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Thiol Derivatization for LC-MS Identification of Microcystins in Complex Matrices

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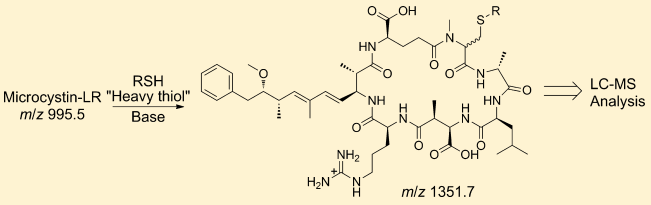
Supporting Information

ABSTRACT: Microcystins are a group of cyclic heptapeptides originating from cyanobacteria. Cyanobacteria also produce a range of peptides and other compounds that can result in complex chromatograms when samples are analyzed by LC-MS. Derivatization with appropriate thiols (e.g., mercaptoethanol) of the olefin in the α,β-unsaturated amide present in most microcystins was shown to simplify analysis of LC-MS chromatograms of sample extracts, making it much easier to identify peaks corresponding to candidate microcystins. Furthermore, interpretation of MS^2 spectra was facilitated by addition of the mass associated with the thiol to the α,β-unsaturated amide of microcystins. Cyanotoxins containing Mdha or Dha reacted readily with thiols, whereas Mser, Ser, Mdhb, and thiol-derivatives of Mdha or Dha did not react under the conditions used. This approach therefore provides a convenient LC-MS method to obtain evidence for the presence of Mdha or Dha and can likely be used to differentiate between the isobaric amino acids Mdha and Dhb in candidate cyanotoxin peaks. When *O*-(2-mercaptoethyl)-*O*′-methyl-hexa(ethylene glycol) (MEMHEG) (M_wt. 356) was used as the thiol, the resulting derivatives eluted in an LC-MS mass window that was largely free of interferences. This approach simplifies detection of candidate microcystin analogues even in the presence of complex mixtures of coeluting components. The method was used for qualitative analysis of a *Microcystis aeruginosa* culture from Lake Naivasha, Kenya, and the results were verified using precursor-ion scanning and high-resolution mass spectrometry.



INTRODUCTION

Microcystins (Figure 1) are potent cyclic heptapeptide hepatotoxins. More than 80 microcystin analogues have been characterized so far.[1] They are produced by certain coccal and filamentous freshwater cyanobacteria belonging to a range of genera including *Microcystis*, *Anabaena*, *Nostoc*, and *Planktothrix*.[2,3] Microcystins are usually cell-bound in healthy cyanobacterial cells. However, in senescent blooms, cell lysis occurs leading to release of toxins. Cyanobacteria and their toxins are ingested by various animals, so microcystins can therefore occur in natural samples of water, bloom material, fish and animal tissues, and many other types of biological materials. Often microcystins co-occur with other compounds from cyanobacterial and other sources as well as the matrix components of the sample being examined. This can make for challenging chemical analysis, and it is easy to overlook minor microcystin components in such circumstances.

Most microcystins contain an α,β-unsaturated carbonyl group in amino acid site-7 which is reactive toward thiols (Figure 2). Derivatization of this moiety with 2-aminoethanthiol (**a** in Figure 2) has been reported as a means of confirming the identity of candidate peaks as microcystins during LC-MS analysis.[4] In addition, this derivatization reaction labels site-7 of the microcystins with extra mass, so that it is easier to identify which fragments in an LC-MS^2 spectrum contain the amino acid originating from site-7 (usually Mdha or Dha). Unfortunately, derivatization with 2-aminoethanthiol also resulted in microcystin derivatives that were doubly charged and had much shorter retention times than their corresponding underivatized microcystin analogues.[4]

We reasoned that derivatization with a thiol that did not contain a charged group, and which was of a similar polarity to the microcystins themselves, would result in derivatives that possessed similar retention times, ionization, and mass spectral fragmentation properties to their corresponding underivatized microcystins. We also anticipated that derivatization of microcystins with a sufficiently high molecular weight thiol of

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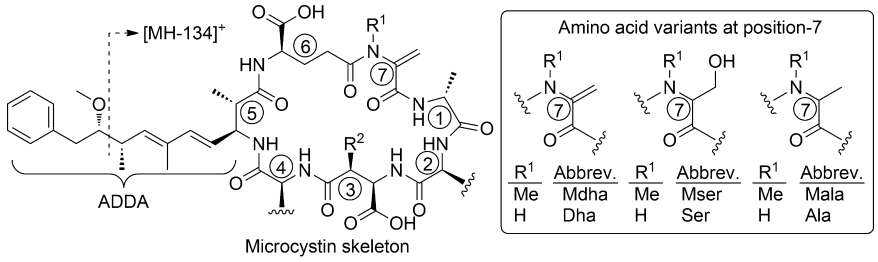

