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Conjugation of Microcystins with Thiols Is Reversible: Base-Catalyzed Deconjugation for Chemical Analysis

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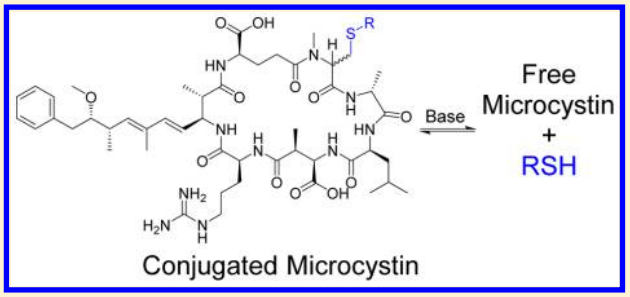
Supporting Information

ABSTRACT: Microcystins are potent cyclic heptapeptide toxins found in many freshwater cyanobacteria. Most microcystins contain an α,β-unsaturated amide that can react with thiol-containing amino acids, peptides, and proteins in vivo and in vitro. While soluble conjugates formed from small peptides can be extracted and analyzed directly by LC−MS, microcystins conjugated to proteins are analyzed after oxidative cleavage of their Adda side chains, but information on which microcystin analogues were present is lost. Observations during the development of thiol-derivatization-based LC−MS methods for microcystin analysis indicated that the reaction of thiols with microcystins was reversible. The kinetics of deconjugation was investigated with mercaptoethanol as a model thiol to identify suitable reaction conditions. A range of microcystins conjugated to mercaptoethanol, methanethiol, cysteine, and glutathione were then successfully deconjugated, demonstrating the feasibility of releasing conjugated forms of microcystins for chemical analysis. Reagents for removing the released thiols or for trapping the released microcystins increased the reaction rate. Optimization of methodologies based on this reaction should increase the method's utility for measuring free and conjugated microcystins. The results also indicate that thiol-conjugated microcystins slowly release free microcystins, even at neutral pH, with consequences for assessment of toxin exposure, metabolism, and trophic transfer. A range of other common natural and environmental toxins, such as deoxynivalenol and acrylamide, also contain α,β-unsaturated carbonyl groups and can be expected to behave in a similar manner.



INTRODUCTION

Microcystins (Figure 1) are potent cyclic heptapeptide toxins found in some of the cyanobacteria that grow in fresh or brackish water.[1,2] Most microcystins contain dehydroalanine (Dha) or *N*-methyldehydroalanine (Mdha) at position-7. Dha and Mdha are 2-substituted acrylamides and contain α,β-unsaturated carbonyl groups that are reactive toward nucleophilic attack, forming conjugates with thiol-containing amino acids, peptides, and proteins (Figure 2).[3−16] Conjugated forms of microcystins are often present in samples from nature, sometimes at much higher concentration than the free microcystins,[4,15,17−23] and may play a role in the ecology and trophic transfer of the toxins or be useful biomarkers of microcystin exposure.[6,9,16,17,20−24]

Soluble microcystin conjugates formed from Cys, GSH, and other small thiol-containing biomolecules can be extracted together with free microcystins and analyzed directly by LC-MS[6,12,15] or immunochemical methods.[6,11,21,25] Nonextractable microcystin conjugates, such as those bound to proteins, can be analyzed by oxidative cleavage of their Adda side chain and quantitation of the resulting oxidative cleavage product by LC−MS or gas chromatography,[18,26,27] or their concentrations can be estimated with immunochemical methods.[17] Alternatively, protein−microcystin conjugates can be hydrolyzed with proteases, and the resulting soluble microcystin-conjugated peptides can be analyzed using an immunoassay with appropriate cross-reactivity.[6] In principle, these approaches can be used to measure the total content of conjugated microcystins (as well as of nodularins) in biological samples, but information as to which analogues are conjugated is lost. Such information could be useful, for example, when trying to compare the microcystin conjugate-profile of intoxicated animals with the microcystin profile in nearby water bodies in order to identify the source of exposure.

Some Michael additions of oxygen, nitrogen, and sulfur nucleophiles to α,β-unsaturated carbonyl compounds have been shown to be reversible,[28−32] and our observations of instability in a standard of a microcystin−Cys conjugate and examination of

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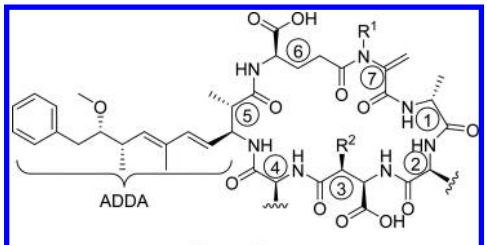

		②	④	R^1	R^2	m/z
1	MC-LR	Leu	Arg	Me	Me	995.5560
2	MC-YR	Tyr	Arg	Me	Me	1045.5353
3	MC-RR	Arg	Arg	Me	Me	1038.5731
4	MC-LA	Leu	Ala	Me	Me	910.4920
5	MC-LF	Leu	Phe	Me	Me	986.5233
6	MC-LY	Leu	Tyr	Me	Me	1002.5183
7	MC-LW	Leu	Trp	Me	Me	1025.5342
8	[Asp^3]MC-RY	Arg	Tyr	Me	H	1031.5197
9	[Dha^7]MC-LR	Leu	Arg	H	Me	981.5404
10	MC-RY	Arg	Tyr	Me	Me	1045.5353

Figure 1. Structures of microcystins used in this study (**1**−**10**). Values for m/z are calculated for $[M + H]^+$.

published kinetic data[3,33] suggested that this might be the case for the reaction of microcystins with thiols *in vitro* (Figure S1). Furthermore, evidence for the reversibility of GSH- and Cys-conjugation to MC-RR *in vivo* has recently been presented.[34] Schmidt et al. suggested that this process might be enzyme-catalyzed because the reverse Michael reaction normally requires low pH and stated that "studies on this reversible conjugation are essential for a more complete understanding of the toxicity, transport, and transformation of microcystins in living cells".[35]

We therefore investigated the kinetics of deconjugation of a range of thiol-conjugated microcystins and found that this reaction is base-catalyzed and can be used to release thiol-conjugated microcystins for LC−MS analysis. Preliminary studies showed that modifications to the procedure have the potential to increase the rate and efficiency of deconjugation. Other methods, such as ELISA and PP2A-inhibition assays, could also be used for analysis of the released microcystins but will not provide information on the toxin profile. The information obtained on the dynamics of the Michael reaction of microcystins provides invaluable information for understanding the behavior of microcystin conjugates *in vivo* and *in vitro*.

MATERIALS AND METHODS

General. Mercaptoethanol, MeSNa, iodoacetamide, and GSH transferase (GST) from rat liver (15 units/mg protein; G8386) were from Sigma−Aldrich (Oslo, Norway). L-Cysteine was from BDH (Auckland, New Zealand), and GSH was from Sigma−Aldrich (Sydney, Australia). A mixed standard consisting of MC-LR, MC-YR, and MC-RR (**1**−**3**) at 0.1 μg/mL and MC-LA, MC-LF, MC-LY, and MC-LW (**4**−**7**) at 0.5 μg/mL in MeOH−H_2O (1:1) was prepared from microcystins from Alexis Biochemicals (Grünberg, Germany) as described previously[3] and supplemented with [Dha^7]MC-LR (**9**) (0.1 μg/mL) from NRC Measurement Science and Standards, Halifax, NS, Canada. MC-RY[36] (**10**) and [Asp^3]MC-RY[37] (**8**) were purified as described elsewhere. Solvents for LC and extraction were of gradient (Romil, Oslo, Norway) or LC/MS quality (Fisher Scientific, Fair Lawn, NJ or Loughborough, United Kingdom). Hypersep C18, (500 mg/3 mL; Thermo Scientific) and Strata-X (200 mg/3 cc or 500 mg/6 cc; Phenomenex) SPE columns were preconditioned according to the manufacturers' recommendations.

