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Identification of Microcystin Toxins from a Strain of *Microcystis aeruginosa* by Liquid Chromatography Introduction into a Hybrid Linear Ion Trap-Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

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The cyclic heptapeptide microcystin toxins produced by a strain of *Microcystis aeruginosa* that has not been investigated previously were separated by liquid chromatography and identified by high-accuracy *m/z* measurements of their $[M + H]^+$ ions and the fragment ions produced by collision-activated dissociation of the $[M + H]^+$ ions. The cyanobacteria B2666 strain was cultured in a standard growth medium, and the toxins were released from the cells, extracted from the aqueous phase, and concentrated using standard procedures. The microcystins were separated by reversed-phase microbore liquid chromatography and introduced directly into a hybrid linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer with electrospray ionization. The known microcystins (MC) MC-LR, MC-LA, [MeSer^7]MC-LR, MC-LL, MC-LF, and MC-L(Aba) were identified along with the two previously unreported structural variants [Asp^3]MC-LA and [Asp^3]MC-LL. In addition to the $[M + H]^+$ ions, accurate *m/z* measurements were made of 12−18 product ions for each identified microcystin. The mean difference between measured and calculated exact *m/z* was less than 2 parts per million, which often allowed assignment of unique compositions to the observed ions. A mechanism is presented that accounts for an important collision-activated dissociation process that gives valuable sequence ions from microcystins that do not contain arginine. The analytical technique used in this work is capable of supporting fairly rapid and very reliable identifications of known microcystins when standards are not available and of most structural variants independent of additional information from other analytical techniques.

Approximately 67 cyclic heptapeptide microcystin (MC) toxins produced by several genera and species of cyanobacteria have been reported during the last ~20 years.[1−3] Considerable information is available about the environmental conditions that promote cyanobacteria growth, the species that produce microcystins, the extraction of toxins from cells and water samples, biological and chromatographic/spectroscopic test methods, mechanisms of toxicity, structures of toxins, water treatment options, and public health issues.[1,2] Despite the significant amount of available information, interest in microcystins continues to be very strong because of the known hazards to farm animals, people, pets, and wildlife through exposure to toxins in drinking and recreational waters. Approximately 50% of the population of the United States receives drinking water from surface sources that could be susceptible to cyanobacteria blooms. Because of generally increasing eutrophication in those bodies of water, blooms are more frequent, especially in the eastern and southern states. Furthermore, the species that inhabit those waters, even within a relatively small geographic area, are often different and produce different mixtures of toxins. Therefore, it is important to have analytical methods to reliably and reasonably quickly determine the distribution of toxins in water samples.

Scheme 1 shows the structure of MC-LR, which is often cited and studied because it may be the most prevalent and one of the most potent toxins of the group. The L-amino acids in ring positions 2 and 4, respectively, are the most variable of the seven amino acids in the microcystins. Accordingly, these cyclic heptapeptides are named by appending the single-letter abbreviations of the amino acids present in positions 2 and 4. The microcystin that has a leucine (Leu) in position 2 and an arginine (Arg) in position 4 is therefore microcystin-LR (Scheme 1). If an amino acid residue in one of these positions is not one of the 20 standard amino acids, the three-letter abbreviation is used. Other amino

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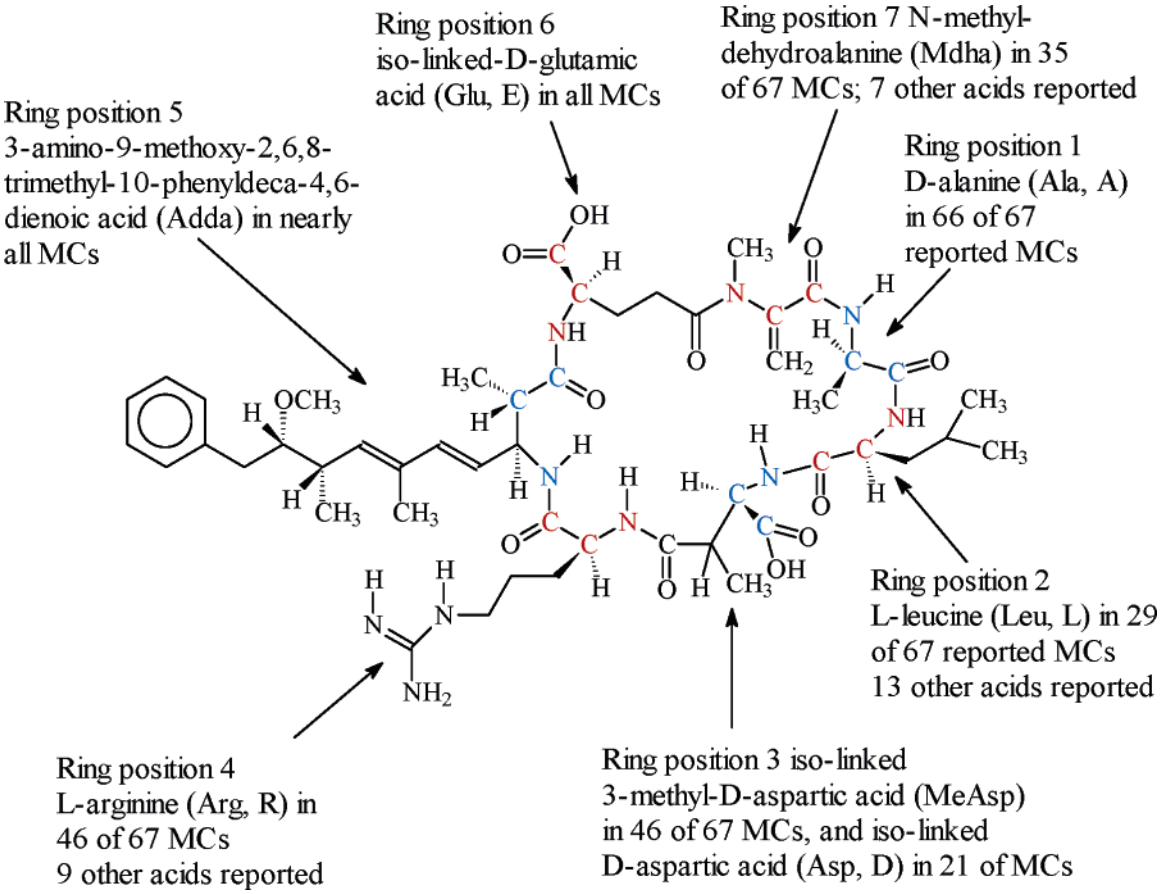
Scheme 1