		②	④	⑦	R^1	R^2	$[MH]^+$ *m/z*
1	MC-LR	Leu	Arg	Mdha	Me	Me	995.5
2	MC-YR	Tyr	Arg	Mdha	Me	Me	1045.5
3	MC-RR	Arg	Arg	Mdha	Me	Me	1038.5
4	MC-LA	Leu	Ala	Mdha	Me	Me	910.5
5	MC-LF	Leu	Phe	Mdha	Me	Me	986.5
6	MC-LY	Leu	Tyr	Mdha	Me	Me	1002.5
7	MC-LW	Leu	Trp	Mdha	Me	Me	1025.5
8	[Dha^7]MC-LR	Leu	Arg	Dha	H	Me	981.5
9	MC-RY	Arg	Tyr	Mdha	Me	Me	1045.5
10	[Asp^3]MC-RY	Arg	Tyr	Mdha	Me	H	1031.5
11	[Asp^3]MC-LY	Leu	Tyr	Mdha	Me	H	988.5
12	[Asp^3]MC-LR	Leu	Arg	Mdha	Me	H	981.5
13	MC-LY(OMe)	Leu	MeOTyr	Mdha	Me	Me	1032.5
14	MC-LL	Leu	Leu	Mdha	Me	Me	952.5
15	[Dha^7]MC-YR	Tyr	Arg	Dha	H	Me	1031.5
16	[Asp^3, $Mser^7$]MC-RY	Arg	Tyr	Mser	Me	H	1049.5
17	[Asp^3, Dha^7]MC-RY	Arg	Tyr	Dha	H	H	1017.5
18	[Asp^3]MC-RF	Arg	Phe	Mdha	Me	H	1015.5
19	[Asp^3, $Mser^7$]MC-LY	Leu	Tyr	Mser	Me	Me	1006.5
20	[Asp^3]MC-YY	Tyr	Tyr	Mdha	Me	H	1038.5
21	[Asp^3, Dha^7]MC-LY	Leu	Tyr	Dha	H	H	974.5
22	[Asp^3]MC-HilY	Hil	Tyr	Mdha	Me	H	1002.5
23	[Asp^3]MC-LF	Leu	Phe	Mdha	Me	H	972.5

Figure 1. Structures of microcystin analogues discussed in the text, with the seven amino acid residues labeled 1−7, and showing the ADDA-fragmentation ($[MH-134]^+$). Amino acid variations in the structures were present at sites 2−4 and 7, and these are indicated with standard 3-letter amino acid abbreviations (with the exception of (MeO)Tyr = methoxytyrosine at position 4, and Mdha = *N*-methyldehydroalanine, Dha = dehydroalanine, and Mser = *N*-methylserine at position 7). **1**−**8** were available commercially as standards, and **9** was isolated and its structure verified by NMR spectroscopy.

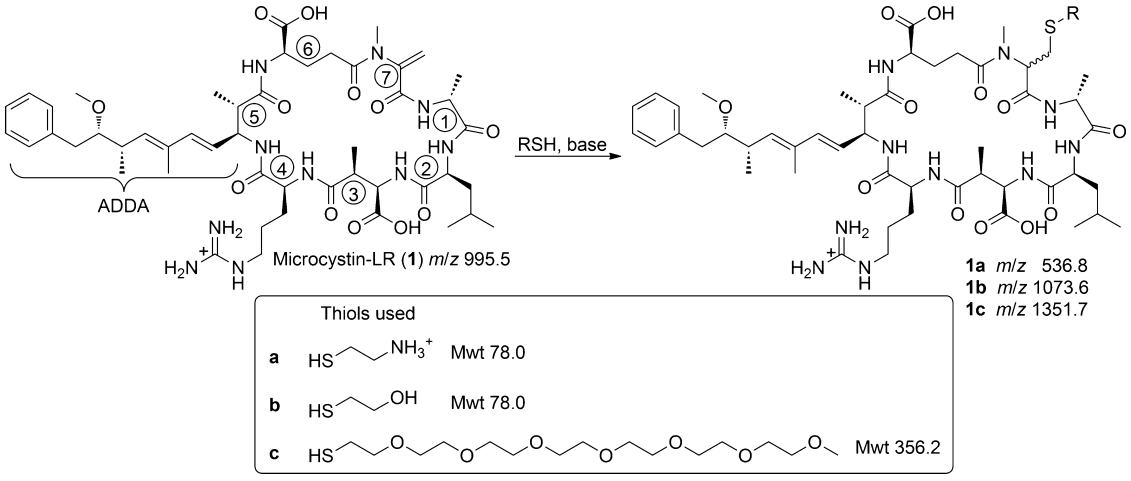

Figure 2. Nucleophilic addition of thiols to the electrophilic olefin in Mdha of MC-LR (**1**), and the thiols discussed in the text: (i) 2-aminoethanthiol (**a**); (ii) mercaptoethanol (**b**); and (iii) *O*-(2-mercaptoethyl)-*O*′-methyl-hexa(ethylene glycol) (MEMHEG) (**c**). Note that the product of the reaction of **1** with thiols **a**, **b**, or **c** are designated **1a**, **1b**, or **1c**, respectively (and that this nomenclature is used to label peaks in Figures 4 and 5), and that two diastereoisomers are formed during the reaction. Position-7 in microcystins corresponds to position-5 in nodularin analogues (see Figure 3).

this type would result in the microcystin derivatives appearing in an LC-MS mass window which was largely free of interferences, resulting in greatly simplified LC-MS chromatograms.

Microcystin profiles in African water bodies have been much less studied than those from Europe and North America. Here we report development of effective thiol-derivatization procedures using microcystin standards, and their application to a microcystin-containing cyanobacterial culture isolated from Lake Naivasha, Kenya. Derivatization was performed with mercaptoethanol (**b** in Figure 2) or *O*-(2-mercaptoethyl)-*O*′-methyl-hexa(ethylene glycol) (MEMHEG) (**c** in Figure 2) for

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LC-MS^2 analysis which, together with interpretation of the MS^2 fragmentation spectra of the underivatized compounds and their thiol derivatives, allowed tentative identification of putative microcystins for which standards were not readily available.