Mercaptoethanol Derivatives. To the mixed standard containing **1**−**7** and **9** (500 μL) was added sodium carbonate buffer (0.2 M, pH 9.7, 2 mL), and then mercaptoethanol (5 μL) was added with brief vortex-mixing, and the sample was placed in the LC−MS sample tray (30 °C). Progress of the reaction was checked periodically by LC-MS^2 analysis (method A). After 3 h, the reaction appeared to be complete for all microcystin analogues, and formic acid in water (1:1, 50 μL) was added to stabilize the adducts. The solution was concentrated under a stream of N_2 to remove most of the MeOH, and water (1 mL) was added. The mixture was applied to a Hypersep C18 SPE column, and eluted successively with 10%, 50%, 60%, 80%, and 100% MeOH (5 mL each). LC−MS (method A) showed mercaptoethanol derivatives of mainly MC-LA, MC-LY, MC-LW, and MC-LF (**4a**−**7a**) in the 50% MeOH fraction, MC-LR, MC-YR, and [Dha^7]MC-LR (**1a**, **2a**, and **9a**) in the 60% fraction, and MC-RR (**3a**) in the 80% fraction. These fractions were combined, evaporated under a stream of N_2, and the residue dissolved in 50% MeOH (1.0 mL) to give a mixture of **1a**−**7a** and **9a**. A specimen of MC-RY (**10**) was separately derivatized with mercaptoethanol and purified to afford **10a** in a corresponding manner.

Methanethiol Derivative. To an aliquot of [Asp^3]MC-RY (**8**) (ca. 10 μg) in MeOH−H_2O was added 1 mg of MeSNa. The reaction was monitored and fractionated as described for the mercaptoethanol derivatives, to yield a solution of ca. 10 μg/mL of **8b** in MeOH−H_2O.

Cysteine and Glutathione Derivatives. Cys and GSH conjugates of [Dha^7]MC-LR (**9c** and **9d**) were prepared after the method of Kondo et al.[5] Briefly, L-cysteine (37 mg) and L-glutathione (80 mg) were each dissolved in 5% K_2CO_3 (2 mL) and added to vials containing [Dha^7]MC-LR (**9**) (3 mg). The vials were flushed with nitrogen, capped, and held at ambient temperature for 3.5 h. Reactions were quenched by the addition of 2 M HCl (370 μL), loaded onto Strata-X SPE columns, washed with H_2O (10 mL), and eluted with MeOH (7 mL). Each load and wash fraction was applied to a second Strata-X SPE column and eluted as described above. The MeOH fractions from the two pairs of columns were each combined, dried under a stream of nitrogen, and dissolved in 80% MeOH (1 mL) for purification by HPLC. Purification was performed by HPLC−UV on a Prodigy ODS(3) column (5 μm, 250 × 10 mm i.d.), eluted isocratically with

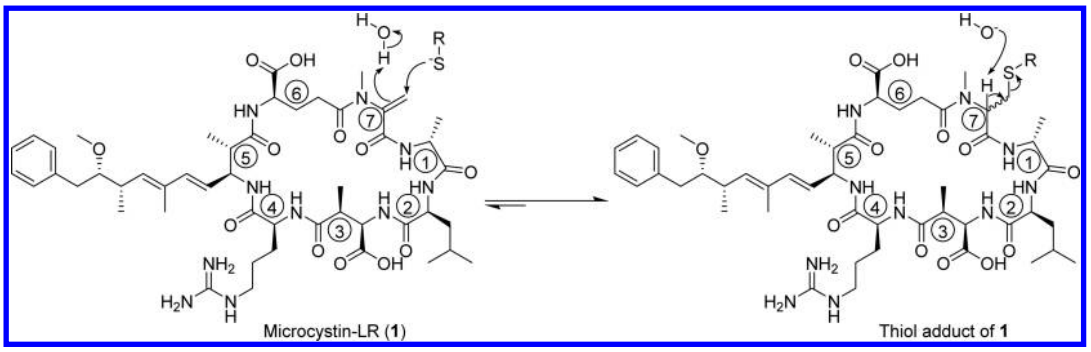

Figure 2. Generic depiction of the base-catalyzed conjugation of the Mdha^7-group of a microcystin with a thiolate (RS^-) in an aqueous solvent, using microcystin-LR (**1**) as a representative example. Microcystins containing a Dha^7-group display similar reactivity. Thiols **a**−**d** used in this study are shown in Figure 3.

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acetonitrile (A) and 0.01 M ammonium acetate (B) at 5 mL/min with detection at 238 nm. The eluent was 28:72 (A–B) for the Cys conjugates (**9c**) and 26:74 (A–B) for the GSH conjugates (**9d**). Fractions containing the products of interest for both **9c** (6.7 min, 9.1 min) and **9d** (5.8 min, 7.2 min) were collected and evaporated in vacuo. The major isomers for Cys (**9c**, isomer 2, 9.1 min) and GSH (**9d**, isomer 2, 7.2 min) were dissolved in H_2O (10 mL), applied to Strata-X SPE columns, washed with H_2O (10 mL), and eluted with MeOH (8 mL). The MeOH fractions were evaporated under a stream of nitrogen and freeze-dried to constant weight to yield pure isomer-2 of **9c** (2.33 mg) and of **9d** (2.69 mg). The minor isomers (isomer-1) for **9c** (6.7 min) and **9d** (5.8 min) contained ca. 40% and 28% of the major isomers, respectively.

Conversion of **9** to **9d** at physiological pH in the presence and absence of GST was based on Habig et al.[38] Reactions were performed in 1 mL of sodium phosphate buffer (pH 7.4; 0.1 M) containing **9** (500 ng/mL), GSH (2 mM), with (15 U/mL) or without the addition of GST, with reaction mixtures held in the LC–MS autosampler at 30 °C and monitored by LC–MS^2 method A.

[D,L-Ala^7]MC-LR. [D,L-Ala^7]MC-LR was prepared from **9** based on a published procedure.[39] Briefly, $NaBH_4$ (35 mg) was added to [Dha^7]MC-LR (**9**) (2 mg) in 80% MeOH (750 μL). After 16 h, the mixture was adjusted to pH 4.5 with 10% acetic acid, loaded onto a preconditioned Strata-X SPE column (200 mg/3 mL), washed with H_2O (5 mL), and eluted with MeOH (3.5 mL). The MeOH fraction was evaporated under a stream of nitrogen, dissolved in 20% MeOH (2 mL), applied to a preconditioned C18 SPE column (500 mg/3 mL), washed with 4 mL of 20% MeOH, and eluted successively with 5 mL aliquots of 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% MeOH. The minor isomer (isomer-1, ca. 20%) eluted in the 30–40% MeOH fractions, and the major isomer (isomer 2, ca. 80%) eluted in the 50–70% MeOH fractions. The appropriate fractions were combined, evaporated in vacuo, and dissolved in 60% MeOH (600 μL) for final purification by HPLC. Purification of [Ala^7]MC-LR was performed as for **9c**, with isomers-1 and -2 eluting at 7.8 and 11.2 min, respectively. Fractions containing the two isomers were recovered with Strata-X SPE columns, as described above for **9c** and **9d**, to yield pure isomer-1 (0.20 mg) and isomer-2 (1.07 mg) of [Ala^7]MC-LR.

Deconjugation Reactions. In a preliminary experiment (Figure S2 and Table S1), **10a** (50 μL, 1:1 MeOH–H_2O) was added to 200 μL of carbonate buffer (pH 10.7, 10.2, 9.7 and 9.2). DMSO (45 μL) was added after 4 h to the three higher pH reactions, and at the start for the pH 9.2 reaction. Iodoacetamide (5 μL; 57 mg/mL in MeCN) was added to the pH 10.7 reaction at 29 h. The four reactions were held together at 30 °C in the autosampler tray and analyzed regularly with 5 μL injections using the LC–MS^2 (method A). A second preliminary trial was performed in the same manner except that **10a** (50 μL) was added to a mixture of the iodoacetamide solution (5 μL) and 50 μL of DMSO in pH 10.7 buffer (200 μL), to test the feasibility of using iodoacetamide to promote the deconjugation reaction (Figure S3).