acids found in positions 2 or 4 include alanine (A), glutamic acid (E), homoarginine (Har), homotyrosine (Hty), methionine (M), phenylalanine (F), tryptophan (W), and tyrosine (Y). Much less variability is found in most other microcystin ring positions. The amino acid residue in position 1 is highly conserved, and D-alanine is present in 66 of the 67 reported microcystins. Erythro-3-methyl-D-aspartic acid (MeAsp) is present in position 3 in most reported microcystins; however, it is replaced by D-aspartic acid (Asp) in about one-third of the reported structures. Those two dicarboxylic acids, and D-glutamic acid in position 6, are inserted into the cyclic peptide structure in the iso configuration, that is, using the carboxylic acid groups remote from carboxylic acid group on the α-carbon.[4−5] The most characteristic amino acid in the microcystin family is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) in ring position 5.[4] The methyl of the 9-methoxy group is either missing or replaced by an acetoxy group in only 10 of the 67 reported microcystins. D-Glutamic acid (Glu) is highly conserved in position 6 with just a few Glu methyl esters reported in that position. The amino acid residues in position 7 are also variable; however, they are not as diverse as those in positions 2 and 4. *N*-Methyldehydroalanine (Mdha) is found in position 7 in 35 of the 67 reported microcystins. Other amino acids reported in position 7 include dehydroalanine (Dha), serine (Ser), dehydrobuterine (Dhb), *N*-methylserine (MeSer), and Ala. Substitutions in positions 1, 3, and 5−7 are designated by the standard amino acid abbreviation and the position number of the amino acid residue, for example, [D-Asp^3]microcystin-LR.

Previous reports state that as many as 12 microcystins are released from lysed cells from a single strain of cyanobacteria.[6−8] The complexity of the mixture of microcystins and other substances that are released from these cells presents a significant analytical challenge. During the early discovery years, considerable effort was expended to isolate and purify sufficient quantities of the individual toxins, and a regimen of analytical techniques was used to determine their structures.[4−8] The amino acids were identified by complete hydrolysis of the peptide bonds of a pure toxin, derivatization of the free amino acids, and separation of the derivatives with liquid chromatography (LC) or gas chromatography (GC). Absolute configurations were determined by LC or GC with chiral columns. Purified toxins were subjected to ring-opening reactions to allow sequencing of the amino acid residues in isolated linear peptides or their derivatives. Sequencing was accomplished by Edman degradation, electron ionization mass

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Table 1. Identification of Microcystins in an Extract of *M. aeruginosa* Strain B2666

retention time (min)	microcystin identified	calculated m/z $[M + H]^+$	mean measured m/z ($n = 8$)	mean error (ppm)	relative amounts[b]
24.63	[MeSer^7]MC-LR	1013.566 62	1013.5663	0.32	0.010
25.15	MC-LR[a]	995.556 05	995.5555	0.55	1.0
27.70	[Asp^3]MC-LA	896.476 40	896.4765	0.11	0.006
28.29	MC-LA[a]	910.492 05	910.4924	0.38	0.133
29.28	MC-L(Aba)	924.507 70	924.5078	0.11	0.003
30.66	[Asp^3]MC-LL	938.523 35	938.5240	0.69	0.004
31.63	MC-LF[a]	986.523 35	986.5241	0.76	0.002
31.89	MC-LL	952.539 00	952.5395	0.53	0.003

[a] Commercial standard available. [b] Estimated by integrating the abundance of the accurately measured $[M+H]^+$ ion ± 0.05 Da over the LC peak and assuming all response factors are unity.

spectrometry (MS),[4] and later by collision-activated dissociation (CAD) of $[M + H]^+$ and $[M + 2H]^{2+}$ ions formed by Cs^+ ion or fast atom bombardment ionization.[5−8] Before about 1993, the toxins, their hydrolysis and degradation products, and derivatives were introduced into low- and high-resolution mass spectrometers with direct insertion probes or flow injection, and molecular compositions were determined by accurate m/z measurements of $[M + H]^+$ and $[M + 2H]^{2+}$ ions. Deuterium exchange reactions and ^1H nuclear magnetic resonance (NMR) or MS were used to determine the iso linkage of the MeAsp and Glu residues.[4−5] Proton and ^{13}C NMR spectra also provided the structure of Adda,[4] confirmation of amino acid residues present,[7−8] and sequence of amino acid residues in microcystins.[9]

During recent years and after the structures of many microcystins were either firmly established or proposed, investigators have tended to rely mainly on LC/MS and LC/MS/MS with electrospray ionization for identifications of microcystins released from cells or found in water samples. This strategy is generally satisfactory for those toxins with well-established structures, especially when analytical or commercial standards are available. However, new structural variants may not be amenable to identification with MS techniques alone, and often the quantities of toxins in a sample are too small to allow isolation, purification, and investigation with other techniques. Electrospray ionization of microcystins yields mainly the $[M + H]^+$ ion and, depending on structure, a $[M + 2H]^{2+}$ ion, and few fragment ions.[3,10] Single-stage MS/MS with low-resolution m/z measurements, for example, with a triple quadrupole or ion trap mass spectrometer, yields insufficient information to unequivocally determine a structure. There are many reports where firm identifications could not be made or structures could not be confirmed with low-resolution LC/MS/MS [11−19] or with LC/MS/MS/MS.[20] One

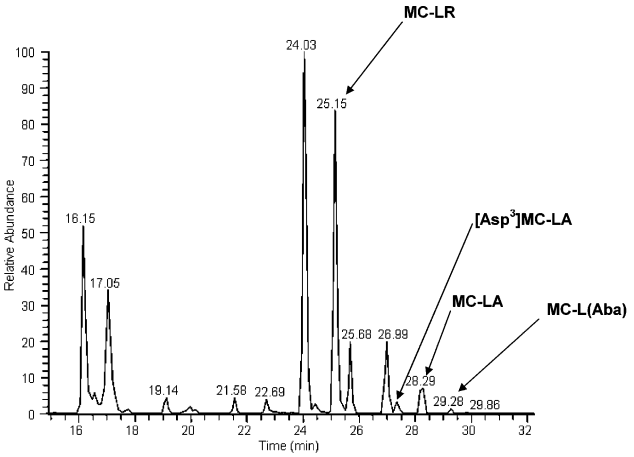

Figure 1. Base peak intensity chromatogram from an extract of *M. aeruginosa* strain B2666 measured on the linear ion trap. The identified microcystins are in Table 1. The peaks that appear before 24.5 min, and a few after, are generally nonmicrocystin sample components that are not identified; however, several of these may also contain microcystins that have not been identified.

significant problem is that different microcystins sometimes have $[M + H]^+$ ions with the same integer m/z. For example, the $[M + H]^+$ ions of the two plausible microcystins MC-ER and [Asp^3]-MC-LW both have an integer m/z of 1011, and their calculated exact m/z differ by only 4 parts-per-million (ppm). However, these ions can be distinguished with m/z measurement accuracy of 2 ppm or less. Another significant problem is that fragment ions obtained by CAD often have multiple plausible compositions with the same integer m/z but different calculated exact m/z. For example, a microcystin m/z 385 fragment ion could be assigned the composition of the protonated tetrapeptide Ala-Leu-MeAsp-Ala, the protonated tripeptide Leu-Asp-Arg, or the protonated dipeptide Ala-Adda. However, these integer isobaric ions can be easily distinguished by high-accuracy m/z measurements.

