **4** (m/z 1049.5633) indicated the presence of four additional hydrogen atoms compared to MC-YR (**3**). Analysis of the MS/MS spectrum showed that all of the ions containing amino acid 2 were shifted to higher mass by 4 Da, while the remaining ions were unchanged, thus locating the additional four H-atoms on amino acid 2 (Tyr in MC-YR). These data are consistent with MC-(H₄)YR, a known MC containing a 1,2,3,4-tetrahydrotyrosine residue.^{36,37}

Compound **5** (m/z 1059.5480) showed an additional CH₂ compared with MC-YR (**3**); this was also located at the amino acid 2 position (Figure 3), suggesting the presence of a homotyrosine residue. Homotyrosine is frequently found in MCs, but its presence (rather than the isomeric *O*-methyl tyrosine that is also found in cyanobacterial peptides)³⁸ is not always confirmed experimentally. In our case, the presence of a homotyrosine residue was confirmed by chemical derivatization as described in the following section.

The new MC **6** (m/z 1073.5630) showed an additional CH_2 compared to MC-HtyR (**5**), and once again, MS/MS fragment ions demonstrated that the additional 14 Da mass belonged to amino acid 2 (Figure 3). However, because all fragment ions (except those involving cleavage of Adda) contained intact amino acids, it was not possible to establish the exact location of this CH_2 group on the homotyrosine residue from MS/MS data alone. Two structures seemed most reasonable, either an *O*-methylated homotyrosine or an *N*-methylated homotyrosine residue at position 2. It was not possible to obtain **6** in sufficient quantity for NMR analysis. Therefore, a microscale *O*-methylation procedure was developed and used to distinguish between the two possible structures.

Methylation of microcystins

(Trimethylsilyl)diazomethane (TMSCHN_2), a less hazardous analogue of diazomethane with essentially the same reactivity, methylates phenols to quantitatively yield the corresponding methyl ethers,¹⁴ and was therefore chosen as the reagent for *O*-methylation of the MCs. Additionally, it was expected that TMSCHN_2 would also react with the two carboxylic groups present in the MCs, and therefore the reagent was preliminary tested with a standard sample of MC-LR (**1**). Surprisingly, even with a very large excess of TMSCHN_2 and long reaction times, only the monomethyl derivative **7** (m/z 1009.5712, $\text{C}_{50}\text{H}_{77}\text{N}_{10}\text{O}_{12}$, additional CH_2 compared to **1**) of MC-LR was obtained. LC-HRMS/MS analysis of the reaction product showed that methylation occurred on the Glu residue, while the MeAsp residue did not react (Figure 4). This can be explained by the formation of a strong ion pair interaction between the MeAsp carboxylate and the Arg guanidinium groups. The reaction was then repeated using MC-YR (**3**), and the bis-methylated product **8** (m/z 1073.5678, $\text{C}_{54}\text{H}_{77}\text{N}_{10}\text{O}_{13}$, additional C_2H_4 compared to **3**) was formed. Compound **8** was shown to be methylated on the Glu and Tyr residues (Figure 4), and confirmed that TMSCHN_2 was capable of reacting with the Tyr phenolic hydroxy group.

Finally, the reaction was performed on two HPLC fractions of the BuOH extract (fractions A and B), which respectively contained MC-HtyR (**5**) and MC-MhtyR (**6**) as the only MCs. The reactions yielded, respectively, the bis-methylated products **9** (m/z 1087.5834, $\text{C}_{55}\text{H}_{79}\text{N}_{10}\text{O}_{13}$, additional C_2H_4 compared to **5**) and **10** (m/z 1101.5979, $\text{C}_{56}\text{H}_{81}\text{N}_{10}\text{O}_{13}$, additional C_2H_4 compared to **6**). Unfortunately, the MS/MS spectra of **9** and **10** displayed low intensity, and most fragment peaks were obscured by noise. However, the diagnostic ions formed by loss of MeAsp (ion *g*, m/z 958.5376 and $\text{C}_{50}\text{H}_{72}\text{N}_9\text{O}_{10}^+$ for **9** and 972.5539 and $\text{C}_{51}\text{H}_{74}\text{N}_9\text{O}_{10}^+$ for **10**) were still detectable, and confirmed that no methylation had occurred at this amino acid residue. These results showed that both MC-HtyR (**5**) and MC-MhtyR (**6**) had a free phenolic OH. This definitely excluded the presence of an *O*-methyltyrosine residue in both compounds, and suggested a homotyrosine-containing structure as the putative structure for **5**. By extension, this analysis also revealed that the homotyrosine residue in **6** is likely to contain an *N*-methyl group.