In subsequent routine reactions for kinetic analysis (Table 2), a solution of the conjugate or conjugate mixture (200 μL, 1:1 MeOH–H_2O) in an HPLC vial was placed in the LC–MS autosampler tray (30 °C) for ca. 15 min prior to the addition of carbonate buffer (0.2 M, 50 μL; also at tray-temperature) of the chosen pH. The vial was capped, and the reaction was followed regularly by LC–MS^2 (method A). Reactions involving DMSO used the same procedure, except that the carbonate buffer was added to a mixture of 180 μL of the conjugate solution and 20 μL of DMSO. Kinetics experiments (Table 2) used A, the mixture of **1a**–**7a** and **9a**; B, **8b**; C, **9c**; D, **9d**; and E, **8b** plus **9d**. In the reaction involving reduction with $NaBH_4$, $NaBH_4$ (1 mg) was added initially with the carbonate buffer to **9d** at the start of the reaction, with a second aliquot of $NaBH_4$ added at 23.4 h. Half-lives of analogues in each reaction were estimated by fitting their relative abundances (LC–MS method A) to 3-parameter exponential curves (SigmaPlot 13.0, Systat Software Inc., San Jose, CA, USA).

LC-MS Analyses: 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) as described previously,[3] eluted with a linear gradient (0.3 mL/min) of acetonitrile (A) and water (B) each containing 0.1% formic acid. The gradient was from 22–75% A over 10 min, to 95% A at 11 min (1 min hold), followed by a return to 22% A with a 3 min hold to equilibrate the column. The LC system was coupled to a Finnigan LTQ ion trap mass spectrometer (Finnigan Thermo Electron Corp., San Jose, CA, USA) operated as described previously.[3] Relative abundances were estimated from the areas of the respective $[M + H]^+$ peaks or, for reactions involving compounds displaying significant $[M+2H]^{2+}$ intensity, from the sum of the areas of the $[M + H]^+$ and $[M+2H]^{2+}$ peaks.

LC-HRMS (Method B). Liquid chromatography with high-resolution MS (LC-HRMS) was as for method A, except that a Waters Acquity UPLC pump and autosampler were used. A Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) was used as detector, with a spray voltage of 3.5 kV, capillary temperature of 350 °C, probe heater at 300 °C, S-lens RF level of 50, and sheath and auxiliary gas at 35 and 10, respectively. The spectrometer was operated in all-ion-fragmentation (AIF) mode (full scan: scanned m/z 500–1400, AGC target 5×10^6, resolution 70,000, and max IT 200 ms; AIF scanned m/z 110–1500, AGC target 3×10^6, resolution 35,000, max IT 200 ms, and normalized collision energy 50).

High Resolution Mass Spectrometry. HR-MS was performed in negative and positive ion modes on a Bruker Daltonics MicrOTOF spectrometer. Purified samples in MeOH were infused via a syringe pump at 4 μL/min. Cluster ions from sodium formate (2 mM) were used for mass-calibration. Mass spectra were acquired with a time-of-flight analyzer over m/z 800–1500. Capillary voltage and skimmer cone voltage were set at −100 V and −50 V for negative ion, and at 150 and 50 V for positive ion spectra.

NMR Spectroscopy. NMR spectra of [Dha^7]MC-LR (**9**) and of isomers-1 and -2 of [Ala^7]MC-LR, **9c** and **9d**, were obtained at 303 K from solutions in CD_3OD (99.8+ atom% D; Aldrich, USA) using a Bruker DRX 400 MHz spectrometer fitted with a 5 mm dual, gradient shielded, inverse probe. NMR assignments for isomer-2 of [Ala^7]MC-LR were obtained by examination of ^1H, ^{13}C, DEPT135, COSY, TOCSY, HSQC, and HMBC NMR spectra. The following NMR spectra were obtained for the remaining compounds: isomer-1 of [Ala^7]MC-LR, ^1H, COSY, and TOCSY; **9c** isomer-1, ^1H; **9c** isomer-2, ^1H, COSY, TOCSY, and HSQC; **9d** isomer-1, ^1H; **9d** isomer-2, ^1H; and **9**, ^1H, COSY, TOCSY, HSQC, HMBC, and ROESY. For **8**, ^1H, ^{13}C, DEPT135, COSY, TOCSY, SELTOCSY, ROESY, HSQC, HMBC, and band-selective HMBC NMR spectra were obtained at 298 K with CD_3OH as solvent using Bruker AVI and AVII 600 MHz NMR spectrometers equipped with TCI cryoprobes and Z-gradient coils. ^1H, COSY, TOCSY, HSQC, and ROESY NMR spectra were obtained with continuous wave presaturation of the OH/H_2O and/or the residual CHD_2OH signals in the spectra. TOCSY and SELTOCSY spectra were acquired with correlation times variously optimized for the detection of short-, medium-, and long-range couplings. The band-selective HMBC NMR spectrum was centered at 171.7 ppm in the carbon axis with a sweep width of 25 ppm for detection of carbonyl resonances. All NMR spectra were calibrated relative to internal CHD_2OD or CHD_2OH (3.31 ppm) and CD_3OD or CD_3OH (49.0 ppm).

RESULTS AND DISCUSSION

Addition of nitrogen, oxygen, and sulfur nucleophiles to electron-deficient double bonds (Michael addition, e.g. Figure 2) can be reversible,[28–32] and information on the reaction kinetics can be used to give control over the extent of conjugation.[29,30] LC–MS analysis of a standard of Cys–[Dha^7]MC-LR (**9c**, isomer-2) stored in 1:1 MeOH–H_2O at −20 °C in our laboratory for 2 years showed ca. 50% deconjugation (data not shown), indicating that the Michael addition of thiols to microcystins was also reversible. Furthermore, when microcystins were reacted with mercaptoethanol,[3,33] small amounts of underivatized microcystins often remained detectable even at long reaction times in the presence of excess thiol (Figure S1), indicating an equilibrium of the type shown in Figure 2.

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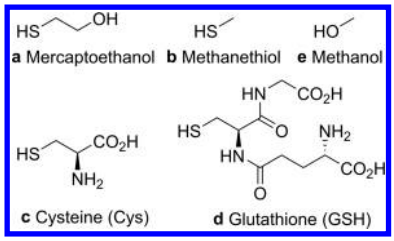
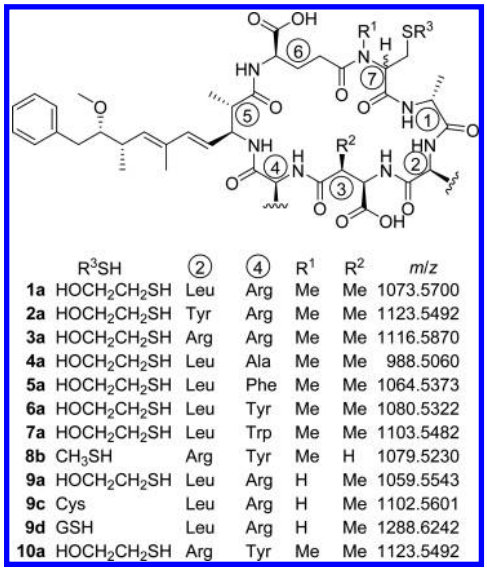


Figure 3. Nucleophiles observed in this study to react with the Mdha[7]/Dha[7]-groups of microcystins **1**–**10** (Figure 1): (a) mercaptoethanol, (b) methanethiol, (c) L-cysteine, (d) glutathione, and (e) methanol. The products from the reactions with thiols a–d are shown in Figure 4 and with MeOH (e) in Figure S5.