EXPERIMENTAL SECTION

Chemicals and Cyanobacterial Samples. Mercaptoethanol, MEMHEG, and 2-aminoethanthiol, were purchased from Sigma–Aldrich, Oslo, Norway. Microcystin (MC-RR, MC-LR, MC-YR, MC-LA, MC-LY, MC-LF, MC-LW) and nodularin (NOD; Figure 3) standards were purchased from Alexis

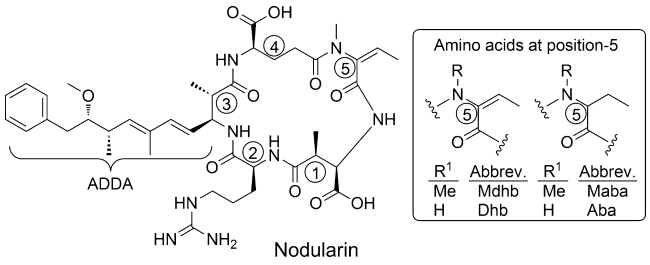

Figure 3. Structure of nodularin (NOD). Note that amino acids 1–5 in nodularin analogues correspond to positions 3–7 in microcystins (Figure 1). NOD and most of its analogues contain Mdhb (*N*-methyldehydrobutyrine) at position-5, although [Dhb^5]-analogues (Dhb = dehydrobutryine) are also known. NOD did not react with mercaptoethanol under the conditions used in this study but did react slowly with $NaBH_4$ to form [Maba^5]NOD.

Biochemicals (Grünberg, Germany), and NMR-quantitated standards of MC-LR, [Dha^7]MC-LR, MC-RR, and NOD were obtained from IMB NRC, Halifax, NS, Canada. A pure specimen of MC-RY (**9**) (manuscript in preparation) was available from a cyanobacterial bloom in Mwanza Gulf in Lake Victoria, Tanzania,[5] in 2010. A microcystin-producing *Microcystis aeruginosa* culture (NIVA-CYA548 = AB2002/40) was obtained from the NIVA (Norwegian Institute of Water Research, Oslo, Norway) culture collection and grown in Z8 medium[6] with a light/dark cycle of 12 h/12 h at ca. 22 °C and a photon flux of 80 mmol m^{-2} s^{-1} for four weeks. NIVA-CYA548 was originally isolated in 2002 from Lake Naivasha, Kenya.[7]

Sample Preparation. Seven microcystin standards were dissolved separately in methanol at ca. 25 μg/mL and then combined and diluted to give a mixed standard containing ca. 0.4–1 μg/mL in MeOH–H_2O (1:1). Aliquots of NIVA-CYA548 (1 mL) were frozen and thawed three times and then ultrasonicated for 10 min. MeOH (1 mL) was then added, and the samples were filtered (0.2 μm, Costar Spin-X Microcentrifuge, Corning, NY, USA). Sodium carbonate buffer (0.2 M, pH 9.7) was added to the microcystin standards mixture and to the filtrate from NIVA-CYA548, in a ratio of 1:4 v/v. To aliquots (200 μL) of the buffered solutions was added mercaptoethanol or MEMHEG (1 μL) or 2-aminoethanthiol (1 mg), and the mixture was vortex-mixed before analysis by LC-MS^2. Underivatized (i.e., no thiol addition) buffered filtrates were used as controls. Carbonate buffers (0.2 M) of pH 9.2, 9.7, 10.2, and 10.7 were tested during method development. A concentrated extract of CYA548 for LC-MS/MS with precursor ion scanning was prepared as follows. The culture (3 L) was frozen, thawed, ultrasonicated (10 min), and then shaken gently overnight with activated HP-20 resin (9 g) to extract microcystins. The resin was recovered by filtration through nylon netting (200 μm mesh), rinsed with water, and eluted slowly with 25 mL of MeOH.[8] The CYA548 concentrate was evaporated to dryness in vacuo and dissolved in MeOH (8 mL), and a specimen was diluted 20-fold for analysis.

Other Reactions. (i) *Reduction*. Solutions of MC-LA, MC-LR, and MC-RY (ca. 0.5 μg/mL) in 1:1 MeOH–H_2O (200 μL) were treated with freshly prepared $NaBH_4$ in MeOH (15 mg/mL; 50 μL), and the reduction was followed by LC-MS^2 (method A). To a mixture of nodularin and [Dha^7]MC-LR (**8**) (both ca. 0.5 μg/mL) in 1:1 MeOH–carbonate buffer (1:1) was added $NaBH_4$ (0.7 mg), and the reduction was followed by LC-MS^2 (method A). (ii) *Esterification*. A solution of MC-LA in MeOH was partially esterified by treatment with diazomethane using an Aldrich diazomethane generator with System 45 connection following the manufacturer's protocol.[9] After reacting for 45 min at 0 °C with occasional swirling, the solution was transferred to a glass vial and evaporated to dryness under a stream of N_2, the residue was dissolved in MeOH (1 mL), and aliquots were diluted to 50% aqueous using either water (control) or carbonate buffer (pH 9.7) and analyzed by LC-MS^2 (method A).

LC-MS Analysis. LC-MS^2 (Method A). Liquid chromatography was performed on a Symmetry C18 column (3.5 μm, 100 × 2.1 mm; Waters, Milford, MA, USA), using a Surveyor MS Pump Plus and a Surveyor Auto Sampler Plus (Finnigan, Thermo Electron Corp., San Jose, CA, USA) eluted with a linear gradient of acetonitrile (A) and water (B) each containing 0.1% formic acid. The gradient was from 22.5% to 42.5% A over 4 min, then to 75% A at 10 min, to 95% A at 11 min (1 min hold) followed by a return to 22.5% A with a 3-min hold to equilibrate the column. The HPLC system was coupled to a Finnigan LTQ ion trap mass spectrometer (Finnigan Thermo Electron Corp., San Jose, CA, USA) operated in full-scan positive ion ESI mode (m/z 500–1600). The ion injection time was set to 100 ms with a total of three microscans. ESI parameters were a spray voltage of 6 kV, a capillary temperature of 375 °C, a sheath gas rate of 55 units N_2 (ca. 550 mL/min), and an auxiliary gas rate of 5 units N_2 (ca. 50 mL/min). ESI settings were optimized while continuously infusing (syringe pump) 0.1 μg/mL of the MC-RR (**3**, m/z 1038.5) standard at 10 μL/min. MS^2 spectra were acquired using the same chromatographic conditions for specified m/z values but with scanning up to m/z 1150, isolation width 2.0, normalized collision energy 50, Activation Q 0.250, and activation time 0.25 ms. The tray temperature was set to 30 °C for determination of relative reaction rates.