The development and commercialization of a hybrid linear ion trap-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer instrument system with the capabilities of microbore

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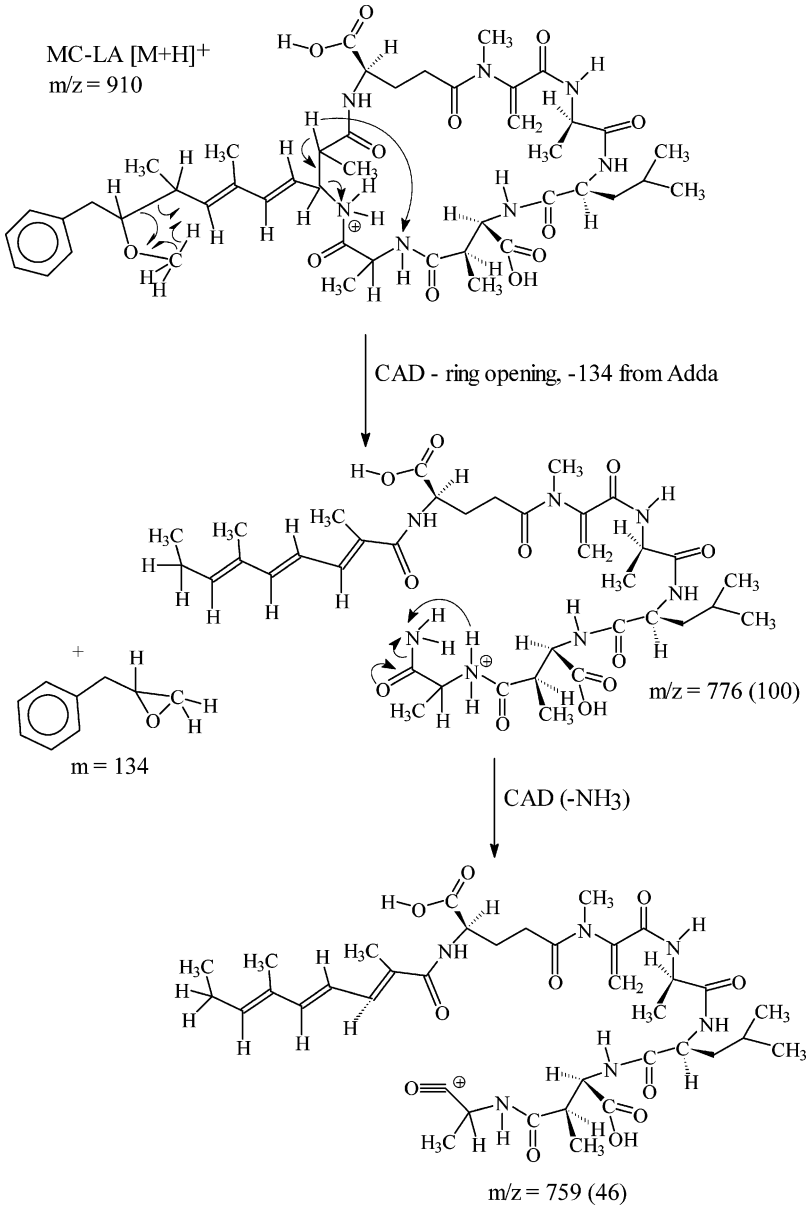
Scheme 2



LC sample introduction, high-accuracy m/z measurements, and multistep tandem mass spectrometry (MS^n) suggested that significantly more information about microcystin structures could be obtained with this instrument system.[3] High-accuracy m/z measurements are defined as generally within a few ppm of the exact m/z values calculated from the nuclide masses and the ionic charge z. The abbreviation MS^n designates multistep tandem mass spectrometry, where n is the number of ionization and ion activation steps in the process. The focus of this work was to determine whether new microcystin structural variants, and microcystins that have no standards available, can be rapidly identified using this instrument system. This potential capability is important because the determination of the microcystins in water samples may be required in emergencies when results are needed within a time period that precludes analyses with other techniques or when the quantities of individual microcystins in a sample are too small for isolation and purification of individual peptides for analyses with other analytical techniques. The toxins investigated were produced by the B2666 strain of *Microcystis aeruginosa* that has not been studied to identify the microcystins it produces. This strain was obtained from The University of Texas algal culture collection, and it was selected by the USEPA as a test system to study treatment options for production of drinking water from cyanobacteria-contaminated source water. Therefore, it was important to have detailed knowledge of the microcystins produced by this strain.

EXPERIMENTAL SECTION

Safety. Microcystins may be fatal if inhaled, absorbed through the skin, or swallowed. They may cause respiratory tract, eye, and skin irritation and liver damage. Dilute solutions should be handled with laboratory gloves, eye protection, and protective clothing. If solids are weighed, this should be done with suitable ventilation and respiratory equipment. Spills may be cleaned with methanol, which is a good solvent for microcystins, and flushed with water.

Table 2. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of MC-LA

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
468.2446	3	Mdha-Ala-Leu-MeAsp-Ala-H	468.24528	1.4
509.2643	7	Adda-Glu-Mdha-H (-NH_3)	509.26462	0.63
559.3123	10	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	0.59
580.3026	9	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	1.5
597.2855	4	Glu-Mdha-Ala-Leu-MeAsp-Ala-H	597.28787	4.0
661.3566	3	Adda-Glu-Mdha-Ala-Leu-H (-CH_3OH) (-NH_3)	661.35959	4.5
693.3860	12	Adda-Glu-Mdha-Ala-Leu-H (-NH_3)	693.38580	0.29
758.4087	22	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-134Adda)(-H_2O)	758.40833	0.49
759.3922	46	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-MeAsp-Ala (-134 Adda) (-NH_3)	759.39235	0.20
776.4188	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-134 Adda)	776.41889	0.12
809.4427	4	Adda-Glu- (-H_2O) | Ala-MeAsp-Leu-Ala-H	809.44438	2.1
860.4571	3	Adda-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-CH_3OH) (-H_2O)	860.45528	2.1
861.4349	5	Adda-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-CH_3OH) (-NH_3)	861.43930	5.1
878.4655	15	Adda-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-CH_3OH)	878.46584	0.39
892.4812	33	Adda-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-H_2O)	892.48149	0.33
893.4656	22	Adda-Glu-Mdha-Ala-Leu-MeAsp-Ala-H (-NH_3)	893.46551	0.10

[a] Relative abundance. [b] Mean error 1.49 ppm.