	R^3SH	②	④	R^1	R^2	*m/z*
1a	$HOCH_2CH_2SH$	Leu	Arg	Me	Me	1073.5700
2a	$HOCH_2CH_2SH$	Tyr	Arg	Me	Me	1123.5492
3a	$HOCH_2CH_2SH$	Arg	Arg	Me	Me	1116.5870
4a	$HOCH_2CH_2SH$	Leu	Ala	Me	Me	988.5060
5a	$HOCH_2CH_2SH$	Leu	Phe	Me	Me	1064.5373
6a	$HOCH_2CH_2SH$	Leu	Tyr	Me	Me	1080.5322
7a	$HOCH_2CH_2SH$	Leu	Trp	Me	Me	1103.5482
8b	CH_3SH	Arg	Tyr	Me	H	1079.5230
9a	$HOCH_2CH_2SH$	Leu	Arg	H	Me	1059.5543
9c	Cys	Leu	Arg	H	Me	1102.5601
9d	GSH	Leu	Arg	H	Me	1288.6242
10a	$HOCH_2CH_2SH$	Arg	Tyr	Me	Me	1123.5492

Figure 4. Structures of the microcystin thiol-derivatives (**1a**–**7a**, **8b**, **9a**, **9c**, **9d**, and **10a**) of microcystins **1**–**10** produced for use in deconjugation experiments. For the thiol derivatives, the structure-number refers to the precursor microcystin (Figure 1), while the letter denotes the adducted thiol shown in Figure 3. Note that an earlier- (usually minor) and a later-eluting (usually major) diastereoisomer are formed for each microcystin–thiol conjugate which, when they need to be discussed separately in the text, are referred to as isomer-1 and isomer-2, respectively. Values for *m/z* are calculated for $[M + H]^+$. NB: The conjugate of Cys–Dha is known as lanthionine (Lan), i.e., **9c** is [Lan[7]]MC-LR.

Using the thiols in Figure 3, we therefore synthesized a range of microcystin–thiol conjugates (Figure 4; accurate masses, Table S1; LC–MS^2 spectra of mercaptoethanol adducts (**1a**–**7a**, **9a**, and **10a**) were essentially identical to those reported previously;[3,33,36] for spectra of **8b**, **9c**, and **9d**, see Figures S58–S60). Because the proposed structure of **8** was only tentative, being based solely on LC–MS/MS data and thiol reactivity,[3,40] its structure was verified by NMR spectroscopy before derivatization. The same strategy was applied as was used previously for NMR analysis of MC-RY,[36] except for the additional use of a band-selective HMBC spectrum to resolve the carbonyl resonances (Figures S18–S34), leading to the assignment of all ^1H and ^{13}C resonances (Table 1) and confirmation of the structure of **8** as shown in Figure 1. The identities of **9** (Figures S52–S57, exhibiting proton and carbon resonances identical to those reported by Harada et al.[41]) and its isomeric pairs of Cys and GSH derivatives **9c** and **9d** (Figures S13, S14, and S44–49) were also confirmed by NMR spectroscopy.

Model Conjugates. The stability of the microcystin–thiol conjugates was studied under basic conditions. A preliminary experiment showed that **10a** was deconjugated upon standing in weakly basic solutions, with the half-life of the conjugate decreasing with increasing pH and with addition of DMSO or iodoacetamide (Figure S2, Table S1). The use of iodoacetamide led to iodination and partial alkylation of **10** and **10a** (Figure S3), and was not investigated further.

More detailed kinetic studies utilized a mixture of mercaptoethanol conjugates of eight representative microcystins with amino acid variations at positions 2, 4, and 7 (**1a**–**7a** and **9a**, Figure 4). These conjugates underwent smooth deconjugation in MeOH–carbonate buffer (Figure 5 and experiment A in Table 2), with the reaction rate following first-order kinetics and increasing markedly with pH (data not shown). At pH 10.7, most of the thiol derivatives (**1a**–**7a**) had half-lives of ca. 30–50 h, but **9a** reacted 4 times slower than *N*-methylated analogue **1a**, presumably due to steric or electronic effects, while **3a** reacted 1.4 times faster than **1a**. Of the pairs of diastereoisomeric sulfide derivatives, the major ones appeared to react faster (e.g., see the ratios of the two peaks for **4a** over time in Figure 5). However, this effect may not be real, as diastereoisomers could potentially undergo scrambling due to the deprotonation at the α-position being followed by reprotonation before elimination of the thiolate can occur (Figure S4).

Deconjugations performed with the methanethiol conjugate of [Asp[3]]MC-RY (**8b**) gave similar results (experiment B in Table 2), with a marked reduction in reaction rate at lower pH consistent with the mechanism in Figure 2. In addition to free **8**, an earlier-eluting isomeric product was also detected (*iso*-**8**, see also Figure 6). *iso*-**8** was also slowly produced with first-order kinetics by treatment of **8** with carbonate buffer at pH 10.7, whereas **2** did not isomerize under the same conditions, and the rate of isomerization to *iso*-**8** was pH-dependent (Figures S6–S9). *iso*-**8** fragmented very similarly to **8** during LC–MS^2 analysis, but unlike **8** and **2**, it did not react with mercaptoethanol under basic conditions. Samples containing a range of other microcystins with Arg at position-2, available in our laboratory from earlier work,[5,6,9,10] showed analogous base-promoted isomerizations. These results suggest that the reactive olefinic Dha[7]/Mdha[7]-group in microcystins containing Arg at position-2 may undergo intramolecular nucleophilic attack by the guanidinium group, although no sign of similar reactivity was observed for MC-RR (**3**) during the experiments described here.

Although the major products corresponded to the free microcystins, small amounts were detected of pairs of compounds corresponding to diastereoisomeric addition of 32 Da to the mass of the microcystins (e.g., **8e** and **9e** in Figures 6, 7, and S6). When excess mercaptoethanol was added, these compounds did not react, whereas the free microcystins reacted rapidly (Figure S7). This indicated an addition of 32 Da to the reactive double bond in the microcystins (Figure S5), and when the reaction was performed in CD_3OH–carbonate buffer, the corresponding products were formed by the addition of 35 Da (addition of CD_3OH to the reactive double bond) (Figure S9). Trace levels of similar isomeric pairs of microcystin adducts containing an extra 18 Da were sometimes also observed that appear to correspond to the addition of H_2O to the reactive double bond (i.e., the corresponding [Mser[7]]- and [Ser[7]]-variants). The problem of alcohol addition to the double bond was significantly reduced when EtOH was used as cosolvent (data not shown) instead of MeOH.

