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# Seven new microcystin variants discovered from a native *Microcystis aeruginosa* strain – unambiguous assignment of product ions by tandem mass spectrometry

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**RATIONALE:** High-resolution mass spectrometry was applied to the study of a *Microcystis aeruginosa* strain previously reported as a [D-Leu[1]]MC-LR producer. Detailed analysis revealed new microcystin (MC) variants produced from the strain, and seven of these were previously unreported variants. This work shows the importance of mass accuracy for the identification of unknown MCs.
**METHODS:** The *M. aeruginosa* strain was isolated from a bloom sample collected from Argentina and acclimated to lab conditions. The MC variants in the strain were separated by UV/Vis detection-guided high-performance liquid chromatography, and their structures were unambiguous determined by tandem mass spectrometry (MS/MS).
**RESULTS:** A simple strategy was developed for quickly locating the low-abundance MC precursors from complex samples. MS/MS anlysis revealed ten MC variants produced from the strain, of which seven have never been reported before.
**CONCLUSIONS:** This work shows the interference of isobarics and isomers in the study of unknown MCs, and, therefore, high mass accuracy is important to avoid false assignments. Moreover, the peak list provided here (30–50 fragments unambiguously assigned for ten MCs) can be used as a reference for the discovery of MCs from environmental samples. 

Microcystins (MCs) are a group of cyanotoxins produced by different cyanobacterial genera such as *Microcystis*, *Nostoc*, *Anabaena*, *Anabaenopsis*, and *Hapalosiphon*.[1] They strongly adapt to environmental changes and rapidly form algal blooms in aquatic habitats.[2,3] MCs consist of a general cyclic heptapeptide structure, D-Ala$^1$-*X*$^2$-D-MeAsp$^3$-*Z*$^4$-Adda$^5$-D-Glu$^6$-Mdha$^7$, with variable amino acids, mostly at positions 2 and 4. Adda ((2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid) is an unusual amino acid, while Mdha is *N*-methyldehydroalanine.[4] The nomenclature of MCs uses the single-letter abbreviations for residues at positions 2 and 4 (e.g., MC-LR; Fig. 1). Accordingly, substitutions of amino acids are designated using standard abbreviations along with the position number; e.g., [Leu[1]]MC-LR refers to Leu at position 1. Over 90 variants of MCs have now been reported.[5–7] The most common structural substitutions occur at positions 2 and 4, and demethylation at positions 3 and 7.[8,9] In other cases, substitutions of Ala[1] for Leu, Ser, and methyl-esterification at Glu[6] have also been observed.[7]

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Studying MCs is important because of their harmful impact on both ecological systems and humans. MCs specifically inhibit protein serine/threonine phosphatases using the Adda and Mdha residues,[10,11] leading to increased phosphorylation in human cells and intrahepatic haemorrhage, haemodynamic shock, heart failure, and death.[4] It has also been reported that MCs inactivate tumor suppressor genes[12] and form nodules associated with morphologic changes in hepatocytes.[13] The lethal dose (LD) of MCs depends on their structures, with MC-LR exhibiting the lowest LD (50 $\mu g \cdot kg^{-1}$, i.p. $LD_{50}$ mouse). Consequently, unambiguous determination of MC variants produced by individual toxin strains is important for the environmental impact assessment and alga blooms control.

Initial attempts at determining the structures of MCs started in the late 1950s;[14] however, full structural identification did not succeed until 1984 using mass spectrometry (MS).[15] Several other analytical techniques have been implemented for the routine analysis of MC residues, including high-performance liquid chromatography (HPLC)-ultraviolet/visible (UV/Vis) detection[16] and nuclear magnetic resonance (NMR).[17] Obviously, the UV/Vis-based method cannot be readily applied to unknown compounds and analytical standards are not available for all microcystin analogues. NMR provides very meaningful data but does not offer the high detection sensitivity of mass spectrometry; MS also provides product ion information from selected precursor

 