Materials. The B2666 toxin-producing strain of *M. aeruginosa* was obtained from The University of Texas algal culture collection. Commercial standards of microcystin-LR, -RR, -YR, -LA, -LF, -LW, and nodularin with a stated purities of 95% were purchased from Calbiochem (San Diego, CA) and used without further purification. Water was from a Millipore (Bedford, MA) Milli-Q water system. Optima grade acetonitrile and methanol with 99.9% stated purity were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid, with 99% purity, was purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of the toxins were prepared by dissolving an appropriate amount of each toxin in a 50% (v/v) methanol−water solution to yield a 250 ng/μL stock solution. The stock solutions were then diluted to prepare the sample solution that contained 1 ng/μL of each toxin. Empore 47-mm C-18 extraction disks and an apparatus to support the disks and provide vacuum assist during the extraction were obtained from Varian Instruments (Walnut Creek, CA).

Cell Culturing and Extraction of Toxins. The B2666 strain was cultured in BG-11 growth media under diurnal illumination. The suspension of cells was washed with dechlorinated tap water and centrifuged, and the toxins were released from the cells by freeze−thaw cycling. The resulting suspension was centrifuged, and the toxins were extracted from 175 mL of the supernatant liquid by filtration through a C-18 silica-impregnated Teflon membrane extraction disk with a slight vacuum assist. The toxins were eluted from the disks with 10 mL of methanol, and the effluent was concentrated to ~1 mL with a gentle stream of nitrogen.

Liquid Chromatography. A Thermo Electron Surveyor autosampler and LC system (San Jose, CA) was used with the hybrid linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer and operated in gradient flow mode at 50 μL/min with a BetaBasic (Thermo Electron, Bellefont, PA) 1 mm (i.d.) × 100 mm C18 column. The binary mobile phase consisted of (A) 0.1% formic acid in H_2O (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). The initial mobile-phase composition of 95% A and 5% B (v/v) was held for 2 min, then the fraction of B was increased linearly to 50% over 20 min, followed by a reduction to 40% B for 3 min. The injection volume was 10 μL.

Mass Spectrometry. The tandem instrument was a LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany), which combines a two-dimensional (2-D) linear ion trap MS [21,22] with a 7-T FT-ICR-MS via a series of transfer octapoles. A diagram of this instrument system has been published.[3] The linear ion trap pressure was maintained at 10^{-5} Torr with helium, which is used for trapping and collisional activation of trapped ions. The linear ion trap was equipped with a dual orthogonal detection system to allow operation as a separate mass spectrometer. A full-scan MS

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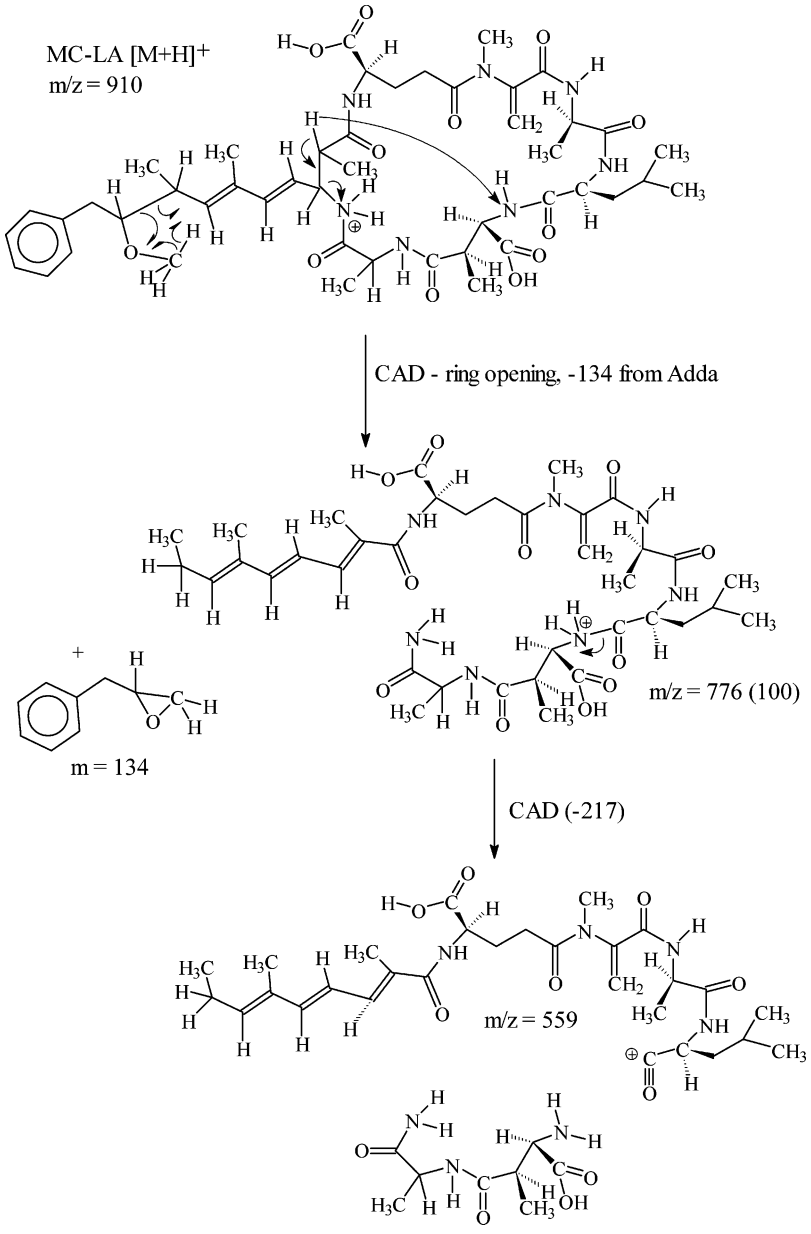
Scheme 3



spectrum generally required 0.2 s, and a full-scan MS/MS event ~0.4 s. The linear ion trap was equipped with automatic gain control to control the ion population in the instrument. This is accomplished with a brief prescan (<5 ms) to determine the ion flux prior to the analytical scan. The measured ion flux was then used to determine the fill time needed for the analytical scan in either the linear ion trap or FT-ICR cell. No internal calibrants were used for measurements in the FT-ICR cell, which was used for the accurate m/z measurements. Full-scan MS data were acquired from m/z 400 to 1200 with a resolving power of 100 000 at m/z 400, while tandem mass spectral data acquired in the ICR cell used a resolving power of 25 000.