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Table 1. NMR Assignments for [Asp^3]MC-RY (8) in CD_3OH and [Ala^7]MC-LR (isomer-2) in CD_3OD[a]

[Asp^3]MC-RY (8)				[Ala^7]MC-LR (isomer-2)			
	atom	^{13}C	^{1}H (multiplicity, J (Hz))		atom	^{13}C	^{1}H (multiplicity, J (Hz))
Ala^1	1	175.1		Ala^1	1	175.2	
	2	49.2	4.52 (dq, 8.9, 7.3)		2	50.4	4.47 (q, 7.3)
	2-NH		7.50 (d, 8.9)		2-NH		
	3	16.7	0.91 (3H, d, 7.3)		3	17.5	1.35 (3H, d, 7.3)
Arg^2	1	173.9		Leu^2	1	174.8	
	2	57.6	4.01 (td, 7.6, 6.5)		2	54.6	4.38 (m)
	2-NH		8.06 (d, 6.5)		2-NH		
	3	29.0	1.94 (2H, m)		3	41.1	1.66 (m)
	4	26.6	1.71 (m)				1.88 (ddd, 13.3, 11.5, 3.8)
			1.74 (m)		4	26.0	1.73 (m)
	5	42.0	3.19 (2H, t, 6.2)		4-Me[b]	21.4	0.90 (3H, d, 6.5)
	6	158.8			5[b]	23.6	0.95 (3H, d, 6.5)
Asp^3	1	176.4		Masp^3	1	176.6	
	2	52.7	4.52 (ddd, 9.4, 4.4, 3.7)		2	57.8	4.49 (d, 4.8)
	2-NH		7.93 (d, 9.4)		2-NH		
	3	39.1	2.83 (dd, 13.7, 4.4)		3	43.2	3.08 (qd, 7.1, 4.8)
			1.91 (dd, 13.7, 3.7)		3-Me	15.0	1.05 (3H, d, 7.1)
	4	175.2			4	177.8	
Tyr^4	1	171.8		Arg^4	1	172.2	
	2	55.8	4.54 (ddd, 11.3, 9.7, 3.1)		2	52.9	4.36
	2-NH		8.69 (d, 9.7)		2-NH		
	3	37.1	3.35 (dd, 14.1, 3.1)		3	29.1	1.53 (m)
			2.48 (dd, 14.1, 11.3)				1.99 (m)
	4	130.0			4	26.3	1.53 (2H, m)
	5, 9	130.9	6.95 (2H, d, 8.6)		5	41.9	3.15 (2H, t, 6.6)
	6, 8	116.2	6.58 (2H, d, 8.6)		6	158.6	
	7	157.2					
Adda^5	1	176.7		Adda^5	1	176.7	
	2	44.9	3.19 (dq, 10.6, 6.9)		2	45.4	2.92 (dq, 10.3, 7.0)
	2-Me	16.1	1.08 (3H, d, 6.9)		2-Me	16.0	1.08 (3H, d, 7.0)
	3	56.7	4.65 (dt, 10.6, 8.9)		3	56.5	4.52 (dd, 10.3, 8.6)
	3-NH		8.11 (d, 8.9)		3-NH		
	4	127.1	5.52 (dd,15.5, 8.9)		4	126.9	5.51 (dd, 15.5, 8.6)
	5	138.5	6.29 (d, 15.5)		5	138.7	6.24 (d, 15.6)
	6	133.9			6	134.1	
	6-Me	12.8	1.64 (3H, d, 1.3)		6-Me	12.9	1.63 (3H, d, 1.2)
	7	136.6	5.45 (dd, 10.0, 1.3)		7	136.8	5.41 (d, 10.0)
	8	37.6	2.61 (dp, 10.0, 6.7)		8	37.7	2.59 (m)
	8-Me	16.5	1.03 (3H, d, 6.7)		8-Me	16.5	1.00 (3H, d, 6.8)
	9	88.3	3.27 (ddd, 7.3, 6.7, 4.5)		9	88.4	3.25 (m)
	9-OMe	58.6	3.25 (3H, s)		9-OMe	58.7	3.14 (3H, s)
	10	38.9	2.69 (dd, 14.0, 7.3)		10	39.0	2.68 (dd, 14.0, 7.3)
			2.84 (dd, 14.0, 4.5)				2.82 (dd, 14.0, 4.7)
	11	140.5			11	140.6	
	12/16	130.5	7.19 (2H, m)		12/16	130.6	7.19 (2H, m)
	13/15	129.1	7.24 (2H, m)		13/15	129.2	7.24 (2H, m)
	14	126.9	7.16 (tt, 7.2, 1.5)		14	127.0	7.17 (m)
Glu^6	1	179.3		Glu^6	1	177.5	
	2	55.6	4.25 (ddd, 9.7, 8.9, 5.0)		2	55.5	4.22 (dd, 7.8, 5.9)
	2-NH		8.31 (d, 8.9)		2-NH		
	3	29.8	1.84 (m)		3	29.0	2.03 (m)
			2.15 (m)				2.14 (m)
	4	33.7	2.50 (ddd, 17.3, 12.2, 3.4)		4	33.9	2.27 (ddd, 14.8, 11.0, 5.0)
			2.61 (ddd, 17.3, 12.0, 5.2)				2.35 (ddd, 14.8, 11.1, 5.9)
	5	177.5			5	175.8	
Mdha^7	1	166.1		Ala^7	1	174.4	
	2	146.4			2	51.1	4.13 (q, 7.0)
	2-NMe	38.4	3.36 (3H, s)		2-NH		
	3	113.6	5.42 (d, 1.0)		3	17.4	1.40 (3H, d, 7.0)
			5.84 (d, 1.0)				

[a] J = coupling constant in Hz; proton signal intensity is 1H unless otherwise indicated. Atom numbering in amino acid units starts from the carbonyl nearest the amino group (Figure S10). Amino acid numbering is as shown in Figure 1. [b] Assignments are interchangeable.

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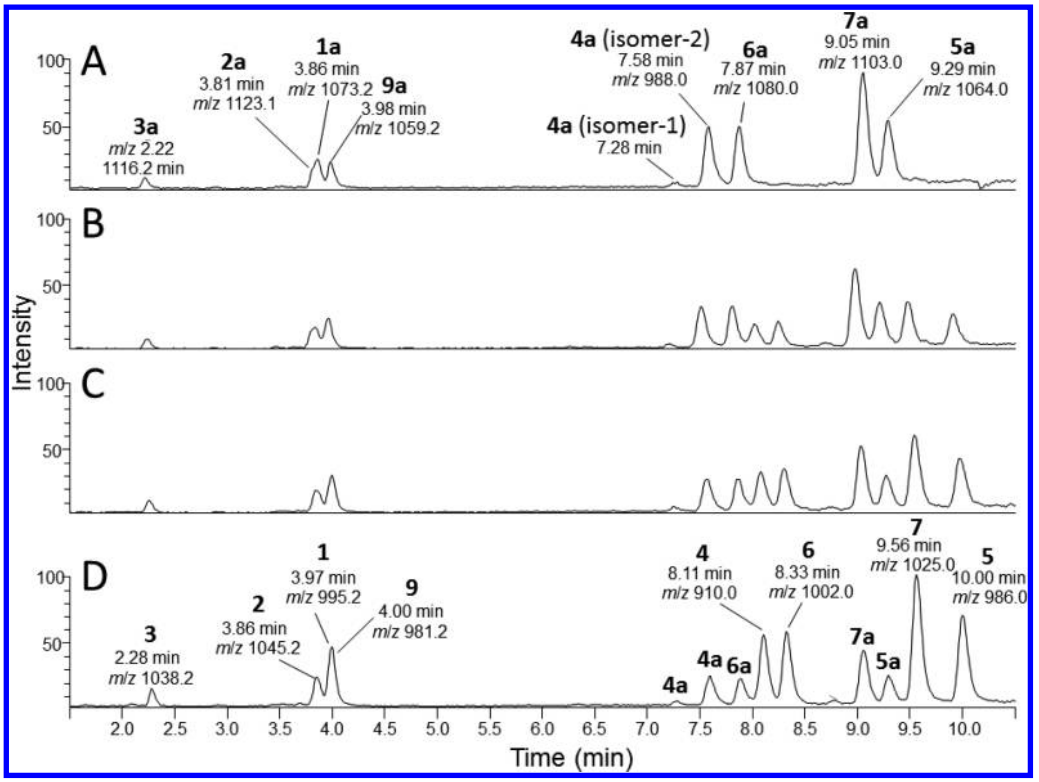

Figure 5. LC−MS^2 analyses (method A) of a mixture of mercaptoethanol derivatives **1a**−**7a** and **9a** in a mixture of pH 10.7 carbonate buffer and MeOH at 30 °C (experiment A, Table 2). A, after 1 h; B, after 24 h; C, after 48 h; and D, after 4 days. The chromatograms are shown using the same absolute vertical scale. Peaks are labeled with m/z for $[M + H]^+$, but the chromatograms were extracted for the sum of the $[M + H]^+ + [M + 2H]^{2+}$ for all the masses indicated in the Figure.