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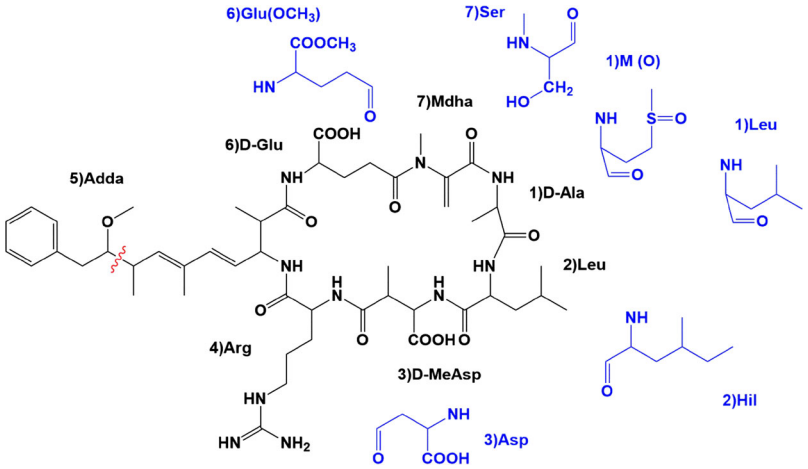


| Microcystin (MC) variants in CAAT 2005-3 | Formula | $[M+2H]^{2+}$ *m/z* | HPLC fraction | Reported before? |
|---|---|---|---|---|
| MC-LR | $C_{49}H_{74}N_{10}O_{12}$ | 498.28166 | 1 | yes |
| [Glu(OCH$_3$)$^6$]MC-LR | $C_{50}H_{76}N_{10}O_{12}$ | 505.28949 | 1 | yes |
| [M(O)$^1$]MC-LR | $C_{51}H_{78}N_{10}O_{13}S$ | 536.28080 | 1 | no |
| [M(O)$^1$,Glu(OCH$_3$)$^6$]MC-LR | $C_{52}H_{80}N_{10}O_{13}S$ | 543.28863 | 1 | no |
| [Leu$^1$]MC-LR | $C_{52}H_{80}O_{12}N_{10}$ | 519.30514 | 2 | yes |
| [Leu$^1$,Glu(OCH$_3$)$^6$]MC-LR | $C_{53}H_{82}O_{12}N_{10}$ | 526.31296 | 2 | no |
| [Leu$^1$,Ser$^7$]MC-LR | $C_{52}H_{82}N_{10}O_{13}$ | 528.31042 | 2 | no |
| [Leu$^1$,Glu(OCH$_3$)$^6$,Ser$^7$]MC-LR | $C_{53}H_{84}O_{13}N_{10}$ | 535.31824 | 2 | no |
| [Leu$^1$,Asp$^3$]MC-LR | $C_{51}H_{78}N_{10}O_{12}$ | 512.29731 | 3 | no |
| [Leu$^1$,Glu(OCH$_3$)$^6$]MC-HilR | $C_{54}H_{84}N_{10}O_{12}$ | 533.32079 | 3 | no |

**Figure 1.** *Top*: structure of MC-LR and numbering scheme for the amino acids (black), with the classical cleavage at the Adda being labelled; substitute units of MC variants found in this research are labelled in blue. *Bottom*: summary for the ten MCs found in CAAT 2005-3.

ions for detailed structural elucidations. For this reason, liquid chromatography/tandem mass spectrometry (LC/MS) utilizing collision-induced dissociation (CID) has become the most common and effective way for the identification of MCs.[7] Several limiting factors exist for MS/MS methods, however: first, variations between MCs are very small, making HPLC separations sometimes difficult.[18] Secondly, MCs are cyclic peptides, which require two backbone cleavages for fragmentation; that is, important predicted fragmentation reactions are not always seen in MS/MS experiments. For example, Mayumi *et al.* failed to determine the methylation of Asp or Leu in a simple MC-LR variant,[19] and Saito *et al.* could not assign the metal-binding site of MC-LR and MC-RR from Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) experiments.[20] Finally, most reported identification routines for MCs rely on only few characteristic product ions, even when using FTICRMS.[20,21] Such structural identification procedures are unreliable, as many of the fragments formed by CID are common among the MCs and multiple ring openings of MCs also lead to isobaric linear peptides, which cannot be resolved by low-resolution MS instruments.[22] In addition, multiple isomeric fragments are formed, which cannot be distinguished by MS (e.g., Mdha-Asp/Dha-MeAsp, Mdha/Dhb (dehydrobutyrine), or MeSer-Asp/Ser-MeAsp).