RESULTS AND DISCUSSION

Figure 1 shows the base peak intensity chromatogram from an extract of *M. aeruginosa* strain B2666 measured on the linear ion trap. Inspection of the mass spectra at the chromatographic peaks revealed eight substances eluting after 24.5 min that can be identified as microcystins. Three of these that elute after 30 min are very small in Figure 1; however, they produced ions that were readily measured. The peaks that appear before 24.5 min, and a few after, are generally non-microcystin sample components that are not identified; however, several of these may also contain microcystins that have not been identified. This report is only concerned with the identified microcystins eluting after 24.5 min that are generally the more hydrophobic components. The available commercial standards MC-LA, MC-LR, and MC-LF eluted in the same region, and the eight substances were tentatively identified by high-accuracy measurements of the m/z of their $[M + H]^+$ ions shown in Table 1. The m/z measurements are the means of eight separate LC injections and FT-ICR-MS m/z measurements. The relative standard deviations of the measurements are all <0.001%, and the mean differences (errors) from the calculated exact m/z are less than 1 ppm. The ions measured

Table 3. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of [MeSer^7]MC-LR

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
393.2019	8	$C_{11}H_{15}O$- Glu-MeSer (-134 Adda) (-NH_3)	393.20202	0.31
470.2729	5	Ala-Leu-MeAsp-Arg-H	470.27216	1.6
527.2759	4	Adda-Glu-MeSer-H (-NH_3)	527.27518	1.4
553.3086	5	MeSer-Ala-Leu-MeAsp-Arg-H (-H_2O)	553.30928	1.23
571.3198	17	MeSer-Ala-Leu-MeAsp-Arg-H	571.31984	0.07
571.3599	10	MeAsp-Arg-Adda-H or Arg-Adda-Glu-H (-CO)	571.36025	0.61
582.3280	6	MeAsp-Arg-Adda-H or Arg-Adda-Glu-H (-NH_3)	582.32862	1.1
599.3548	45	MeAsp-Arg-Adda-H or Arg-Adda-Glu-H	599.35517	0.62
710.3862	5	MeAsp-Arg-Adda-Glu-H (-H_2O)	710.38719	1.4
862.4646	5	$C_{11}H_{15}O$-Glu-MeSer-Ala-Leu-MeAsp-Arg (-134 Adda)(-NH_3)	862.46691	2.7
884.5206	6	Arg-Adda-Glu-MeSer-Ala-Leu-H or MeSer-Ala-Leu-MeAsp-Arg-Adda-H or Ala-Leu-MeAsp-Arg-Adda-Glu-H (-CO)	884.52402	3.9
964.5128	6	Adda-Glu-MeSer-Ala-Leu-MeAsp-Arg-H (-NH_3) (-CH_3OH)	964.51385	1.1
995.5557	100	Adda-Glu-MeSer-Ala-Leu-MeAsp-Arg-H (-H_2O)	995.55605	0.35

[a] Relative abundance. [b] Mean error 1.26 ppm.

in all cases are the monoisotopic ions containing the isotopes with the highest natural abundances. These identifications were confirmed by an analysis of the CAD spectra of the $[M + H]^+$ ions, with emphasis on high-accuracy m/z measurements of 12 or more of the most abundant fragment ions in the MS/MS spectra. Generally these fragment ions had ion count abundances of >200 (arbitrary scale) and relative abundances greater than 2%. Relative amounts of the microcystins were estimated by integrating the abundance of the accurately measured $[M + H]^+$ ion ± 0.05 Da over the LC peak and assuming all response factors are unity. Absolute amounts could not be measured because either insufficient standards were available to calibrate the instrument response or, in most cases, no standards were available.

The frequently detected and reported MC-LR (second entry Table 1) was readily identified in the chromatogram (Figure 1) by the accurate measurement of its $[M + H]^+$ ion and confirmed by the compositions of 16 accurately measured ions in the product ion spectrum of its $[M + H]^+$ ion.[3] This product ion spectrum has been discussed in some detail.[3] The product ion spectra of the $[M + H]^+$ ions of MC-LR, MC-YR-, MC-RR, and MC-LA have also been discussed, however without the benefit of high-accuracy m/z measurements.[23]

The measured m/z and relative abundances (RAs) of the ions in the product ion spectra of the $[M + H]^+$ ions of the other seven MCs in Table 1 are in Tables 2−8. The compositions of the ions in Tables 2−8 are expressed in terms of the abbreviations of amino acid residues found in MCs, and the sequences are in accordance with the general structure of MCs (Scheme 1), and data presented in this report. The compositions of the ions in Tables 3−8 were determined by comparing the accurately measured m/z with calculated exact m/z of various combinations of amino acid residues, and certain fragments, and selecting the composition with the minimum m/z difference, or error, as shown in Tables 2−8. Most of these compositions are unique because of the frequent sub-2 ppm differences between measured and calculated m/z. With few exceptions, the amino acid residues considered in these calculations were those that have been reported in MC structures. The content of each residue is restricted to a specific number of each of the nuclides ^{12}C, ^{1}H, ^{14}N, ^{16}O, and ^{32}S. Some fragments of these residues are also considered in the exact m/z calculations, specifically those that correspond to commonly observed losses of CO, H_2O, NH_3, methanol, and the $C_9H_{10}O$ terminus of the Adda residue. When the loss of one of these small molecules is included in the calculated m/z, the molecule or molecules lost are shown in parentheses in Tables 2−8; however, the small molecule losses are not shown in the sequences of the amino acid residues. The exception to this convention is the loss of $C_9H_{10}O$ (134 Da) or ($C_9H_{10}O + NH_3$) from the Adda residue as shown in Scheme 2. In these ions, the modified composition of the Adda residue is shown in the sequence of amino acid residues. Finally, amino acid residues that are isomers cannot be distinguished by m/z measurements, and the positions of those residues in a sequence are based on the general patterns of residues in known MCs (Scheme 1). Isomeric pairs found in MC structures include MeAsp and Glu, and Mdha and Dhb. Also threonine (Thr), which has not been reported present in any microcystin, is an isomer of MeSer.

(23) Yuan, M.; Namikoshi, M.; Otsuki, A.; Rinehart, K. L.; Sivonen, K.; Watanabe, M. F. *J. Mass Spectrom.* **1999**, *34*, 33−43.

Table 4. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of the Previously Unreported [Asp3]MC-LA

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
446.2299	2	$C_{11}H_{15}O$- Glu-Mdha-Ala (-134 Adda) (-NH_3)	446.22857	3.0
454.2289	3	Mdha-Ala-Leu-Asp-Ala-H	454.22963	1.6
509.2644	8	Adda- Glu-Mdha-H (-NH_3)	509.26462	0.43
559.3130	13	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	0.66
580.3016	8	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	0.22
583.2728	2	Glu-Mdha-Ala-Leu-Asp-Ala-H	583.27222	0.99
661.3583	7	Adda-Glu-Mdha-Ala-Leu-H (-CH_3OH) (-NH_3)	661.35959	2.0
693.3858	16	Adda-Glu-Mdha-Ala-Leu-H (-NH_3)	693.38580	0.00
727.3682	3	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu-Asp-Ala (-134 Adda) (-NH_3) (-H_2O)	727.36613	2.8
744.3929	18	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-Asp-Ala-H (-134 Adda) (-H_2O)	744.39268	0.30
745.3764	59	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-Asp-Ala (-134 Adda) (-NH_3)	745.37670	0.40
762.4029	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-Asp-Ala-H (-134 Adda)	762.40324	0.45
795.4321	3	Adda-Glu (-H_2O) | Ala-Asp-Leu-Ala-H	795.42873	4.2
846.4362	5	Adda-Glu-Mdha-Ala-Leu-Asp-Ala-H (-CH_3OH) (-H_2O)	846.43963	4.1
847.4231	11	Adda-Glu-Mdha-Ala-Leu-Asp-Ala-H (-CH_3OH) (-NH_3)	847.42365	0.65
864.4499	16	Adda-Glu-Mdha-Ala-Leu-Asp-Ala-H (-CH_3OH)	864.45019	0.34
878.4658	22	Adda-Glu-Mdha-Ala-Leu-Asp-Ala-H (-H_2O)	878.46584	0.046
879.4496	27	Adda-Glu-Mdha-Ala-Leu-Asp-Ala-H (-NH_3)	879.44986	0.30

[a] Relative abundance. [b] Mean error 1.25 ppm.