Thiol Derivatization

A micro-scale derivatization technique has been recently reported,³⁹ which allows differentiation between MCs containing the isomeric amino acids Mdha and Dhb (dehydrobutryne, or 2-aminocrotonic acid), which have both been reported at position 7 of

MCs. The method involves the reaction of MCs with a thiol (e.g. β -mercaptoethanol) followed by LC-MS analysis. When Mdha-containing MCs are present, the reaction is fast (several hours), but when Dhb-containing MCs are present, complete derivatization takes several days. The presence of an Mdha residue in all the MCs **1–6** from the Green Lake extract was confirmed by derivatization of the microcystins with β -mercaptoethanol and LC-HRMS analysis showing complete reaction after 12 h (see the Supporting Information section for details).

Ferintoic acids

The ferintoic acids are a sub-class of the anabaenopeptins, cyclic hexapeptides⁴⁰ that are synthesized by NRPSs.⁴¹ Anabaenopeptins contain six amino acids, five of which form a cyclic structure with an amide bond involving the ϵ -amino group on an invariant Lys residue. The sixth amino acid is attached to the Lys α -amino group through a unique ureido bridge; variants of the anabaenopeptins with L-Tyr, L-Arg, L-Phe, L-Ile, L-Glu, and L-Lys at this position have been reported. For historical reasons, anabaenopeptins with an L-Trp residue as the exocyclic amino acid are known as ferintoic acids. Two types of ferintoic acids have been described, ferintoic acid A (**11**), and ferintoic acid B, in which the Ile residue is replaced by Val.²⁶ Anabaenopeptins, including the ferintoic acids, have been reported to inhibit protein phosphatase 1⁴² as well as proteases such as carboxypeptidase A.⁴³

The ferintoic acid cluster (Figure 5) contained three nodes, suggesting the presence of two analogues of ferintoic acid A (**11**, m/z 867.4423) which we name here as ferintoic acid C (**12**, m/z 899.4143) and D (**13**, m/z 915.4069). The relative amounts of **11–13** (Figure 5) were estimated from the areas of the relevant peaks in the extracted-ion chromatograms. The molecular formulas of **12** ($C_{46}H_{57}N_8O_9S$, one additional S atom compared ferintoic acid A) and **13** ($C_{46}H_{58}N_8O_{10}S$, one additional S and O atom) did not match any known anabaenopeptin analogue.

The fragmentation patterns of the new compounds were compared with that of ferintoic acid A (**11**) (Figure 6). The fragment ion at m/z 591.2941 ($C_{31}H_{39}N_6O_6^+$) found in the MS/MS spectrum of **11** and due to the loss of amino acids 3 and 4, was also present in the MS/MS spectra of **12** and **13**. This located the difference between the three compounds to be on one of these two amino acids. The ion at m/z 690.3629 ($C_{36}H_{48}N_7O_7^+$), formed by the loss of Hty at position 4, was shifted to m/z 722.3352 ($C_{36}H_{48}N_7O_7S^+$) for **12** and m/z 738.3306 ($C_{36}H_{48}N_7O_8S^+$) for **13**. Therefore, the additional atoms in **12** and **13** must be located on amino acid 3. All of the other fragment ions detected in the MS/MS spectra were fully consistent with this conclusion. Considering that substitution of methionine (or its oxidized form methionine sulfoxide) for valine has been previously observed in several anabaenopeptins, these data indicated the presence of a Met residue at position 3 in ferintoic acid C (**12**) and a methionine sulfoxide (MetO) residue at position 3 in ferintoic acid D (**13**).