Table 2. Half-Lives (h) at 30 °C for the Deconjugation of Microcystin Derivatives (Figure 4) Under a Range of Reaction Conditions

experiment →	A								B	C[a]	D[a]	E[a]	
compound →	**1a**	**2a**	**3a**	**4a**	**5a**	**6a**	**7a**	**9a**	**8b**	**9c**	**9d**	**8b**	**9d**
pH 9.2									305				
pH 10.7	38	52	28	54	45	44	49	156	22	49	13	27	29
pH 10.7 + DMSO										41	9.7		

[a]These experiments were conducted with purified isomer-2 of specified derivatives.

Cys- and GSH-Conjugates. Cys- and GSH-conjugates of microcystins, such as [Mlan^7]MC-LR (**9c**),[5] have been detected in animals dosed with MCs and in environmental samples,[4,10,15] as have conjugates of the related pentapeptide nodularin toxins.[21] Cys-conjugates are generally thought to be breakdown products of GSH conjugates[38] but, as has been pointed out, proteolysis of protein−microcystin conjugates could also lead to Cys-conjugated microcystins.[6] We found no difference in the rate of GSH conjugation to **9** in the presence or absence of rat liver GST at pH 7.4 and 30 °C ($t_{1/2}$ 4.1 h), nor was there any difference in the ratio of the two diastereoisomeric products between the reactions with or without GST (Figure S15−S17). This suggests that the GST-catalyzed reaction may be of minor importance for **9** under normal physiological conditions, paralleling the findings of Buratti et al.[7] for **3**. We observed the same products and diastereoisomer ratios when the conjugation was performed in the carbonate buffers or at physiological pH, so the deconjugation reactions reported here with isomer-2 of **9c** and **9d** are representative of those that would occur with Cys- and GSH-conjugates formed in vivo.

Base-promoted deconjugation of isomer-2 of [Lan^7]MC-LR (**9c**) and GSH−[Dha^7]MC-LR (**9d**) proceeded without problems (Figures 6, 7, and 8A−C) apart from the formation of small amounts of the aforementioned methanol adducts, although cleavage of the GSH-conjugate appeared to be somewhat faster than that for the Cys-conjugate (experiments C and D, Table 2). Significant amounts of isomer-1 of **9c** and **9d** appeared during the deconjugation reactions. When the deconjugation reaction was performed on a mixture of **8b** and **9d**, these early eluting isomers were also produced, but no **8d** or **9b** were detectable (Figure 6). This suggests that the isomers in this reaction are formed primarily through base-promoted isomerization α to the amide carbonyl in the Mdha^7/Dha^7-residue (via deprotonation/reprotonation), rather than through deconjugation followed by reaction of the freed microcystin with the released thiol (Figure S4). As the pairs of isomers are in equilibrium and undergo deconjugation by the same reaction mechanism (Figures 2 and S4), this isomerization should not affect the outcome of the deconjugation reaction. That this is the case can be seen in Figure 8, where the concentration of isomer-1 increases initially and then decreases toward the end of the reaction.

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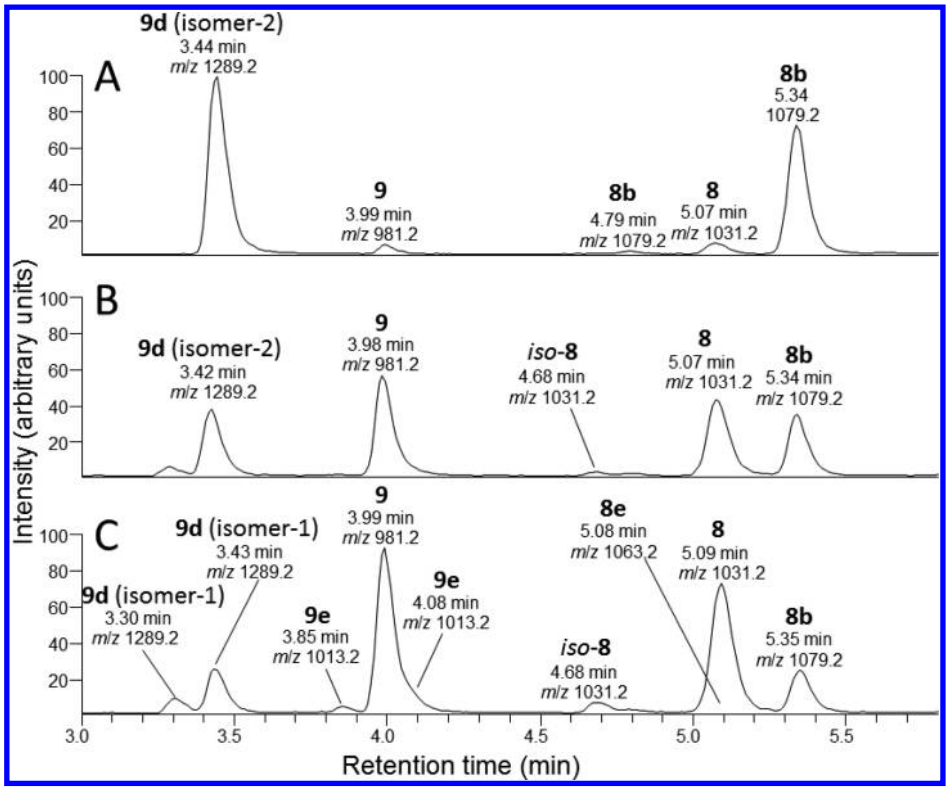

Figure 6. LC-MS^2 chromatograms (method A) of a mixture of the methanethiol derivative of [Asp^3]MC-RY (**8b**) and isomer-2 of the GSH derivative of [Dha^7]MC-LR (**9d**) in a mixture of pH 10.7 carbonate buffer and MeOH at 30 °C (experiment E, Table 2). A, after 1 h; B, after 21 h; and C, after 48 h. The chromatograms are shown using the same absolute vertical scale. Peaks are labeled with m/z for $[M + H]^+$, but the chromatograms were extracted for the sum of the $[M + H]^+ + [M + 2H]^{2+}$ for all of the masses shown in the Figure. Note the slow formation of *iso*-**8** with a retention time of 4.68 min.

Increasing the Rate of Deconjugation. Because the microcystin conjugation/deconjugation reaction (Figure 2) is an equilibrium process, it should be possible to drive the reaction in a chosen direction by manipulating the reaction conditions. When conjugation is desired, a large excess of thiol is used together with a moderately basic pH (up to or slightly higher than the pK_a of the thiol),[3,33] and under these conditions, the deconjugation reaction is scarcely discernible (Figure S1). Although increasing the temperature can be expected to increase the rate at which equilibrium is achieved, it will probably have only a minor effect of the position of the equilibrium. However, three simple strategies can be envisaged to favor the deconjugation reaction.

(1) Increasing the pH should assist deconjugation, as the rate of the conjugation reaction ceases to increase significantly around 1 pH unit above the thiol's pK_a, whereas the rate of deconjugation should continue to increase in proportion to the hydroxide ion concentration up until close to the pK_a of the weakly acidic proton α to the amide carbonyl in residue-7 of the conjugate. The effect of pH on the deconjugation of **8b** is clearly evidenced in Table 2. A similar effect was seen for mercaptoethanol adduct **10a** (Figure S2 and Table S1), and it is for this reason that pH 9.7 was chosen for performing conjugations of microcystins to thiols for LC−MS analysis[3,33] (i.e., very rapid thiol conjugation and slow deconjugation rates). High pH can lead to problems with analyte stability, such as isomerization of amino acids and other unwanted reactions. At pH 10.7, the only significant side reactions we observed were slow formation of isomers of some microcystins containing Arg at position-2 and addition of MeOH to the reactive double bond in the Dha^7/Mdha^7-group. So, it may be possible to increase the rate of deconjugation by using a higher pH than in the present study, provided side reactions remain at an acceptable level.