Table 2 shows the measured *m/z* and RAs of 16 ions in the CAD spectrum of the $[M + H]^+$ ion of MC-LA,[4] which is the fourth entry in Table 1. The dominant fragmentation in the product ion spectrum of the $[M + H]^+$ ion of MC-LA, and generally the MCs that we have studied that do not contain Arg, is the loss of 134 Da from the Adda residue, concurrent or stepwise ring opening, and loss of an ammonia molecule as shown in the proposed mechanism in Scheme 2. The loss of 134 Da from the Adda residue in a $[M + H]^+$ ion is thought to occur by charge-remote homolytic cleavage of the Adda C8−C9 bond with transfer of a hydrogen atom from the Adda methoxy methyl group to C8 of Adda (Scheme 2).[3] The presence of a $C_{11}H_{15}O$ residue in ions from the CAD of the $[M + H]^+$ ions of MC-RR, MC-YR, MC-LR, and MC-LA was noted as a characteristic feature of the these spectra, but no mechanism for their formation was proposed.[23] The *m/z* 893 (22% RA), 776 (100% RA), and 759 (46% RA) ions in Table 2 suggest a stepwise process beginning with either ring opening and loss of ammonia or loss of $C_9H_{10}O$ (134 Da). Protonation of the Adda amide nitrogen, which has a calculated energy not much different from protonation of the Adda methoxy nitrogen,[10] leads to ring opening driven by the formation of the extended conjugated system. If proton transfer is to the [Ala[4]] amide nitrogen, as shown in Scheme 2, elimination of ammonia gives the *m/z* 893 ion. The loss of $C_9H_{10}O$, either before, concurrent with, or after ring opening but before loss of ammonia, gives the base peak *m/z* 776 ion. The *m/z* 759 ion (Scheme 2) is formed by loss of ammonia to give the b-type linear peptide ion, which has several possible structures in addition to the one shown in Scheme 2. If proton transfer during ring opening is randomly to any other amide nitrogen, heterolytic cleavage of that peptide bond to give the b-type peptide ion would account for the presence of $C_{11}H_{15}O$-terminated b-type sequence ions in these spectra. This is illustrated in Scheme 3, where the proton is transferred to the amide nitrogen of the Leu-MeAsp peptide bond, and heterolytic cleavage of that bond gives the *m/z* 559 ion in Table 2. A complete set of these ions without relative abundances has been reported for MC-LA and three other microcystins.[23] Four ions in Table 2 have compositions that show losses of methanol, which likely comes from a charge-remote fragmentation of an intact Adda residue by loss of the methoxy group and a hydrogen on an adjacent carbon. The sources of the water molecules are less clear, but likely are the free carboxylic acid groups on the Glu and MeAsp residues. The other sequence ions in Table 2, in the range of *m/z* 468−693, are b- or y-type ions, sometimes with intact Adda

Table 5. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of MC-LL

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
509.2647	5	Adda-Glu-Mdha-H (-NH_3)	509.26462	0.16
510.2921	11	Mdha-Ala-Leu-MeAsp-Leu-H	510.29223	0.25
559.3133	9	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	1.2
580.3025	7	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	1.3
639.3338	4	Glu- Mdha-Ala-Leu-MeAsp-Leu-H	639.33482	1.6
661.3597	2	Adda-Glu-Mdha-Ala-Leu-H (-NH_3) (-CH_3OH)	661.35959	0.17
693.3863	13	Adda-Glu-Mdha-Ala-Leu-H	693.38580	0.72
783.4251	2	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-MeAsp-Leu (-134Adda) (-NH_3) (-H_2O)	783.42873	4.6
800.4562	20	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Leu-H(-134Adda)(-H_2O)	800.45528	1.2
801.4390	60	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-MeAsp-Leu (-134 Adda)(-NH_3)	801.43930	0.37
818.4657	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-134 Adda)	818.46584	0.17
851.4899	5	Ala-Leu-MeAsp-Leu (-H_2O) | H-Glu-Adda	851.49133	1.7
902.5026	3	Adda-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-CH_3OH) (-H_2O)	902.50223	0.41
903.4855	6	Adda-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-CH_3OH) (-NH_3)	903.48625	0.83
920.5130	9	Adda-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-CH_3OH)	920.51279	0.23
934.5286	37	Adda-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-H_2O)	934.52844	0.17
935.5135	35	Adda-Glu-Mdha-Ala-Leu-MeAsp-Leu-H (-NH_3)	935.51246	1.1

[a] Relative abundance. [b] Mean error 0.95 ppm.

Table 6. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of the Previously Unreported [Asp^3]MC-LL

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
496.2754	4	Mdha-Ala-Leu-Asp-Leu-H	496.27658	2.4
509.2651	6	Adda-Glu-Mdha-H (-NH_3)	509.26462	0.94
559.3115	9	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	2.0
580.3024	3	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	1.2
693.3844	10	Adda-Glu-Mdha-Ala-Leu-H (-NH_3)	693.38580	2.0
786.4401	19	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-Asp-Leu-H (-134 Adda) (-H_2O)	786.43963	0.60
787.4230	51	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu-Asp-Leu (-134 Adda) (-NH_3)	787.42365	0.83
804.4502	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-Asp-Leu-H (-134 Adda)	804.45019	0.012
889.4727	3	Adda-Glu-Mdha-Ala-Leu-Asp-Leu-H (-CH_3OH) (-NH_3)	889.47060	2.4
906.4991	9	Adda-Glu-Mdha-Ala-Leu-Asp-Leu-H (-CH_3OH)	906.49714	2.2
920.5132	30	Adda-Glu-Mdha-Ala-Leu-Asp-Leu-H (-H_2O)	920.51279	0.45
921.4953	28	Adda-Glu-Mdha-Ala-Leu-Asp-Leu-H (-NH_3)	921.49681	1.6

[a] Relative abundance. [b] Mean error 1.39 ppm.