16S-ITS rRNA Metagenomic Analysis

The microbiome associated with the Green Lake bloom was probed using a cultivation-independent approach in order to make a preliminary survey of the identity of the cyanobacteria inhabiting this field sample. A 16S-ITS (internal transcribed spacer) rRNA

amplicon library was prepared from metagenomic DNA extracted from the collected samples. PCR products were subcloned via TOPO TA cloning and five representative plasmids were sequenced. All of the cyanobacterial sequences were identical at a 99% sequence identity threshold, and were assigned to *Microcystis aeruginosa* based on the top matching sequences from a BLASTn search (Table S1 in Supporting Information). Therefore, a *M. aeruginosa* strain (named *M. aeruginosa* SEAGL14 in this paper) is the predominant cyanobacterial species present in the 2014 Green Lake bloom, and this strain is the likely producer of the MCs and ferintoic acids detected in these field samples.

Signature sequences within adenylation domains and correlation with the synthesis of MC variants

One of the most frequent structural variations in MCs is due to amino acid substitutions at position 2.⁴⁴ This amino acid is selected and activated by the adenylation domain *mcyBA*d1. *McyBA*d1 fragments were amplified by PCR from the metagenomic DNA using *mcyB*-specific primers and then subcloned. Eight representative clones were sequenced that were identical at the 99% sequence identity threshold. The top matching sequences from a BLASTx search were *mcyBA*d1 genes from *M. aeruginosa* (Table S2 in Supporting Information). Therefore, only one genotype of *mcyBA*d1 (*mcyBA*d1_SEAGL14) was detected by PCR-based metagenomic screening, and this was clearly associated with a *Microcystis* species.

The nine amino acids lining the putative binding pocket of the adenylation domain⁴⁵ of this variant of *McyBA*d1 were identified as DGWTIGAVKK. This signature sequence shared almost complete identity with the sequence (DGWTIGAVEK) of the binding pocket of the *McyBA*d1 genotype from a number of *Microcystis* strains,⁴⁶ which are reported to simultaneously produce MCs containing Leu, Arg, or Tyr at position 2. It can be assumed that *mcyBA*d1_SEAGL14 should have a similarly relaxed substrate selectivity, and is therefore consistent with our findings here of MC variants with Leu, Phe or Tyr-related residues at position 2. It is worth noting that no MC variant with an Arg residue at position 2 was detected in the Green Lake bloom extract. This is presumably related to the fact that Glu331 is replaced by Lys331 in *McyBA*d1_SEAGL14. Glu331 (or Asp331) is thought to play a key role in the accommodation of basic amino acid side chains (i.e. Arg);⁴⁵ therefore, it can be argued that the presence of Lys331 instead of a negatively charged amino acid does not allow for recognition of Arg. The Glu→Lys mutation at position 331 of *McyBA*d1 has not been previously observed.

Signature sequences within adenylation domains and correlation with the synthesis of ferintoic acids

No specific primers for adenylation domains of anabaenopeptins (and therefore of ferintoic acids) are described in the literature; therefore, the metagenomic DNA from the Green Lake sample was amplified by PCR using more general primers, targeting highly conserved motifs in adenylation domains of NRPS. The PCR products were subcloned and six plasmids sequenced. All six were related to adenylation domains of NRPSs, however, only one (*ferB*_Ad) shared high similarity with AptB adenylation domains from the anabaenopeptin synthetase gene cluster (Table S2 in Supporting Information). The AptB

adenylation domain is known to recruit the amino acid residue in position 3 of the anabaenopeptins. The signature sequence of the binding pocket of FerB_Ad (DMWFLGGAI) shares almost complete identity with the substrate specificity code (DMWFMGGVI) of AptB_Ad from the nodulapeptin synthetase cluster.⁴⁹ In nodulapeptin, the AptB_Ad protein selects for Met, MetO or Ile as the substrate. Therefore, FerB_Ad is expected to be responsible for the activation of Met, MetO, Ile or Val in the production of the ferintoic acids from this Green Lake population.