LC-MS/MS with Precursor-Ion Scanning (Method B). Liquid chromatography was performed on the same HPLC column as used in method A (above), using an Acquity UPLC module (Waters, Milford, MA) eluted with the same gradient as for method A. The UPLC system was coupled to a Quattro Ultima triple-quadrupole mass spectrometer (Waters, Milford, MA) operated in positive ion ESI mode. Precursor-ion scanning (m/z 900–1100) for m/z 135 was performed with collision energy at 50 eV and a collision cell Pirani pressure of 1.7×10^{-3} mbar. ESI parameters were a capillary voltage of 3.5 kV, a source temperature of 100 °C, a desolvation temperature of 300 °C, and a desolvation gas flow of 600 L/h of N_2.

High-Resolution LC-MS and LC-MS^2 (LC-HRMS) (Method C). LC-HRMS spectra were obtained with ESI in positive ion mode on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) connected to an HP 1100 Series HPLC pump and an Agilent 1200 Series autosampler.

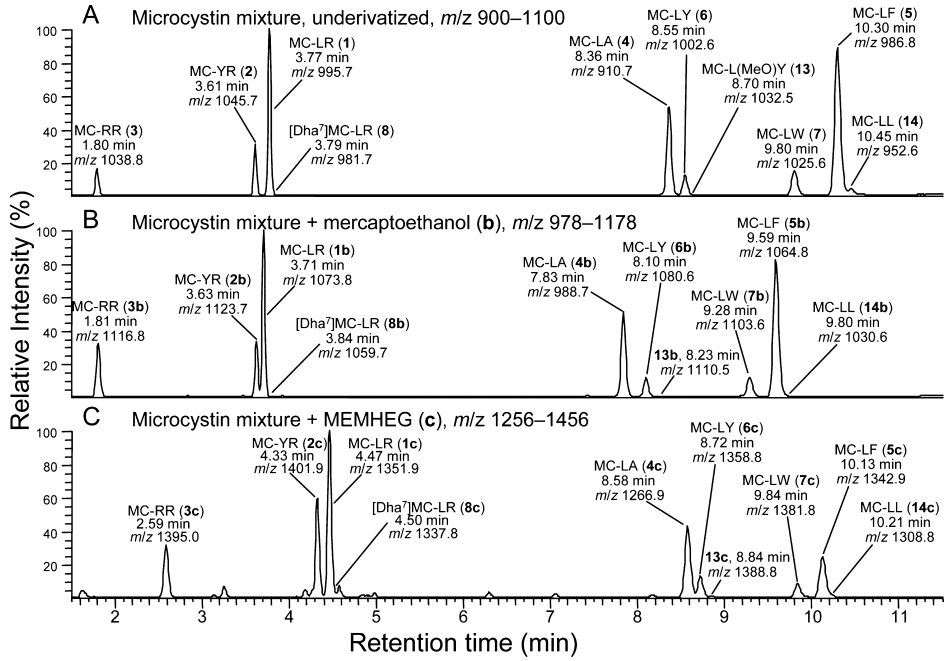

Figure 4. LC-MS^2 (method A) analysis of a mixture of commercially available microcystins: A, underivatized standards; B, standards derivatized with mercaptoethanol (adds 78 Da); and C, standards derivatized with MEMHEG (adds 356 Da). Peaks are marked with the compound's number (see Figures 1 and 2 and Table 1), R_t and m/z for $[MH]^+$.

The elution gradient and HPLC column were identical to those used for method A. ESI parameters were a spray voltage of 6 kV, a capillary temperature of 375 °C, a sheath gas flow of 16 units N_2 (ca. 160 mL/min), and an auxiliary gas flow of 6 units N_2 (ca. 60 mL/min). LC-MS scans were acquired over m/z 900−1100, and data-dependent (m/z 900−1150) LC-MS^2 scans were obtained for selected samples with CID settings as for method A.

RESULTS AND DISCUSSION

Derivatization of Standards, Reaction Kinetics, and Mass-Spectral Analysis. Derivatization of MC-LR (**1**) with 2-aminoethanthiol (**a**) proceeded smoothly to yield **1a**, but, as expected,[4] the product eluted much earlier than **1** and displayed both singly- and doubly charged molecular ions, and this reaction was not investigated further.

The rate of thiol derivatization of MC-RY (MH^+ m/z 1045.5) with mercaptoethanol was followed by LC-MS^2 in weakly basic solutions. The half-life ($t_{1/2}$) of MC-RY (**9**) was 16 min at pH 9.2, and, although too fast to measure accurately, $t_{1/2}$ was estimated to be 2−3 min at pH 9.7−10.7. This is consistent with the thiolate form of mercaptoethanol (pK_a = 9.88 in ethanol−water[10]) being the reactive species, and buffer at pH 9.7 was used for the remainder of the studies reported here. LC-MS^2 analysis showed essentially complete conversion of **9** to its mercaptoethanol derivative (**9b**, MH^+ m/z 1123.5), which was present as a major (ca. 99%) and a minor stereoisomer, at all tested pH values.

