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Rapid and Convenient Oxidative Release of Thiol-Conjugated Forms of Microcystins for Chemical Analysis

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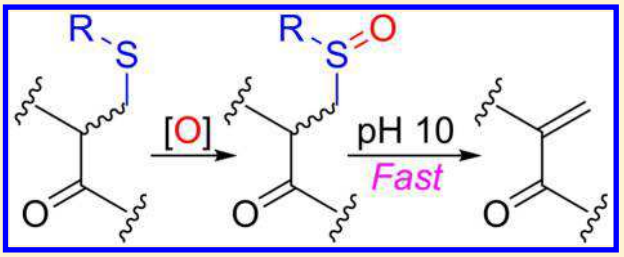
Supporting Information

ABSTRACT: Microcystins are potent cyclic heptapeptide toxins found in some cyanobacteria, and usually contain an α,β-unsaturated carbonyl group that is readily conjugated to thiol-containing amino acids, peptides, and proteins in vivo and in vitro. Methods for deconjugating these types of adducts have recently been reported, but the reactions are slow or result in derivatized microcystins. Mercaptoethanol derivatives of a range of microcystins were therefore used as model compounds to develop deconjugation procedures in which the dialkyl sulfide linkage was oxidized to a sulfoxide or sulfone that, when treated with base, rapidly eliminated the adducted thiol as its sulfenate or sulfinate via β-elimination to afford free microcystins with the α,β-unsaturated carbonyl group intact. These free microcystins can be analyzed by LC/MS to determine the toxin profile of bound microcystins. The method was tested on Cys- and GSH-derivatives of [Dha^7]MC-LR. In solution, the deconjugation reactions were complete within minutes at pH 10.7 and within a few hours at pH 9.2. Oxidation of sulfides to sulfoxides is easier and more rapid than oxidation to sulfones, allowing the use of milder oxidants and shorter reaction times. Oxidation of any methionine residues present in the microcystins occurs inevitably during these procedures, and interpretation of the microcystin profile obtained by LC/MS analysis needs to take this into account. Oxidation of tryptophan residues and degradation of microcystins by excess oxidant were circumvented by the addition of Me_2SO as a sacrificial reducing agent. These methods may be useful for other compounds that undergo conjugation via thia-Michael addition, such as acrylamide and deoxynivalenol. Oxidation of sulfides to sulfoxides can occur in vivo and could affect the bioavailability of toxins and drugs conjugated via thia-Michael addition, potentially exacerbating oxidative stress by catalytically converting GSH to its sulfenate via conjugation, oxidation, and elimination to regenerate the free toxin.



INTRODUCTION

Microcystins (Figure 1) are a large group of cyclic heptapeptide toxins found in some cyanobacteria that grow in fresh water.[1] Most microcystins contain 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) at position-5 and an α,β-unsaturated carbonyl group, usually as part of a dehydroalanine (Dha) or *N*-methyldehydroalanine (Mdha) group at position-7 that is highly reactive toward nucleophilic attack and rapidly forms adducts with thiol-containing amino acids, peptides, and proteins when ingested.[2−8] These conjugates can be present in tissue samples at much higher concentrations than the free microcystins and may represent a useful biomarker indicative of previous exposure.[6,9−13] The soluble conjugates formed from cysteine, glutathione, and other small thiol-containing biomolecules can be extracted and analyzed directly by LC/MS, whereas nonextractable microcystin conjugates, such as those bound to proteins, can be analyzed by oxidative cleavage of the 6,7-oelfinic group in their Adda side chains (Figure 1), with chromatographic quantitation of the resulting oxidative cleavage product.[14−16] Alternatively, the 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.[5] In principle, these procedures can be used to measure the total content of protein-conjugated microcystins (as well as of nodularins) in tissue samples, but all information as to which microcystin analogues are present as conjugates is lost.[2,11] Such information could be useful, for example, when trying to compare the microcystin conjugate profile of intoxicated animals with the microcystin profiles of nearby water bodies in order to identify the source of exposure.[2]