DISCUSSION

Freshwater, marine, and terrestrial cyanobacteria produce a wide array of toxic metabolites, the most common of which are the MCs.⁵⁰ The presence of MCs can limit the use of lakes and reservoirs for recreational activities, as drinking water reservoirs, and for irrigation. Concentrations of MC-LR are highly variable in phytoplankton communities and are greatly influenced by the composition of phytoplankton species present. The World Health Organisation (WHO) has set a provisional guideline value for MC-LR of 1.0 µg/L in drinking water. More generally, WHO established a level of 100,000 cyanobacterial cells/mL (equivalent to approximately 50 µg chlorophyll-a/L if cyanobacteria dominate) as a guideline value for a moderate health alert in recreational waters.⁵⁰ These provisions for adequate public warnings are the only short-term measure to avoid large scale intoxications and allergies. We are evaluating the feasibility of early detection of cyanobacterial blooms using similar remote and proximal sensing tools as those used to monitor contamination of surface waters.⁵¹

Nutrient enrichment has been proved to be the most important factor responsible for cyanobacterial blooms,¹⁰ even if this is a topic still opened for further studies. Similarly, our knowledge is fragmentary concerning the structures and amounts of cyanotoxins produced, the fate of these cyanotoxins in the food chain, and the influence of human activities on these events. Even if national authorities are generally aware of the MC problem, present regulations are generally at the provisional state and will need to change as knowledge increases on the structure, distribution, and toxicology of MCs.

A general method for the rapid detection of known or novel (and potentially more toxic) cyanotoxins is an urgent need to properly manage cyanobacterial blooms, and should be considered in health control programs at the private, municipal, and state levels. The large structural variability of cyanotoxins, and the frequent occurrence of new congeners, makes the problem of developing such a method a challenging one. The Green Lake bloom is illustrative in this respect: the new MC-MhtyR was by no means a minor compound, and comprised about half of the total MC content in the collected samples. In contrast, MC-LR, the only MC whose concentration is considered in WHO guidelines, represented only about 7% of the total MCs. Similarly, the new ferintoic acids C and D predominated over the known ferintoic acid A.

Currently, cyanotoxins are predominately monitored using reversed-phase HPLC combined with MS and MS/MS. The current MS-based methods are fast and sensitive, but require the availability of standards, or rely on assumptions concerning the structures of the analyzed

MCs. This approach can fail in the identification of new cyanotoxins. For example, the most general method which can be used for the MS/MS identification of MCs by MS/MS relies on the fragment peak at m/z 135 arising from side-chain fragmentation of the Adda residue. While this residue is generally conserved in MCs, some examples of MCs are known in which a free OH or an OAc group replaces the OMe group in Adda.⁵² Such MCs or others with a modification to the Adda residue would not be identified by this MS/MS method.

This combined high-resolution LC-MS and automated molecular networking data analysis approach may be used for the rapid identification of both known as well as new variants of the MCs. The method does require a library of reference MS/MS spectra, but due to its inherent capacity to show the similarities between structurally related molecules, can be highly effective to identify even previously undescribed variants. Moreover, even though the GNPS website has only a few cyanotoxins at the present time, it was highly effective in helping to identify these cyanotoxins in the Green Lake bloom. It can be anticipated that future depositions to GNPS of cyanotoxin high resolution MS/MS spectra will further improve the effectiveness of the method. We are currently working to build such a library using both standards and the cyanotoxins present in cultured and field samples.

In this study, we were able to determine the putative structure of the new MC-MhtyR (6) even though the compound was present in the sample in insufficient quantities for NMR analysis. This took advantage of a microscale methylation reaction with (trimethylsilyl)diazomethane that was used here for the first time in the structural analysis of a natural product.

The predominant cyanobacterium in the 2014 Green Lake bloom was identified as a strain of *Microcystis aeruginosa*. Fragments of the putative adenylation domains belonging to the microcystin and ferintoic acid gene clusters were also identified. Their sequences, especially those that determine substrate selectivity, matched well with the structures of the identified cyanotoxins from the bloom sample. Therefore, it appears very likely that the fragments found in the metagenomic DNA from the Green Lake blooms are indeed part of the microcystin and ferintoic acid gene clusters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Molecular network from Seattle Green Lake organic extract with a cosine similarity score cutoff of 0.60. Nodes are labeled with parent m/z ratio; edge thickness is related to cosine similarity score.

Figure 2.