In our previous work, we described the precise localization of metal-binding sites for MCs using high-resolution FTICRMS.[23] Here, we applied detailed CID experiments on a native *Microcystis aeruginosa* strain, which was initially identified as a [D-Leu$^1$]MC-LR producer by Orbitrap mass spectrometry.[24] The analytical strategy[25,26] comprised purification of the extract by UV/Vis detection-guided fraction collection,[24] yielding several fractions that were subsequently analyzed by detailed CID experiments using high-resolution mass spectrometry. The product ions produced were examined with the support of previously reported fragmentation pathways as well as the characterization of a major component in the present fractions. FTICRMS enabled us to assign more than 30 unique product ions for each precursor ion, and we identified a total of ten MCs from the investigated toxic strain; seven of them were novel, previously unreported MC variants.

## EXPERIMENTAL

### CAAT 2005-3 sample

The bloom sample was collected from the drainage canal of a sewage treatment facility in Pila town (35°59′49″S, 58° 08′11″W), Buenos Aires province, Argentina. The *M. aeruginosa* strain was isolated from the bloom sample, acclimated to lab conditions and initially identified as a [D-Leu$^1$]MC-LR producer (CAAT 2005-3). Details of isolation and characterization can be found elsewhere.[24]



  

### Liquid chromatography

The extract was separated and concentrated by semi-preparative HPLC (HP Agilent 1100 system) on a Thermo Scientific Hyperprep HS $C_{18}$ column (250 × 10 mm, 5 µm) equilibrated with a mobile phase of deionized water (+0.05% trifluoroacetic acid (TFA), v/v)/35% acetonitrile (+TFA 0.05%) at a flow rate of 3 mL/min, and detected by UV (λ = 238nm). Identification was based on retention time and UV spectrum. Any peaks exhibiting MC-like behavior were collected. The fractions were collected, concentrated, desalted with a $C_{18}$ SPE cartridge (Thermo Scientific) and diluted 50-fold with methanol/water/formic acid (50:50:1, v/v/v) prior to offline mass spectrometric analysis.

### High-resolution mass spectrometry

The samples were analyzed by electrospray ionization (ESI)-FTICRMS on a solariX 7 Tesla instrument (Bruker, Bremen, Germany) equipped with an Infinity cell.[27] Sixteen individual transients were collected and co-added for each spectrum to enhance the signal-to-noise (S/N).[28] In MS/MS mode, precursor ions were isolated first in the quadrupole, externally accumulated in the hexapole for 0.1–2 s, and 4–28 eV collision energy was applied for CID. Peak assignment was based on matching both theoretical mass and isotopic patterns.

## RESULTS AND DISCUSSION

### CID method validation using the abundant main component

After separation and purification by HPLC, three 'MC-like' peaks (fractions 1–3) were collected for detailed MS analysis (the preparative HPLC separation is shown in Supplementary Fig. S1, Supporting Information). These fractions exhibited a complex composition in the initial full scan screening analysis: no MC peaks were seen in fraction 1 in the expected *m/z* region and the previously reported [Leu$^1$]MC-LR variant appeared as the major component (*m/z* 519.3051, $[M+2H]^{2+}$) in fraction 2. In addition, several tentative MC peaks were observed in both fractions 2 and 3, with doubly charged ions dominant over singly charged ions (Supplementary Fig. S2, Supporting Information, summarizes screening analyses of fractions 1–3). CID was used in our previous work to determine the structure of [Leu$^1$]MC-LR by means of six characteristic product ions.[24] The main [Leu$^1$]MC-LR species was initially investigated here and used to validate the structural elucidation scheme for novel MCs. MS/MS analysis of the isolated doubly charged precursor ion generated multiple product ions with significant signal-to-noise (S/N) ratios. As most of these species potentially have diagnostic value, efforts were made to assign as many of these peaks as possible (e.g., Supplementary Fig. S3, Supporting Information, illustrates the CID spectrum of the [Leu$^1$]MC-LR $[M+2H]^{2+}$ species). In this study, over 50 [Leu$^1$]MC-LR product ions were confidently identified by accurate mass measurements with relative mass errors <0.3 ppm. Most of them were complementary fragment pairs; detailed assignments are summarized in Supplementary Table S1, Supporting Information. The nomenclature was based on the general numbering scheme for MCs (Fig. 1). For example, in Supplementary Table S1 (Supporting Information), the fragment '1~4+$NH_2$' at *m/z* 529 designates that the fragment contains the amino acids at positions 1 to 4 in [Leu$^1$]MC-LR plus the '$NH_2$' group; the fragments '3~5-134' or '4~6-134' at *m/z* 465 refer to amino acids at positions 3 to 5 or 4 to 6 (these are isomers with same *m/z* value), minus the classical fragment seen with Adda: $[Phe\text{-}CH{=}CH\text{-}OMe]^+$ (*m/z* 134.0726). Overall, CID was an effective method for producing sufficient structural information from MCs, and, therefore, it was applied for studying other, less abundant MC variants in CAAT 2005-3.