The Michael addition of thiols to the α,β-unsaturated carbonyl moiety in microcystins is a reversible reaction.[2,12] The deconjugation reaction of microcystins was recently investigated with model thiols, showing that the reaction can be reversed under basic conditions.[2,11] Although this approach can be performed successfully by using reaction conditions that thermodynamically favor deconjugation, deconjugation occurred relatively slowly under the conditions tested or resulted in derivatized microcystins and some degradation products due to the high pH and extended reaction times.[2,11] For routine LC/MS analysis, it is desirable to have rapid

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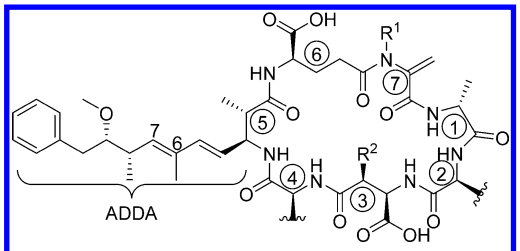

		②	④	R^1	R^2	*m/z*
1	MC-LR	Leu	Arg	Me	Me	995.5560
2	MC-YR	Tyr	Arg	Me	Me	1045.5353
3	MC-RR	Arg	Arg	Me	Me	1038.5731
4	MC-LA	Leu	Ala	Me	Me	910.4920
5	MC-LF	Leu	Phe	Me	Me	986.5233
6	MC-LY	Leu	Tyr	Me	Me	1002.5183
7	MC-LW	Leu	Trp	Me	Me	1025.5342
8	[Asp^3]MC-RY	Arg	Tyr	Me	H	1031.5197
9	[Dha^7]MC-LR	Leu	Arg	H	Me	981.5404
10	MC-RY	Arg	Tyr	Me	Me	1045.5353
11	[Asp^3]MC-LY	Leu	Tyr	Me	H	988.5026

Figure 1. Structures of microcystins used in this study (**1**−**11**). Values for *m*/*z* are calculated for $[M + H]^+$. **1**−**11** were derivatized with the thiols in shown in Figure 2 to produce a range of sulfide derivatives that were then oxidized to afford the corresponding sulfoxides and sulfones shown in Figure 4.

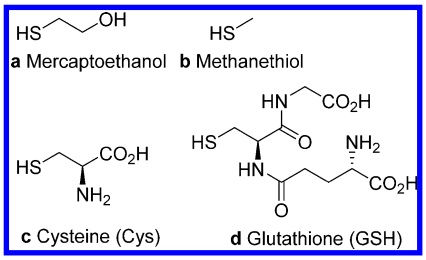

Figure 2. Thiols conjugated to the Mdha^7/Dha^7-groups of microcystins **1**−**11** (Figure 1) for the development of deconjugation methods: (**a**) mercaptoethanol, (**b**) methanethiol, (**c**) L-cysteine, and (**d**) glutathione. The products from the reactions of **1**−**11** with thiols **a**−**d**, as well as their oxidation products (the corresponding sulfoxides and sulfones), are shown in Figure 4.

and mild deconjugation conditions so as to avoid such problems. Ideally, such a method should not only be rapid but also be artifact free, convenient, and safe and yield underivatized microcystins to obviate the need for standards of derivatized microcystins for confirmation of identity and quantitation.

Here, we report using microcystins (Figure 1) derivatized with model thiols (Figure 2) to develop alternative procedures in which the sulfide linkage is oxidized to a sulfoxide or sulfone prior to deconjugation (Figure 3) and then testing the method on semisynthetic Cys- and GSH-conjugates of [Dha^7]MC-LR. Sulfoxides are also produced naturally in vivo by oxidation of sulfides, and the implications of this for the toxicology of compounds that undergo thia-Michael addition are briefly considered.

MATERIALS AND METHODS

General. Potassium peroxymonosulfate (Oxone) (technical grade from Sigma−Aldrich, Steinheim, Germany) was dissolved in water at 10 mg/mL. Hydrogen peroxide (30%) was from Merck (Darmstadt, Germany). Solvents for LC and extraction were of gradient (Romil, Oslo, Norway) or LC/MS quality (Fisher Scientific, Fair Lawn, NJ, or Loughborough, United Kingdom). Me_2SO was from Merck (Darmstadt, Germany). Preparation of microcystins and their thiol conjugates, their characterization by NMR and LC/MS, and preparation of working solutions and mixtures for LC/MS and reactivity studies were as described by Miles et al.[2] The identities of sulfoxide and sulfone derivatives (Figure 4) were verified by LC/HRMS (Table 1) and LC/MS^2 (Supporting Information) analysis. Hypersep C18 solid-phase extraction (SPE) columns (100 mg) from Thermo Scientific (Bellefonte, PA) were washed with MeOH (2 mL) and water (2 mL) immediately prior to use.