(a) Structures of MC congeners found in Green Lake, Seattle. (b) The MC cluster from the Green Lake extract. Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; MeAsp is β -methyl aspartic acid; Mdha is *N*-methyldehydroalanine; (H₄)Tyr is 1,2,3,4-tetrahydrotyrosine; Htyr is homotyrosine; Mhtyr is *N*-methylhomotyrosine.

fragment ion	MC-LR (1)	MC-FR (2)	MC-YR (3)	MC-(H ₄)YR (4)	MC-HtyR (5)	MC-MhtyR (6)
[M+H] ⁺	995.5535 C ₄₉ H ₇₅ N ₁₀ O ₁₂ ⁺	1029.5361 C ₅₂ H ₇₃ N ₁₀ O ₁₂ ⁺	1045.5320 C ₅₂ H ₇₃ N ₁₀ O ₁₃ ⁺	1049.5633 C ₅₂ H ₇₇ N ₁₀ O ₁₃ ⁺	1059.5480 C ₅₃ H ₇₅ N ₁₀ O ₁₃ ⁺	1073.5630 C ₅₄ H ₇₇ N ₁₀ O ₁₃ ⁺
a	861.4803 C ₄₀ H ₆₅ N ₁₀ O ₁₁ ⁺	895.4702 C ₄₃ H ₆₃ N ₁₀ O ₁₁ ⁺	911.4591 C ₄₃ H ₆₃ N ₁₀ O ₁₂ ⁺	915.4967 C ₄₃ H ₆₇ N ₁₀ O ₁₂ ⁺	925.4768 C ₄₄ H ₆₅ N ₁₀ O ₁₂ ⁺	939.4926 C ₄₅ H ₆₇ N ₁₀ O ₁₂ ⁺
b	553.3073 C ₂₄ H ₄₁ N ₈ O ₇ ⁺	587.2954 C ₂₇ H ₃₉ N ₈ O ₇ ⁺	603.2861 C ₂₇ H ₃₉ N ₈ O ₈ ⁺	607.3217 C ₂₇ H ₄₃ N ₈ O ₈ ⁺	617.3033 C ₂₈ H ₄₁ N ₈ O ₈ ⁺	631.3190 C ₂₉ H ₄₃ N ₈ O ₈ ⁺
c	470.2704 C ₂₀ H ₃₆ N ₇ O ₆ ⁺	504.2580 C ₂₃ H ₃₄ N ₇ O ₆ ⁺	520.2494 C ₂₃ H ₃₄ N ₇ O ₇ ⁺	524.2840 C ₂₃ H ₃₈ N ₇ O ₇ ⁺	534.2662 C ₂₄ H ₃₆ N ₇ O ₇ ⁺	548.2818 C ₂₅ H ₃₈ N ₇ O ₇ ⁺
d	599.3529 C ₃₁ H ₄₇ N ₆ O ₆ ⁺	599.3568 C ₃₁ H ₄₇ N ₆ O ₆ ⁺	599.3529 C ₃₁ H ₄₇ N ₆ O ₆ ⁺	599.3570 C ₃₁ H ₄₇ N ₆ O ₆ ⁺	599.3542 C ₃₁ H ₄₇ N ₆ O ₆ ⁺	599.3542 C ₃₁ H ₄₇ N ₆ O ₆ ⁺
e	682.3899 C ₃₅ H ₅₂ N ₇ O ₇ ⁺	682.3944 C ₃₅ H ₅₂ N ₇ O ₇ ⁺	682.3899 C ₃₅ H ₅₂ N ₇ O ₇ ⁺	682.3945 C ₃₅ H ₅₂ N ₇ O ₇ ⁺	682.3913 C ₃₅ H ₅₂ N ₇ O ₇ ⁺	682.3915 C ₃₅ H ₅₂ N ₇ O ₇ ⁺
f	753.4269 C ₃₈ H ₅₇ N ₈ O ₈ ⁺	753.4319 C ₃₈ H ₅₇ N ₈ O ₈ ⁺	753.4266 C ₃₈ H ₅₇ N ₈ O ₈ ⁺	753.4315 C ₃₈ H ₅₇ N ₈ O ₈ ⁺	753.4284 C ₃₈ H ₅₇ N ₈ O ₈ ⁺	753.4289 C ₃₈ H ₅₇ N ₈ O ₈ ⁺
g	866.5109 C ₄₄ H ₆₈ N ₉ O ₉ ⁺	900.5008 C ₄₇ H ₆₆ N ₉ O ₉ ⁺	916.4899 C ₄₇ H ₆₆ N ₉ O ₁₀ ⁺	920.5272 C ₄₇ H ₇₀ N ₉ O ₁₀ ⁺	930.5075 C ₄₈ H ₆₈ N ₉ O ₁₀ ⁺	944.5231 C ₄₉ H ₇₀ N ₉ O ₁₀ ⁺
h	882.4693 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺	882.4753 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺	882.4692 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺	882.4752 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺	882.4714 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺	882.4709 C ₄₃ H ₆₄ N ₉ O ₁₁ ⁺
i	728.3953 C ₃₆ H ₅₄ N ₇ O ₉ ⁺	728.4000 C ₃₆ H ₅₄ N ₇ O ₉ ⁺	728.3950 C ₃₆ H ₅₄ N ₇ O ₉ ⁺	728.4000 C ₃₆ H ₅₄ N ₇ O ₉ ⁺	728.3969 C ₃₆ H ₅₄ N ₇ O ₉ ⁺	728.3969 C ₃₆ H ₅₄ N ₇ O ₉ ⁺
j	783.4740 C ₄₀ H ₆₃ N ₈ O ₈ ⁺	817.4633 C ₄₃ H ₆₁ N ₈ O ₈ ⁺	833.4529 C ₄₃ H ₆₁ N ₈ O ₉ ⁺	837.4896 C ₄₃ H ₆₅ N ₈ O ₉ ⁺	847.4701 C ₄₄ H ₆₃ N ₈ O ₉ ⁺	861.4859 C ₄₅ H ₆₅ N ₈ O ₉ ⁺
k	866.5109 C ₄₄ H ₆₈ N ₉ O ₉ ⁺	900.5008 C ₄₇ H ₆₆ N ₉ O ₉ ⁺	916.4899 C ₄₇ H ₆₆ N ₉ O ₁₀ ⁺	920.5272 C ₄₇ H ₇₀ N ₉ O ₁₀ ⁺	930.5075 C ₄₈ H ₆₈ N ₉ O ₁₀ ⁺	944.5231 C ₄₉ H ₇₀ N ₉ O ₁₀ ⁺
l	446.2271 C ₂₃ H ₃₂ N ₃ O ₆ ⁺	446.2301 C ₂₃ H ₃₂ N ₃ O ₆ ⁺	446.2267 C ₂₃ H ₃₂ N ₃ O ₆ ⁺	446.2298 C ₂₃ H ₃₂ N ₃ O ₆ ⁺	446.2028 C ₂₃ H ₃₂ N ₃ O ₆ ⁺	446.2278 C ₂₃ H ₃₂ N ₃ O ₆ ⁺
m	375.1903 C ₂₀ H ₂₇ N ₂ O ₅ ⁺	375.1926 C ₂₀ H ₂₇ N ₂ O ₅ ⁺	375.1902 C ₂₀ H ₂₇ N ₂ O ₅ ⁺	375.1929 C ₂₀ H ₂₇ N ₂ O ₅ ⁺	375.1910 C ₂₀ H ₂₇ N ₂ O ₅ ⁺	375.1910 C ₂₀ H ₂₇ N ₂ O ₅ ⁺