The reaction with mercaptoethanol was then tested on a semiquantitative blend of commercially available microcystin analogues (**1**−**7**, Figure 4) with pH 9.7 buffer at 30 °C. Under these conditions, MC-RR (**3**) reacted with $t_{1/2}$ 6 min, MC-LR (**1**) $t_{1/2}$ 7 min, MC-YR (**2**) $t_{1/2}$ 9 min, MC-LF (**5**) $t_{1/2}$ 20 min, and MC-LA (**4**) $t_{1/2}$ 21 min. Again, reaction was essentially complete, although it was necessary to wait several hours for full conversion of the nonarginine-containing microcystins (all of which reacted more slowly than the tested arginine-containing analogues in the mixture). As with MC-RY, two isomers of the mercaptoethanol derivatives formed, but one of them was always very minor (typically 1−5% of the major isomer). Similar results were observed for a standard of [Dha^7]MC-LR (**8**) (data not shown), although here the minor isomer was typically ca. 5−10% of the major isomer, presumably due to reduced steric effects resulting from absence of the *N*-methyl group in the thiol-reactive Dha residue at site-7. Under the LC-MS^2 conditions used (method A), mercaptoethanol derivatives of arginine-containing microcystins eluted about 0.1 min earlier than their parent toxins (**1**−**3**, Figure 4a,b), whereas derivatives of microcystins without an arginine residue typically eluted ca. 0.5 min earlier than their parent toxins (**4**−**7**, Figure 4a,b).

No detectable isomerization or other reactions were observed under the derivatization conditions used in this study over a period of at least 2 days, both for microcystin standards and for the culture material. However, a number of 9-acetoxydesmethylAdda (ADMAdda) derivatives of microcystins are reported in the literature,[11] as are esters of the free carboxylic acid group of the Glu^6 residue.[12,13] We produced a partially esterified sample of MC-LA and found that the basic reaction conditions used for derivatization (pH 9.7 buffer) caused slow but steady hydrolysis ($t_{1/2}$ ca. 4 h) of the ester group to produce MC-LA. We anticipate that other esterified analogues of microcystin may also be hydrolyzed under the derivatization conditions used, but the presence of these in a sample can readily be detected by comparing LC-MS chromatograms of the sample in MeOH−H_2O with those of samples treated with base with and without addition of the thiol.

Replacement of mercaptoethanol (**b**, Figure 2) with MEMHEG (**c**, Figure 2) resulted in a similarly effective derivatization of microcystins **1**−**9**, with similar proportions for the pairs of diastereoisomers. In this case, derivatives of

Table 1. Microcystins Detected by LC-MS^2 (Method A) with and without Thiol Derivatization in Standards and in an Extract from an *M. aeruginosa* Culture Isolated from Africa (CYA548)[a]

underivatized sample				mercaptoethanol derivative		MEMHEG derivative		abundance in sample	
R_t (min)	$[MH]^+ m/z$	microcystin	status	R_t (min)	$[MH]^+ m/z$	R_t (min)	$[MH]^+ m/z$	mixed standard	culture CYA548
1.80	1038.5	MC-RR (**3**)	confirmed	1.81	1116.5	2.59	1394.5	major	ND
3.59	1031.5	[Dha^7]MC-YR (**15**)	tentative	3.49	1109.5	—	1387.5	minor	ND
3.61	1045.5	MC-YR (**2**)	confirmed	3.63	1123.5	4.33	1401.5	major	ND
3.68	981.5	[Asp^3]MC-LR (**12**)	tentative	3.69	1059.5	4.26	1337.5	minor	ND
3.77	995.5	MC-LR (**1**)	confirmed	3.71	1073.5	4.47	1351.5	major	ND
3.79	981.5	[Dha^7]MC-LR (**8**)	confirmed	3.84	1059.5	4.50	1337.5	minor	ND
4.79	1049.5	[Asp^3, Mser^7]MC-RY (**16**)	tentative	NR	—	NR	—	ND	minor
4.85	1017.5	[Asp^3, Dha^7]MC-RY (**17**)	tentative	4.69	1095.5	5.40	1373.5	ND	minor
5.01	1031.5	[Asp^3]MC-RY (**10**)	tentative	4.86	1109.5	5.41	1387.5	ND	major
5.29	1045.5	MC-RY (**9**)	confirmed	5.22	1123.5	5.79	1401.5	ND	ND
6.07	1015.5	[Asp^3]MC-RF (**18**)	tentative	5.90	1093.5	6.53	1371.5	ND	minor
7.60	1006.5	[Asp^3, Mser^7]MC-LY (**19**)	tentative	NR	—	NR	—	ND	minor
7.75	1038.5	[Asp^3]MC-YY (**20**)	tentative	7.59	1116.5	8.36	1394.5	ND	minor
7.94	974.5	[Asp^3, Dha^7]MC-LY (**21**)	tentative	7.09	1052.5	7.82	1330.5	ND	minor
8.15	988.5	[Asp^3]MC-LY (**11**)	tentative	7.76	1066.5	8.55	1344.5	ND	major
8.36	910.5	MC-LA (**4**)	confirmed	7.83	988.5	8.58	1266.5	major	ND
8.40	1002.5	[Asp^3]MC-HilY (**22**)	tentative	7.97	1080.5	8.75	1358.5	ND	minor
8.55	1002.5	MC-LY (**6**)	confirmed	8.10	1080.5	8.72	1358.5	major	ND
8.70	1032.5	MC-LY(OMe) (**13**)	tentative	8.23	1110.5	8.84	1388.5	minor	ND
9.80	1025.5	MC-LW (**7**)	confirmed	9.28	1103.5	9.84	1381.5	major	ND
9.83	972.5	[Asp^3]MC-LF (**23**)	tentative	9.21	1050.5	9.91	1328.5	ND	minor
10.30	986.5	MC-LF (**5**)	confirmed	9.59	1064.5	10.13	1342.5	major	ND
10.45	952.5	MC-LL (**14**)	tentative	9.80	1030.5	10.21	1308.5	minor	ND

[a]NR, no reaction; ND, not detected. Identities were considered confirmed only when peaks possessed identical retention time and mass spectral fragmentation to authentic standards and were considered tentative when these properties were consistent with the proposed structure in the absence of a standard. Retention times for the thiol derivatives are for the most abundant diastereoisomer. Compounds were denoted as "Major" when they constituted more than ca. 10% of the most abundant microcystin in the sample. MS^2 spectra for the analogues and their mercaptoethanol derivatives are available in the Supporting Information.

arginine-containing microcystins eluted about 0.8 min later than the parent toxins (**1**–**3**, Figure 4a,c), while derivatives of nonarginine-containing microcystins eluted with about the same retention times as the parent toxins (**4**–**7**, Figure 4a,c).