Deconjugation of Thiol-Conjugated Microcystins. Experiment A: Deconjugation of 10a via Its Sulfone (10a(O_2)). To the mercaptoethanol conjugate of MC-RY (**10a**) in MeOH−H_2O (1:1, 200 μL) was added carbonate buffer (50 μL; pH 10.7, 0.05 M) and Oxone (50 μL; 10 mg/mL) with vortex mixing. A second reaction was performed in an identical manner, except that the carbonate buffer was only added after oxidation to **10a(O_2)** was judged to be complete. A solution of [Dha^7]MC-LR (**9**) in MeOH−H_2O was treated with Oxone but without the addition of carbonate buffer for comparison. Reactions were followed by LC/MS^2 (method A) (Figure 5).

Experiment B: Reanalysis of the Kinetics of Base-Promoted Deconjugation of 10a. The data from the first preliminary experiment of Miles et al.[2] for deconjugation of **10a** (see also Figure S2 and Table S1 of Miles et al.[2]) was reanalyzed to include **10a**-sulfoxide (**10a(O)**) (Figures 6 and S4, and Table 2).

Experiment C: Deconjugation of Model Conjugates via H_2O_2-Oxidation to Sulfoxides. To a mixture of mercaptoethanol-derivatized microcystins (**1a**−**7a** and **9a**) containing **11** as a trace contaminant (200 μL)[2] was added H_2O_2 (30%; 25 μL). After 2 h, water (1 mL) was added, and the solution was applied to an SPE column. The column was washed with 10% MeOH (2 mL) and eluted with 80% MeOH (2 mL). An aliquot (50 μL) of the eluate was evaporated to dryness under a stream of N_2 and dissolved in MeOH−H_2O (1:1; 200 μL) to give a solution containing a mixture of the sulfoxides of **1a**−**7a** and **9a** (**1a(O)**−**7a(O)** and **9a(O)**). The same procedure was used to oxidize **8b** to produce a solution containing the **8b**-sulfoxide (**8b(O)**. Deconjugation reactions were initiated by the addition of carbonate buffer (0.05 M; pH 10.7; 50 μL) to the sulfoxide-containing solutions in LC vials in the LC/MS sample tray, and the reactions were followed by LC/MS^2 (method A) (Table 3 and Figures S5−S7).

Experiment D: Deconjugation of Model Conjugates via Oxone-Oxidation to Sulfoxides. To a mixture of mercaptoethanol-derivatized microcystins (**1a**−**7a** and **9a**) containing **11** as a trace contaminant[2] (200 μL) and Me_2SO (20 μL) in an LC vial, was added Oxone (20 μL; 10 mg/mL in water) with vortex mixing, and then 110 min later, carbonate buffer (50 μL; pH 10.7; 0.05 M) was added. Progress of the reactions was followed by LC/MS^2 (method A) (Figure 7).

Experiment E: Deconjugation of Cys-Conjugate 9c via Oxone Oxidation to Its Sulfoxide. To a solution of **8b** and isomer-2 of **9c** in MeOH−H_2O (200 μL; 1:1)[2] and Me_2SO (20 μL) in an LC vial was added Oxone in water (50 μL, 10 mg/mL), and then, after 1.37 h, carbonate buffer (50 μL; pH 10.7; 0.05 M) was added. Progress of the reactions was followed by LC/MS (method A) (Table 4 and Figure S8).

Experiment F: Deconjugation of GSH-Conjugate 9d via Oxone Oxidation to Its Sulfoxide. To a solution of **8b** and isomer-2 of **9d** in MeOH−H_2O (200 μL; 1:1)[2] and Me_2SO (20 μL) in an LC vial was added Oxone in water (50 μL, 10 mg/mL), and then, after 49 min, carbonate buffer (50 μL; pH 10.7; 0.05 M) was added. Progress of the reactions was followed by LC/MS (method A) (Table 4 and Figure 8).