Figure 3. High-resolution MS/MS fragmentations of MCs. Fragments which are mass shifted and therefore contain the variable amino acid residue are labeled in red, while the non-shifting fragments are labeled in blue.

fragment ion	7	8
$[M+H]^+$	1009.5712	1073.5678
<i>a</i>	875.4977	939.4926
<i>b</i>	553.3085	617.3035
<i>c</i>	470.2712	534.2662
<i>d</i>	613.3700	613.3701
<i>e</i>	696.4069	696.4069
<i>f</i>	767.4443	767.4451
<i>g</i>	880.5282	944.5233
<i>h</i>	896.4866	896.4882
<i>i</i>	742.4127	N/D
<i>j</i>	N/D	N/D
<i>k</i>	N/D	N/D
<i>l</i>	460.2435	460.2437
<i>m</i>	389.2067	389.2067

Figure 4.

Structures of methylated MCs obtained by methylation of standard samples of MC-LR (1) and MC-YR (3) and of MC-HtyR (5) and MC-MhtyR (6) from the Green Lake extract, and high-resolution MS/MS fragmentations of the methylated standards which were used to localize the sites of methylation. Fragments that are mass shifted and therefore contain the variable amino acid are labeled in red whereas the non-shifting fragments are labeled in blue. Fragment ions are denoted as shown in Figure 3.