(2) Removal of the free thiol formed during the reaction should force the equilibrium over toward deconjugation, but the reagents used need to be compatible with the analytes. Dimethyl sulfoxide (DMSO) oxidizes thiols to disulfides,[42] and addition of DMSO to deconjugation reactions of **9c**, **9d** and **10a** significantly increased the deconjugation rate (Tables 2 and S1, and Figure S2) without side reactions (Figure 8). Addition of iodoacetamide, which reacts rapidly with thiols under neutral to basic conditions, also appeared to increase the reaction rate (Figure S2 and Table S1) but resulted in iodination and partial esterification of some microcystins (Figure S3). Addition of other reagents containing thiol-reactive functional groups, such as epoxides or electron-deficient olefins, could also be effective but was not tested in this study.

(3) Conversion of the freed microcystins into a nonthiol-reactive derivatives would prevent reconjugation, as well as minimize the isomerization of [Arg^2]-microcystins and the addition of MeOH to the Dha^7/Mdha^7 groups. One such approach is to perform the deconjugation in the presence of a large excess of a non-natural thiol, such as mercaptoethanol, with the released microcystins analyzed as their mercaptoethanol derivatives by suitable LC−MS[3,33] or ELISA[37,43] methods. While this approach was partially successful with **9c** and **9d** (data not shown), there is a potential for autoxidation of the added thiol over the course of the reaction. Another alternative is to use $NaBH_4$ to reduce the reactive double bonds in the [Dha^7]- and

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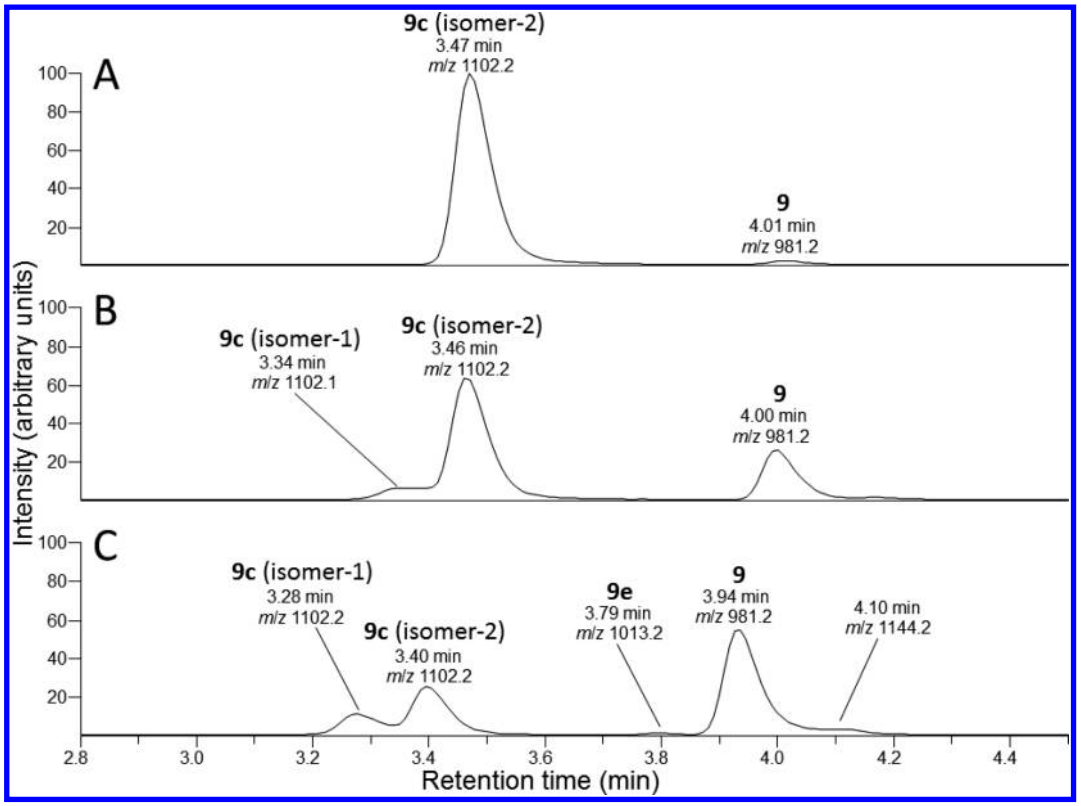

Figure 7. LC−MS^2 analysis (method A) of Cys derivative **9c** (isomer-2) in a mixture of pH 10.7 carbonate buffer and MeOH at 30 °C (experiment C, Table 2), after A, 40 min; B, 21 h; and C, 65 h. The chromatograms are shown using the same absolute vertical scale. Peaks are labeled with m/z for $[M + H]^+$, but the chromatograms were extracted for the sum of the $[M + H]^+ + [M + 2H]^{2+}$ for all of the masses shown in the Figure.

[$Mdha^7$]-groups of the released microcystins to give the corresponding nonthiol-reactive [Ala^7]- and [$Mala^7$]-groups (e.g., **9d** + $OH^- \rightleftharpoons GS^- + H_2O$ + **9** + $NaBH_4 \rightarrow$ [Ala^7]MC-LR; Scheme S1). This reaction is compatible with microcystins[39] and is irreversible, and $NaBH_4$ can be used in basic solutions (although it reacts slowly with hydroxylic solvents). The identity of the $NaBH_4$ reduction products of **9** was verified by LC−MS^2 and NMR spectroscopy (Tables 1 and S3; Figures S35−S43, S50 and S51) prior to attempting deconjugation with $NaBH_4$ reduction. When this reductive deconjugation approach was used on **9d**, the reaction was essentially complete in less than 2 days, whereas a control reaction (without $NaBH_4$) at the same pH was only about 55% deconjugated (Figure 9). The reduction products were readily analyzed by LC−MS, although they were present as pairs of diastereoisomers (Figures S11 and S12) which could make the analysis more difficult for complex samples.

Conclusions. The results reported here indicate that even at neutral pH, there is a significant uncatalyzed reaction of thiols with microcystins, and a slow equilibrium between conjugated and free microcystins. Thus, conjugated microcystins are expected to be readily formed in vivo and to be potentially bioavailable both from the free and conjugated forms. Although this is entirely consistent with the observation of reversible MC-RR conjugation with Cys and GSH in vivo in bighead carp,[34] it should be noted that based on the results obtained here, the treatment of the extracts from the fish with base (15% aqueous ammonia) prior to analysis could be expected to have caused partial deconjugation of the conjugated microcystins present in the samples. Microcystins conjugated to structural proteins in an organism could result in exposure of that organism to a low steady-state concentration of free microcystins over an extended time, to a degree that would depend on temperature, pH, and the concentration and pK_a values of the thiols present. Microcystins conjugated via thiols to proteins that were consumed as part of the diet could also be expected to yield some free microcystin over time, in addition to peptide−microcystin conjugates produced via digestive proteolysis. The environmental and toxicological consequences of these effects may warrant further investigation and may be of particular significance in alkaline lake systems such as those found in Africa's Rift Valley.

Sometimes, studies of the metabolic fate of microcystins utilize strongly basic conditions for tissue digestion.[44,45] The results presented here suggest that digestion under these conditions could release a significant proportion of any conjugated microcystins that were present. This is desirable when assessing the total content of free and bound toxin, and presents no problem when using radio labeling or Lemieux oxidation for detection. It is, however, necessary to know to what degree the conjugated microcystins are affected by such treatments, both in terms of their deconjugation and the subsequent stability of the free toxins, before drawing conclusions about the proportions of free and conjugated toxin in environmental samples. This is especially so if an analysis method is used that is sensitive to modifications to the chemical structure of the toxins, such as ELISA, protein phosphatase inhibition, or LC−MS.