### Discovery of new MC variants

Full scan mass spectra of the three isolated fractions exhibited complex compositions, from which several tentative MC compounds were assigned (Supplementary Fig. S2, Supporting Information). It is likely that further low-abundance MC species existed in these fractions, but their signal intensities were of too low S/N or they were suppressed by co-components in the complex extracts.[29,30] Here, a simple strategy was developed for quickly deducing the *m/z* values of low-abundance MCs in complex mass spectra. The method utilized the classic MC side-chain cleavages seen for Adda or demethyl-Adda (DMAdda).[31] Species with *m/z* values between 440 and 580 were isolated and accumulated in the linear ion trap first, because all doubly charged MCs exhibit peaks in this *m/z* region. Subsequently, CID at 10 eV was applied, which was sufficient to cleave Adda or DMAdda (Fig. 1) without triggering extensive fragmentation. The product ions at *m/z* 135 and 121 (corresponding to Adda and DMAdda, respectively) and signals of ions >*m/z* 800 were monitored. For example, in fraction 1, the spectra in the high *m/z* range were very simple after precursor separation and fragmentation (Fig. 2), and the peaks probably originated from MCs minus the Adda/DMAdda residues. As can be seen in Fig. 2, the product ions at *m/z* 135, 861, 875, 937, and 951 indicated four

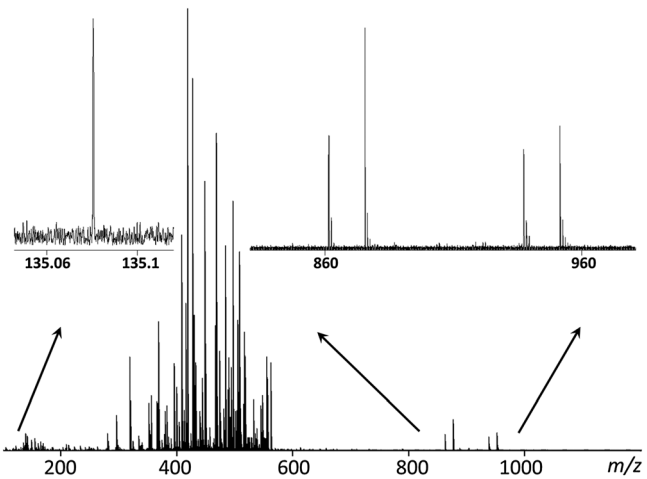


**Figure 2.** CID spectrum of HPLC fraction 1 (precursor ions from *m/z* 440–580 were isolated in the quadrupole). The insets show the x-axis expansion of the Adda fragment (left) and the product ions seen for higher *m/z* values, due to the loss of Adda (right).