Figure 5.
(a) Structures of ferintoic acids. (b) The ferintoic acid cluster from the Green Lake extract. MetO is methionine sulfoxide.

fragment ion	ferintoic acid A (11)	ferintoic acid C (12)	ferintoic acid D (13)
$[M+H]^+$	867.4423 $C_{46}H_{59}N_8O_9^+$	899.4140 $C_{46}H_{59}N_8O_9S^+$	915.4087 $C_{46}H_{59}N_8O_{10}S^+$
<i>a</i>	690.3629 $C_{36}H_{48}N_7O_7^+$	722.3352 $C_{36}H_{48}N_7O_7S^+$	738.3306 $C_{36}H_{48}N_7O_8S^+$
<i>a</i> - H_2O	672.3522 $C_{36}H_{46}N_7O_6^+$	704.3246 $C_{36}H_{46}N_7O_6S^+$	720.3197 $C_{36}H_{46}N_7O_7S^+$
<i>b</i>	591.2941 $C_{31}H_{39}N_6O_6^+$	591.2943 $C_{31}H_{39}N_6O_6^+$	591.2945 $C_{31}H_{39}N_6O_6^+$
<i>b</i> - H_2O	573.2834 $C_{31}H_{37}N_6O_5^+$	573.2836 $C_{31}H_{37}N_6O_5^+$	573.2838 $C_{31}H_{37}N_6O_5^+$
<i>c</i> + CO + H_2O	405.2144 $C_{20}H_{29}N_4O_5^+$	405.2145 $C_{20}H_{29}N_4O_5^+$	405.2147 $C_{20}H_{29}N_4O_5^+$
<i>c</i> + H_2O	379.2352 $C_{19}H_{31}N_4O_4^+$	379.2352 $C_{19}H_{31}N_4O_4^+$	379.2354 $C_{19}H_{31}N_4O_4^+$
<i>d</i>	782.3896 $C_{42}H_{52}N_7O_8^+$	814.3619 $C_{42}H_{52}N_7O_8S^+$	830.3572 $C_{42}H_{52}N_7O_9S^+$
<i>d</i> - H_2O	764.3792 $C_{42}H_{50}N_7O_7^+$	796.3512 $C_{42}H_{50}N_7O_7S^+$	812.3464 $C_{42}H_{50}N_7O_8S^+$
<i>e</i>	362.2087 $C_{19}H_{28}N_3O_4^+$	394.1807 $C_{19}H_{28}N_3O_4S^+$	410.1759 $C_{19}H_{28}N_3O_5S^+$
<i>f</i>	663.3518 $C_{35}H_{47}N_6O_7^+$	695.3242 $C_{35}H_{47}N_6O_7S^+$	711.3193 $C_{35}H_{47}N_6O_8S^+$
<i>f</i> - CO	637.3725 $C_{34}H_{49}N_6O_6^+$	669.3448 $C_{34}H_{49}N_6O_6S^+$	685.3401 $C_{34}H_{49}N_6O_7S^+$

Figure 6.

High-resolution MS/MS fragmentations of ferintoic acids A, C and D. Fragments that are mass shifted and therefore contain the variable amino acid are labeled in red, while the non-shifting fragments are labeled in blue.

Adenylation domains of the *Microcystis* present in the Green Lake bloom sample and other reported *Microcystis* strains.

Table 1

<i>Microcystis</i> sp.	Substrates	Binding pocket signatures identified in McyBAd1 (residue position ^a)									
		235	236	239	278	299	301	322	330	331	
SEAGL14	Leu, Phe, Tyr, (H ₄)Tyr, Mhty, Hty,	D	G	W	T	I	G	A	V	K	
N-C 171/10 ^b	Leu, Tyr, Arg	D	G	W	T	I	G	A	V	E	
NIES102 ^b	Leu, Tyr, Arg	D	G	W	T	I	G	A	V	E	
HUB 5-2-4-like ^c	Leu, Arg	D	G	W	T	I	G	A	V	E	

^a As described by Conti *et al.*⁴⁷

^b Data reported by Tooming-Klunderud *et al.*⁴⁶

^c Data reported by Mikalsen *et al.*⁴⁸