Although the application of the deconjugation methods to real samples was not part of this study, the approaches described here are clearly applicable to soluble low molecular

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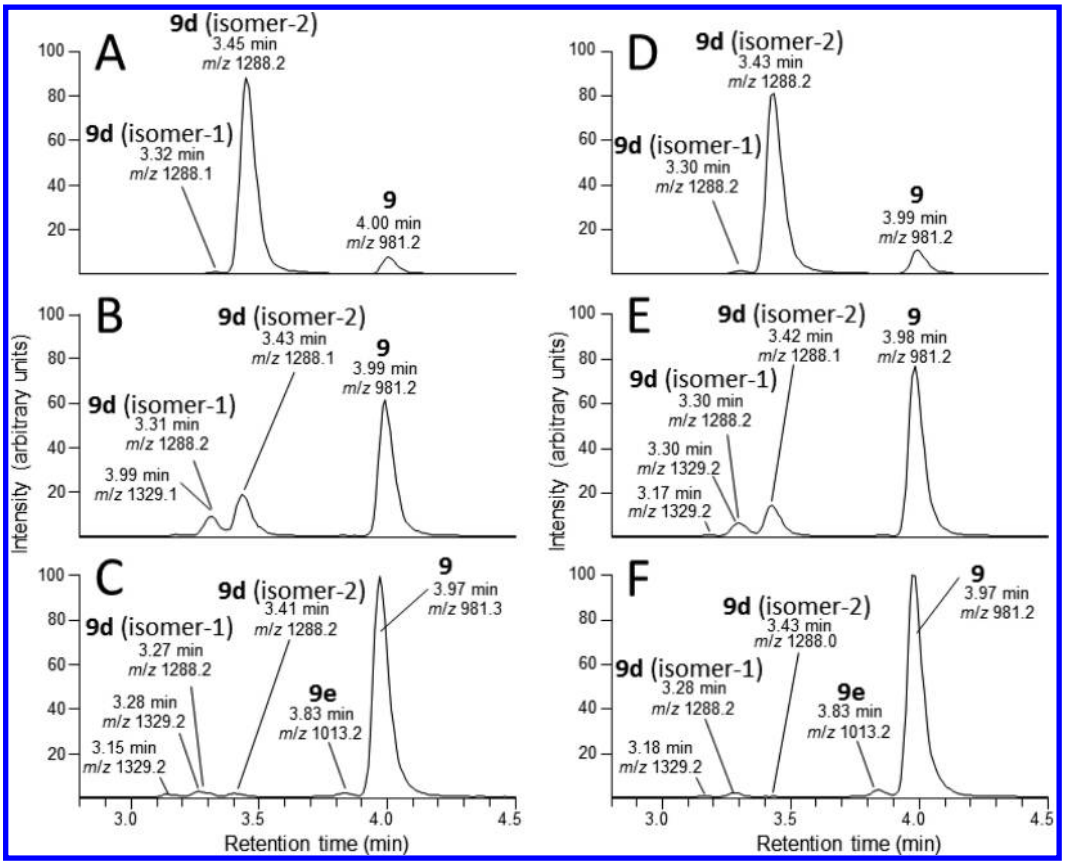

Figure 8. LC-MS^2 chromatograms (method A) of isomer-2 of the GSH derivative of [Dha^7]MC-LR (**9d**) in a mixture of pH 10.7 carbonate buffer and MeOH at 30 °C (experiment D, Table 2): A, after 1.2 h; B, after 22.0 h; and C, after 65.4 h. On the right are the corresponding chromatograms (D–F) for an identical mixture run in parallel but containing 8% DMSO v/v and analyzed at nearly the same reaction times (1.4, 22.2, and 65.7 h). Each set of chromatograms (A–C, and D–F) is shown using the same absolute vertical scale. Peaks are labeled with m/z for $[M + H]^+$, but the chromatograms were extracted for the sum of the $[M + H]^+ + [M + 2H]^{2+}$ for all the masses shown in the Figure.

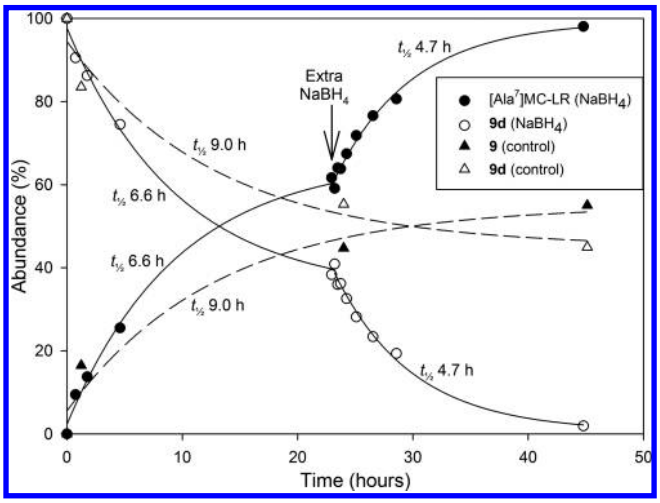

Figure 9. LC-MS^2 analysis (method A) of deconjugation reactions of GSH–[Dha^7]MC-LR (**9d**, isomer-2): (1) reductive deconjugation in pH 10.7 buffer with the addition of $NaBH_4$ at times 0 and 23.4 h (circles, solid lines), with the percentage of **9d** (sum of isomer-1 and isomer-2) shown with hollow circles and the percentage of [Ala^7]MC-LR (sum of isomer-1 and isomer-2) shown with filled circles, and (2) control reaction (no $NaBH_4$) in pH 10.7 buffer (triangles, dashed lines), with the percentage of **9d** (sum of isomer-1 and isomer-2) shown with hollow triangles and the percentage of **9** shown with filled triangles. Reaction 1 is as shown in Scheme S1, while reaction 2 is as depicted in generic form in Figure 2. No significant amounts of other microcystin derivatives were detected during either of the two reactions, which were run concurrently.

weight conjugates (e.g., with Cys, GSH, *N*-acetylCys) of the type studied here in vitro. Strategies 2 and 3 are available for promoting deconjugation, even in samples containing biological thiols that would otherwise compete with the deconjugation reaction. Some proteins containing conjugated microcystins could potentially denature in the basic conditions necessary for deconjugation, but such samples could, if necessary, be digested with proteases to produce soluble peptide–microcystin conjugates prior to deconjugation. A further important point to note is that the reverse Michael reaction (deconjugation) of microcystins is promoted by base, so samples and extracts that are to be analyzed for microcystins and microcystin-conjugates should probably have a small amount of a weak acid (e.g., acetic acid) added to prevent spontaneous deconjugation during storage.

The reversibility of thiol conjugation demonstrated here indicates that the toxicokinetics and toxicology of microcystins may be more complex than previously realized. Furthermore, it has important implications for other toxic compounds containing electron-deficient double bonds, such as type-B trichothecenes (e.g., deoxynivalenol), which was recently shown to undergo the reversible thia-Michael reaction,[28] acrylamide, and brevetoxins. Further development of the approaches described here should lead to practical methods for studying the conjugated forms of such toxins and to a better understanding of their behavior in exposed organisms, food, feed, and water.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00028.

Accurate masses and LC−MS/MS spectra of new microcystin derivatives, schemes showing detailed reaction mechanisms, related LC−MS chromatograms, NMR spectra, plots of reaction progress, and tabulated reaction rates (PDF)

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Notes

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■ ABBREVIATIONS

Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; Dha, dehydroalanine; GST, glutathione transferase; Lan, lanthionine; Mala, *N*-methylalanine; MC, microcystin; Mdha, *N*-methyldehydroalanine; Mlan, *N*-methyllanthionine; Mser, *N*-methylserine; SPE, solid-phase extraction

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