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possible MC variants. Their corresponding doubly charged species were expected at *m/z* 498, 505, 536, and 543. These predicted species were isolated and accumulated next, to increase the S/N. As a result, abundant product ions were observed from the CID experiments (Supplementary Tables S2–S5, Supporting Information). The four species were unambiguously identified as MC-LR, [Glu$(OCH_3)^6$]MC-LR, [M$(O)^1$]MC-LR, and [M$(O)^1$,Glu$(OCH_3)^6$]MC-LR (for the structures, see Fig. 1). To our knowledge, the latter two species are entirely new, unreported MC variants. The same methodology was then applied to HPLC fractions 2 and 3, and, surprisingly, five further novel MC variants were discovered (Supplementary Tables S6–S10, Supporting Information). These newly discovered MCs were not reported in the previous study on CAAT 2005-3,[24] because their signal intensities were too low for discovery in full scan experiments. The strategy used here proved to be an efficient way for rapidly discovering low-abundance MCs from a complex environmental sample.

### Unambiguous assignment of product ions

Most LC/MS routines used today for the discovery of new MC variants identify compounds by means of 5–10 unique product ions from CID.[7] In our opinion, it is not sufficient to use only the few most abundant product ions for this purpose. First, differences in molecular weights of MCs can be very small, as seen, for example, for the investigated precursor ions of [Leu$^1$,Glu$(OCH_3)^6$]MC-HilR, [Leu$^1$,Glu$(OCH_3)^6$,Ser$^7$]MC-LR, and [M$(O)^1$]MC-LR (Fig. 1). Precursor ions with close *m/z* values are easily co-isolated prior to MS/MS and may then produce false positive fragment identifications. Secondly, multiple ring openings on MCs lead to isobaric linear peptides, which complicate the assignment of product ions. Figure 3 exemplifies this situation, where the *m/z* values of two signal duplets for CID fragments were only 3.3 and 5.8 ppm apart, and these

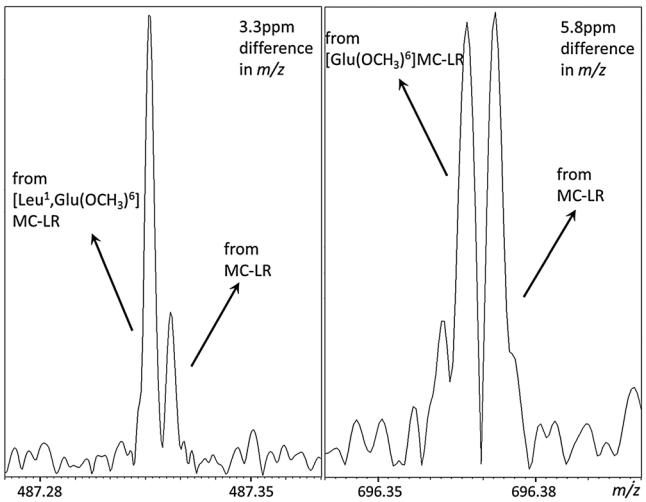


**Figure 3.** CID spectra showing close product ions from different MC precursors. *Left*: $[M–C_2H_6O_3]^+$ ion from [Leu$^1$,Glu$(OCH_3)^6$]MC-LR and fragment [1~4+$NH_2$] from MC-LR (3.3 ppm *m/z* difference). *Right*: fragment [6~4] from [Glu$(OCH_3)^6$]MC-LR and fragment [3~6-134] from MC-LR (5.8 ppm *m/z* difference). See Supporting Information for more details.

cannot be distinguished by low-resolution, and even many high-resolution time-of-flight mass spectrometers. These isobaric product ions originate from different MC precursor species, and this easily leads to false negative assignments. For these reasons, complete and unambiguous product ion assignments with high mass accuracy are recommended for the unequivocal identification of novel MCs. In this work, more than 30 fragments were unambiguously assigned for the 10 MC variants found in CAAT 2005-3. Moreover, the peak list provided in the Supporting Information can be used as a useful template of diagnostic ions for identifying MCs or similar compounds in other MC studies.

## CONCLUSIONS

A detailed HRMS study of a *M. aeruginosa* strain revealed nine new MC variants, seven of which have not been reported in the literature previously. The study demonstrated a simple routine to quickly identify low-abundance MCs from complex samples. The provided fragment list can serve as a useful reference for other researchers.

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## SUPPORTING INFORMATION

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