The mass spectra of both thiol derivatives were essentially identical to the parent toxin, displaying a molecular ion cluster with a dominant MH^+ ion and a series of up to 4–5 Na^+/NH_4^+ adduct-ions, together with a weak fragment with m/z of $[MH-134]^+$ (i.e., Adda fragmentation), except that m/z for MH^+ was increased by the mass of the adducted thiol (78 Da for mercaptoethanol and 356 Da for MEMHEG) derivatives of microcystins **1**–**9**. No doubly charged ions were observed for any of the microcystins under the LC-MS^2 conditions (method A) used in this study, even for analogues containing two arginyl residues (e.g., **3**).

Ion trap LC-MS^2 spectra of microcystins are structurally informative and relatively easy to interpret by comparing spectra with fragmentation patterns from microcystin standards.[14] Examination of the LC-MS^2 spectra showed that fragmentation of the mercaptoethanol derivatives occurred in a similar manner to the parent toxins, except that all fragments containing Mdha or Dha were increased by the mass of the adducted thiol. This was confirmed by comparison with MS^2 spectra of several reduced microcrocystin derivatives ([Mala^7]-MC-LR,[14] -LA, and -RY, Figure 1). Thus, comparison of the MS^2 spectra of the parent microcystin with those of their thiol derivatives can facilitate interpretation of the MS^2 spectra (Supporting Information). Furthermore, fragmentation spectra of the $[MH-134]^+$ ion (from fragmentation of the Adda moiety) can be obtained during LC–MS^n analysis, assisting in identification of the Adda-containing fragments in the MS^2 spectra.[15]

A clear effect of Arg-substitution was observed on retention times for microcystins during LC-MS^2 analysis (Table 1). Analogues containing two Arg groups (Arg^2 and Arg^4) eluted around 2 min (e.g., **3**). For microcystins containing a single Arg, those containing an Arg^4 group (e.g., **1**) eluted at 3.3–4.1 min, whereas those containing an Arg^2 group (e.g., **9**) eluted at 4.8–6.4 min. Microcystins without Arg groups (e.g., **4**) eluted at 7.6–10.5 min.

The pentapeptide cyanotoxin nodularin which—although structurally related to the microcystins—contains Mdhb in the location where MC-LR (**1**) contains Mdha (Figure 3) did not react with mercaptoethanol under the conditions used in our study. Because our investigations with MC-LR and [Dha^7]MC-LR standards showed that the presence or absence of an *N*-methyl group in the reactive amino acid moiety had minimal effect on its reactivity toward thiols, it seems likely that Dhb-containing cyanotoxins will be as unreactive as the Mdhb-group was in nodularin. The thiol derivatization procedure thus provides a convenient way to verify whether or not an Mdha/Dha group, rather than an Mdhb/Dhb group, is present in a candidate microcystin/nodularin peak simply by LC-MS analysis, without having to resort to purification followed by NMR or hydrolytic amino acid analysis. Partial reduction of the olefin in Mdha-, but not in Dhb-containing peptides, was

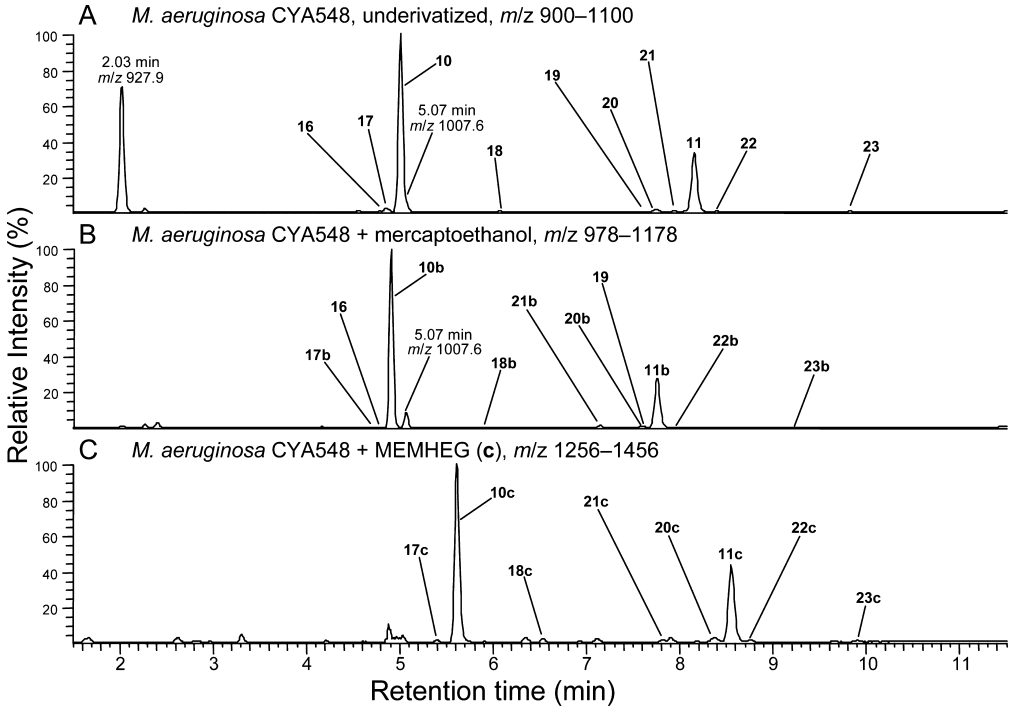

Figure 5. LC-MS^2 chromatograms (method A) of an extract from a culture (CYA548) of *M. aeruginosa* isolated from Lake Naivasha, Kenya: A, underivatized extract; B, extract derivatized with mercaptoethanol (adds 78 Da); and C, extract derivatized with MEMHEG (adds 356 Da). Microcystin peaks are marked with the compound's number (see Figures 1 and 2 and Table 1), and nonmicrocystin peaks are marked with R_t and m/z for the most prominent ion.