Table 7. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of MC-LF

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
509.2639	4	Adda-Glu-Mdha-H (-NH_3)	509.26462	1.4
544.2772	10	Mdha-Ala-Leu-MeAsp-Phe-H	544.27658	1.1
559.3129	8	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	0.48
580.3014	6	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	0.57
693.3852	11	Adda-Glu-Mdha-Ala-Leu-H (-NH_3)	693.38580	0.87
751.4045	4	$C_{11}H_{17}NO$-Glu (-134 Adda) (-H_2O) | Phe- MeAsp-Leu-Ala-H	751.40252	2.6
834.4398	28	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Phe-H(-134Adda)(-H_2O)	834.43963	0.20
835.4234	41	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-MeAsp-Phe (-134 Adda) (-NH_3)	835.42365	0.30
852.4503	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-134 Adda)	852.45019	0.13
885.4711	5	Adda-Glu (-H_2O) | Phe- MeAsp-Leu-Ala-H	885.47568	5.2
936.4852	6	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-CH_3OH) (-H_2O)	936.48658	1.5
937.4701	4	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-CH_3OH) (-NH_3)	937.47060	0.53
951.4842	3	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-H_2O) (-NH_3)	951.48624	2.1
954.4982	13	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-CH_3OH)	954.49714	1.1
968.5130	40	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-H_2O)	968.51279	0.22
969.4978	19	Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-H (-NH_3)	969.49681	1.0

[a] Relative abundance. [b] Mean error 1.2 ppm.

residues, and often accompanied by the loss of a small molecule or molecules.

Table 3 shows the measured *m/z* and RAs of 13 ions in the CAD spectrum of the $[M + H]^+$ ion of [MeSer[7]]MC-LR, which is the first entry in Table 1. This MC was reported in 1992 and assigned a structure based on an amino acid analysis, accurate measurement of the *m/z* of the $[M + H]^+$ ion, integer accuracy MS/MS of the $[M + H]^+$ ion, and a ^1H NMR spectrum.[24] However, since methylamine and not *N*-methylserine was observed in the amino acid analysis, and the NMR spectrum was not definitive, the MS evidence was crucial to this structural assignment. The significant MeSer-containing ions were reported to be $[MeSer - Ala - H]^+$ at *m/z* 173, $[Glu - MeSer - H]^+$ at *m/z* 231, a weak $[CO - Glu - MeSer (-H)]^+$ ion at *m/z* 257, and a $[C_{11}H_{14}O - Glu - MeSer]^+$ ion at *m/z* 393. In the present work, the former three ions were not observed; however, the *m/z* 393 ion was measured and it has the composition $[C_{11}H_{15}O - Glu - MeSer]^+$ (Table 3). This ion is formed by the mechanism in Scheme 3, with transfer of the proton to the amide N of the Ala−MeSer peptide bond, and heterolytic cleavage of that bond to give the type-b *m/z* 393 ion. The ion at *m/z* 862 (Table 3) is produced by the mechanism in Scheme 2, but it is also of low abundance because the proton transfer in the first step is more likely to the strongly basic guanidinyl portion of the Arg residue than to an amide nitrogen. Other MeSer-containing ions in Table 3 that confirm this structure are *m/z* 527, 553, 571, 862, 884, 964, and 995. The base peak in the product ion spectrum of the $[M + H]^+$ ion is *m/z* 995, which corresponds to loss of a water molecule, possibly the dehydration of MeSer to Mdha. This is consistent with our general observation that CAD spectra of microcystin $[M + H]^+$ ions that contain Arg have base peaks that are caused by the loss of a small molecule such as water, ammonia, or carbon monoxide, and the ion from the loss of 134 Da from Adda is of low abundance or is not observed. In microcystins that do not contain Arg, the ion produced by the loss of 134 Da from Adda is the base peak (Tables 2, 4−8).

Table 4 shows the measured *m/z* and RAs of 18 ions in the CAD spectrum of the $[M + H]^+$ ion of the previously unreported [Asp[3]]MC-LA, which is the third entry in Table 1. A difference of 14.0159 Da between the *m/z* of the $[M + H]^+$ ion of this compound and the *m/z* of the $[M + H]^+$ ion of MC-LA (Table 1) clearly indicated the difference was due to a methylene group (calculated for CH_2 14.015 65). The three most likely structural changes in MC-LA that would account for this 14-Da mass loss are replacement of MeAsp in position 3 by Asp (Scheme 1), replacement of Mdha in position 7 by Dha, and replacement of the Adda C-9 methoxy group by a hydroxyl group. Comparison of the data in Tables 2 and 4 reveals that the nine ions from *m/z* 758 to 893 in Table 2 (MC-LA) have the same compositions as

(24) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Stotts, R. R.; Dahlem, A. M.; Beaslet, V. R.; Carmichael, W. W.; Evans, W. R. *J. Org. Chem.* **1992**, *57*, 866−872.

Table 8. Ions in the Collision-Activated Dissociation Spectrum of the $[M + H]^+$ Ion of MC-L(Aba)

measured m/z	RA[a] (%)	composition and sequence	calculated m/z	error[b] (ppm)
482.2610	8	Mdha-Ala-Leu-MeAsp-Aba-H	482.26093	0.15
509.2642	9	Adda-Glu-Mdha-H (-NH_3)	509.26462	0.83
559.3128	11	$C_{11}H_{15}O$- Glu-Mdha-Ala-Leu (-134 Adda) (-NH_3)	559.31263	0.30
580.3014	10	Adda-Glu-Mdha-Ala-H (-NH_3)	580.30173	0.57
611.3023	5	Glu-Mdha-Ala-Leu-MeAsp-Aba-H	611.30352	2.0
693.3864	14	Adda-Glu-Mdha-Ala-Leu-H (-NH_3)	693.38580	0.87
772.4237	23	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-134 Adda) (-H_2O)	772.42398	0.36
773.4071	46	$C_{11}H_{15}O$-Glu-Mdha-Ala-Leu-MeAsp-Aba (-134 Adda) (-NH_3)	773.40799	1.2
790.4345	100	$C_{11}H_{17}NO$-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-134 Adda)	790.43454	0.051
875.4544	6	Adda-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-CH_3OH) (-NH_3)	875.45494	0.62
892.4834	11	Adda-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-CH_3OH)	892.48149	2.1
906.4961	27	Adda-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-H_2O)	906.49714	1.1
907.4882	26	Adda-Glu-Mdha-Ala-Leu-MeAsp-Aba-H (-NH_3)	907.48115	7.8

[a] Relative abundance. [b] Mean error 1.38 ppm.

the nine ions from *m/z* 744 to 879 in Table 4 except for the replacement of MeAsp by Asp, the replacement of Mdha by Dha (not shown), or the replacement of the Adda C-9 methoxy group by a hydroxyl group (not shown). The three ions at *m/z* 744, 745, and 762 in Table 4, which correspond to *m/z* 758, 759, and 776 in Table 2, include losses of 134 Da from the Adda residue, which confirms that the C-9 methoxy is present. The ions at *m/z* 446, 509, 559, 580, 661, and 693 in Table 4 all have a Mdha residue and neither a MeAsp nor a Asp, which strongly implies that the Mdha residue is present in this microcystin. That Asp is also present is confirmed by the ion at *m/z* 795 in Table 4, which contains Asp and not Mdha. Therefore, this new microcystin is [Asp^3]MC-LA. The base peak in this spectrum is the expected *m/z* 762 ion from the loss of $C_9H_{10}O$ (134 Da), and $C_{11}H_{15}O$-terminated sequence ions formed by the mechanisms in Schemes 2 and 3 are at *m/z* 745, 727, 559, and 446.

The cyclic peptide MC-LL was discovered in *M. aeruginosa* PCC 7820 cultures and first reported in 2000.[13] We found only one subsequent report of MC-LL, and this was based entirely on a low-abundance ion with a *m/z* of 950.5381 in the negative ion electrospray of a cyanobacterial extract introduced by flow injection.[25] The original identification was based on a matrix-assisted laser desorption/ionization time-of-flight mass spectrum obtained in the collision-activated dissociation postsource decay mode. Those investigators identified ions at *m/z* 510, 559, 639, 801, and 818 and assigned the same compositions that are given for those ions in Table 5, which shows the measured *m/z* and RAs of 17 ions in the CAD spectrum of the $[M + H]^+$ ion of MC-LL (Table 1, last entry). They also identified sequence ions at *m/z* 155, 213, 375, and 397 that we did not observe. There are 12 ions in Table 5 that have compositions containing two Leu residues, and these plus the other sequence ions at *m/z* 693, 661, 580, 559, and 509 provide strong confirmation for the structure of MC-LL. The base peak in this spectrum is the expected *m/z* 818 ion from the loss of $C_9H_{10}O$ (134 Da), and $C_{11}H_{15}O$-terminated sequence ions formed by the mechanisms in Schemes 2 and 3 are at *m/z* 801, 783, and 559.

Table 6 shows the measured *m/z* and RAs of 12 ions in the CAD spectrum of the $[M + H]^+$ ion of the previously unreported [Asp^3]MC-LL, which is the sixth entry in Table 1. Just as with MC-LA and [Asp^3]MC-LA, a difference of 14.0155 Da in the measured *m/z* of the $[M + H]^+$ ions of MC-LL and the suspected [Asp^3]MC-LL strongly suggested that one of the three methyl groups was absent in this compound. Comparison of the data in Tables 5 and 6 reveals that the seven ions from *m/z* 800 to 935, except for the ions at *m/z* 851 and 902, in Table 5 (MC-LL) have the same compositions as the seven ions from *m/z* 786 to 921 in Table 6 except for the replacement of MeAsp by Asp, the replacement of Mdha by Dha (not shown), or the replacement of the Adda C-9 methoxy group by a hydroxyl group (not shown). The three ions at *m/z* 786, 787, and 804 in Table 6, which correspond to *m/z* 800, 801, and 818 in Table 5, include losses of 134 Da from the Adda residue, which confirms that the C-9 methoxy is present. The ions at *m/z* 509, 559, 580, and 693 in Table 6 all have a Mdha residue and neither a MeAsp nor a Asp, which strongly implies that the Mdha residue is present in this microcystin. We could not find an ion that contains Asp but not Mdha to confirm the presence of Asp; however, the evidence is strong that this compound is the new microcystin [Asp^3]MC-LL.

(25) Zeck, A.; Weller, M. G.; Niessner, R. *Anal. Chem.* **2001**, *73*, 5509−5517.

The base peak in this spectrum is the expected m/z 804 ion from the loss of $C_9H_{10}O$ (134 Da), and $C_{11}H_{15}O$-terminated sequence ions formed by the mechanisms in Schemes 2 and 3 are at m/z 787 and 559.

The cyclic peptide MC-LF was discovered in a *M. aeruginosa* PCC 7813 laboratory culture and in a PCC 7820 extract and first reported in 1995.[11,12] This structure was based on an amino acid analysis, an integer accuracy measurement of the $[M + H]^+$ ion, and its integer accuracy product ion spectrum. Table 7 shows the measured m/z and RAs of 16 ions in the CAD spectrum of the $[M + H]^+$ ion of MC-LF, which is the seventh entry in Table 1. Table 7 includes the compositions of 13 ions that were not reported by previous investigators and confirms the compositions of the ions at m/z 544, 835, and 852 that were previously reported. The compositions of the ions in Table 7 have a pattern that is similar to ions in the CAD spectra of the other microcystin $[M + H]^+$ ions that do not contain Arg. Twelve of the 16 ions include Phe in their compositions, and the sequence ions at m/z 509, 580, 693, 751, and 885 strongly support the structure of MC-LF. The base peak in this spectrum is the expected m/z 852 ion from the loss of $C_9H_{10}O$ (134 Da), and $C_{11}H_{15}O$-terminated sequence ions formed by the mechanisms in Schemes 2 and 3 are at m/z 835 and 559.

We found only one literature citation for MC-L(Aba), where Aba is reported to be aminoisobutyric acid.[26] Those investigators did not report any mass spectrometric data for this substance and no other information to support its structure. Table 8 shows the measured m/z and RAs of 13 ions in the CAD spectrum of the $[M + H]^+$ ion of MC-L(Aba), which is the fifth entry in Table 1. In addition to the $[M + H]^+$ ion in Table 1, there are nine ions in the CAD spectrum that strongly support the inclusion of an aminobutyric acid; however, we cannot find any ions that indicate side-chain fragmentation that might give evidence of the structure of the Aba residue. The sequence ions at m/z 482, 509, 559, 580, 611, and 693 support the structure with Aba in the variable ring position 4 (Scheme 1). Again the pattern of compositions of the ions in Table 8 is similar to the pattern of ions in the CAD spectra of the other microcystin $[M + H]^+$ ions that do not contain Arg. The base peak in this spectrum is the expected m/z 790 ion from the loss of $C_9H_{10}O$ (134 Da), and $C_{11}H_{15}O$-terminated sequence ions formed by the mechanisms in Schemes 2 and 3 are at m/z 773 and 559.

CONCLUSION

The liquid chromatography/hybrid linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer technique described in this paper is capable of supporting fairly rapid and reliable identifications of known microcystins when standards are not available. Few, if any, commercial or analytical standards of the nearly 70 known microcystins have been available. This technique is also capable of supporting identifications of new structural variants when sufficient material is not available for study with other analytical techniques. This capability is made possible by the high-accuracy m/z measurements that can be obtained even with the variable conditions of microbore column liquid chromatography introduction of samples and electrospray ionization. The mean difference between measured and calculated exact m/z was less than 2 ppm, which often allowed assignment of unique compositions to the observed ions. Separate amino acid analyses may be required for some samples when there is the indication that isomeric amino acids are present in mircocystins. Amino acid residues that are isomers cannot yet be distinguished by m/z measurements, and the positions of those residues in a sequence are usually based on the general patterns of residues in known microcystins. For example, glutamic acid and 3-methylaspartic acid are isomers that have well-defined and conserved positions in microcystin structures, and the isomers *N*-methyldehydroalanine and dehydrobuterine have been reported in ring position 7.

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