recently reported with 1,5-diaminonaphthalene during matrix-assisted laser desorption/ionization (MALDI) MS, and this method could also therefore be used to differentiate microcystin analogues containing Dhb and Mdha groups.[16] However, only partial reduction was observed for Mdha-derivatives, the mass of the derivatives only changes by 2 Da, and this approach is not as well suited to rapid analysis of complex mixtures of microcystins as LC-MS with thiol derivatization. We found Mdha- and Dha-containing microcystins were also conveniently derivatized for LC-MS^2 analysis by reduction of the olefin in residue-7 with $NaBH_4$. However, the Mdhb^5 group in NOD was also reduced with $NaBH_4$ to form [Maba^5]NOD under these conditions,[17] albeit four times more slowly than the Dha^7-group in **8**, so this procedure is less effective than thiol derivatization for differentiating between Mdha/Dha- and Mdhb/Dhb-containing analogues.

[Dha^7]MC-LR (**8**) was identified as a minor contaminant in the standards mixture, together with several minor components that were tentatively identified as [Asp^3]MC-LR (**12**), MC-LL (**14**), [Dha^7]MC-YR (**15**), and an analogue with MS and retention time characteristics (extra CH_2O in Tyr^4, and slightly longer R_t than MC-LY) consistent with a methoxyTyr^4-derivative of MC-LY (i.e., **13**) rather than a hydroxyHty^4 derivative. Proposed identities of microcystin contaminants detected in standards, and of microcystins detected in the algal sample (CYA548), were based on LC-MS^2 analysis and thiol-derivatization, aided by comparison with published data, and are presented in Table 1. Observed MS^2 spectra for **1**–**9**, **10**, **12**, **14**, **15**, and **23** were consistent with published mass spectral information,[12,14,18−27] and all compounds displayed the expected molecular ions during high-resolution MS (Supporting Information). It should be noted that mass spectrometric methods alone cannot differentiate between isobaric amino acids (e.g., Aba and isoAba) or stereochemistry (e.g., *E*- vs *Z*-Adda, or between L- and D-amino acids). Therefore, compounds in Table 1 are listed as tentative unless an authentic standard was used to establish its identity by both retention time and MS/MS comparisons.

Derivatization of CYA548 and Data Analysis. CYA548 is a strain of *M. aeruginosa* isolated from Lake Naivasha in Kenya's Rift Valley, ca. 350 km northeast of Lake Victoria. Haande et al.[7] found desmethylMC-YR (**15** or [Asp^3]MC-YR) as the only microcystin in strain CYA548 (i.e., AB2002/40) using MALDI−time-of-flight MS analysis with postsource decay and collision-induced dissociation, although the site of demethylation was not reported.

However, LC-MS^2 analysis (method A) of CYA548 (Figure 5a) revealed two main peaks, together with a range of minor peaks. Most of the peaks were derivatized by treatment with mercaptoethanol (Figure 5b), although several (notably those at 2.03 (MH^+ m/z 928) and 5.07 (MH^+ m/z 1007) were not. The main peak (R_t 5.01 min) displayed MH^+ m/z 1031 and reacted with mercaptoethanol, and its retention time and MS^2 fragmentation corresponded to [Asp^3]MC-RY (**10**). No MC-LR (**1**), MC-YR (**2**), desmethylMC-YR (**15** or [Asp^3]MC-YR) (precursors at m/z 995, 1031 and 1045, filtered for fragments of m/z 599), or MC-RY (**9**) (precursor at m/z 1045, filtered for fragments of m/z 440) were detected by LC-MS^2 analysis. The second major, later eluting, microcystin peak (MH^+ m/z 988, R_t 8.15 min) eluted slightly earlier than MC-LY and gave MS^2 spectra consistent with [Asp^3]MC-LY (**11**). A number of minor microcystin components were also tentatively identified, including [Mser^7]- and [Dha^7]-analogues of the two major [Asp^3]-analogues (Table 1). The [Mser^7]-containing analogues (**16** and **19**) did not react with mercaptoethanol, and it should be noted that this type of analog could be overlooked if thiol-

reactivity was used as the sole criterion for identifying peaks as microcystins.

Derivatization of the extract of CYA548 with MEMHEG proceeded smoothly (Figure 5c), and the mass range for typical microcystins was changed from m/z 900−1100, to m/z 1256−1456, a region of the chromatogram that was relatively free from interfering compounds. Nonmicrocystin analogues (e.g., the peaks at 2.03 and 5.07 min) were not derivatized and so did not appear in the mass window used for analysis of the MEMHEG derivatives. Consequently, the chromatogram in Figure 5c is dominated by peaks originating from microcystins, whereas the chromatograms in Figures 5a and 5b also contain other components (probably peptides), and it was relatively easy to identify candidate peaks of minor microcystin analogues. The [Mser^7]-microcystin analogues in the sample (**16** and **19**) did not react and were absent in this chromatogram, because they fall outside the mass range used to detect the derivatized microcystins (m/z 1256−1456); however, they were nonetheless still present in the chromatogram at their original (underivatized) m/z values and retention times.

The presence in Ugandan lakes of MC-RY (**9**) and [Asp^3]MC-RY (**10**) has recently been reported, based on analysis of fractionated extracts using medium-resolution MS/MS,[23] although their identities were not confirmed by methods capable of discriminating structural and stereochemical isomers, such as NMR spectroscopy or amino acid analysis. The MC-RY (**9**) standard used in the present study had been purified and its structure verified by one- and two-dimensional NMR spectroscopy (manuscript in preparation), thus greatly strengthening interpretation of its MS^2 fragmentation patterns (which were consistent with those reported by Okello et al.[23]). This in turn strengthens the interpretation of the MS^2 spectra (Supporting Information) leading to the tentative identification of the less common Arg^2-containing analogues for which standards are not available, such as the [Asp^3]- and [Mser^7]-congeners of MC-RY.

Confirmation by LC-MS/MS with Precursor-Ion Scanning and High-Resolution LC-MS. Underivatized samples of microcystin standards and CYA548 were analyzed by LC-HRMS (method C) using the same column and gradient elution as was used for the LC-MS^2 studies (method A). All peaks reported in Table 1 were also detected by LC-HRMS (method C), and their MH$^+$ ions were found to have m/z values corresponding to those calculated for the atomic compositions of the standards or for the proposed tentative structures (observed deviations, Δ = 1.3 to −3.0 ppm, Supporting Information).

Most microcystins contain the unusual β-amino acid Adda at position 5 (Figure 1). During CID in positive ion mode, the Adda side chain cleaves to give a characteristic fragment ion at m/z 135,[15] a reaction commonly exploited during MRM LC-MS analysis of microcystins with triple-quadrupole instruments. A concentrated extract of CYA548 was analyzed by LC-MS/MS with precursor-ion scanning for m/z 135 using a triple-quadrupole instrument (method B) using the same HPLC column and gradient elution as had been used for the LC-MS^2 (method A) analyses. The resulting chromatogram (Figure 6) shows the retention times and m/z for precursor ions giving rise to product ions of m/z 135. Such precursor ions probably contain Adda and are therefore likely to be microcystins.

It is apparent that most of the proposed microcystins identified by LC-MS^2 (method A) with the aid of thiol

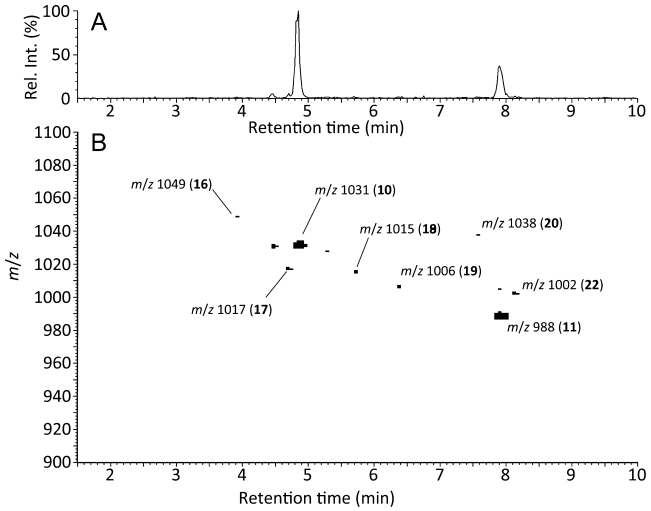

Figure 6. LC-MS/MS (method B), with precursor-ion-scanning for m/z 135 (indicative of the presence of Adda), of extract from a culture (CYA548) of *M. aeruginosa* isolated from Lake Naivasha, Kenya. A, chromatogram for precursors of m/z 135; B, precursor-ion map for precursors of m/z 135. Identified microcystin peaks are marked with the compound's number (see Figure 1 and Table 1), and unidentified peaks are marked with m/z for the precursor ion.

reactivity (Table 1 and Figure 5) were also identified by LC-MS/MS with precursor-ion scanning (method B). Furthermore, retention times observed for the peaks in Figure 6 closely matched those listed in Table 1. This provides strong independent evidence that the compounds listed in Table 1 are Adda-containing compounds and is consistent with the presence of prominent [MH−134]$^+$ fragments in the MS^2 spectra of **1**−**23** during LC-MS^2 (method A) analysis. LC-MS/MS with precursor-ion scanning for m/z 135 also readily identified microcystins which contained modifications at position-7 that render them unreactive toward thiols, such as [Mser^7]-derivatives **16** and **19**, making this approach highly complementary to thiol derivatization with LC-MS^2 (method A) when a sufficiently concentrated sample is available.

In summary, the combination of derivatization and LC-MS methods reported here provides a valuable suite of tools for detecting and confirming the presence of microcystins in difficult matrices and for determining their probable structures. Comparison of chromatograms with and without mercaptoethanol derivatization, or examination of chromatograms after derivatization with MEMHEG, readily identified peaks arising from Mdha/Dha^7-containing microcystins. The ability to differentiate between microcystins containing Mdha and Dhb at position-7 through microscale derivatization followed by LC-MS analysis is particularly useful. Application of thiol derivatization methods lead to identification of MC-RY derivatives in an African *Microcystis* culture. Extension of the thiol-derivatization methodology to quantitative analysis may be possible, and preliminary trials with fluorescent thiols were promising.

■ ASSOCIATED CONTENT

Supporting Information

Tabulated HR-MS for [MH]$^+$ of **1**−**23**. LC-MS^2 chromatograms of [Dha^7]MC-LR (**8**) and its mercaptoethanol and MEMHEG derivatives. LC-MS^2 chromatograms and MS of **8** and NOD before and after treatment with mercaptoethanol.

$LC\text{-}MS^2$ spectra for $[MH]^+$ of **1**−**23**, mercaptoethanol derivatives of **1**−**23**, and reduced derivatives of **1**, **4**, and **9**. Excel spreadsheet tabulating fragments for **1**, **2**, and **4**−**23** and reduced derivatives of **1**, **4**, and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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