Kinetic Analyses. Half-lives of sulfoxide- and sulfone-conjugates in each deconjugation reaction and for the oxidation of **8b(O)** to **8b(O_2)** with Oxone were estimated by fitting their relative abundances (LC/MS method A) to 2-parameter exponential curves (SigmaPlot 13.0, Systat Software Inc., San Jose, CA, USA). However, production of **10** (experiment B) was fitted to a 5-parameter exponential rise to a maximum to extract independent half-lives for the production of alkene-**10** from sulfide-**10a** and sulfoxide-**10a(O)** (Figure 6).

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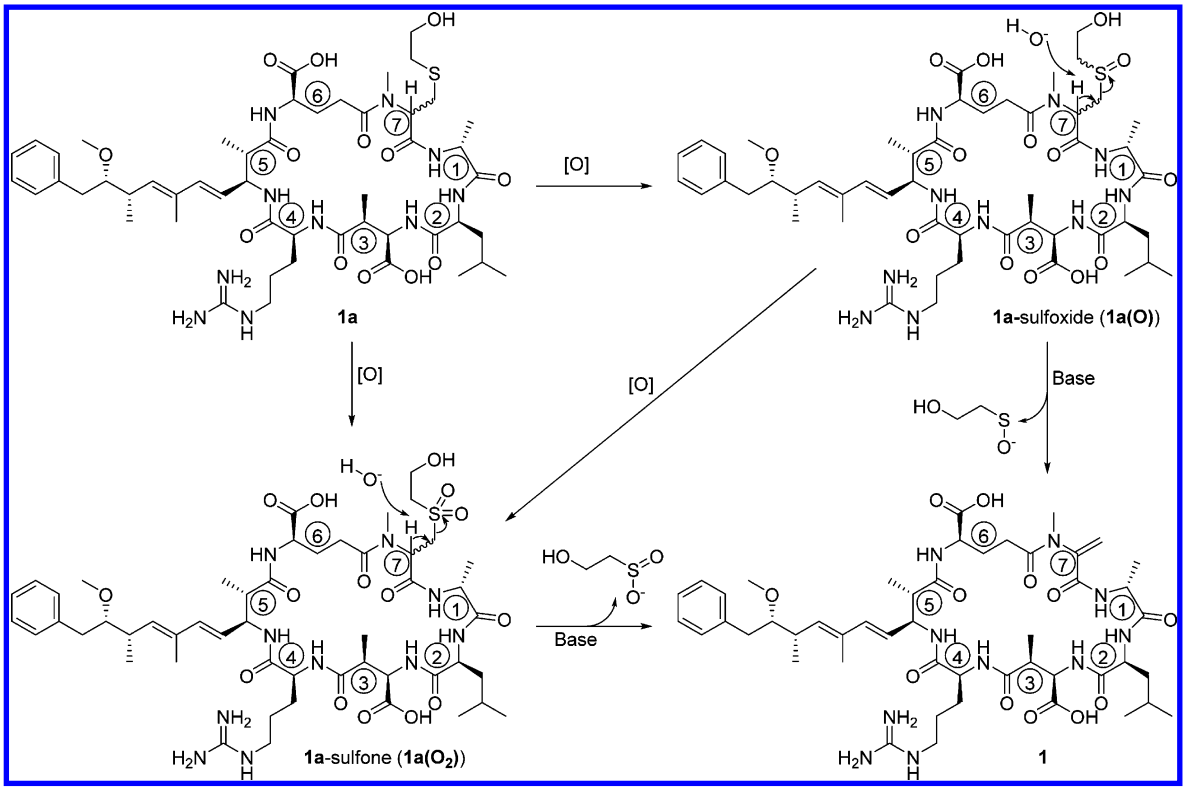

Figure 3. Oxidation of the mercaptoethanol conjugate of MC-LR (**1a**) to its sulfoxide (**1a(O)**) and sulfone (**1a(O_2)**) derivatives, followed by base-catalyzed elimination of the corresponding sulfenates and sulfinates, respectively, to yield MC-LR (**1**). All microcystin derivatives (**1**–**11**, Figure 1) of thiols **a**–**d** (Figure 2) used in this study (Figure 4) behaved in a similar manner.

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,[17] 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.[17] Briefly, the mass spectrometer was operated in full-scan positive ion ESI mode (m/z 500–1600) with the ion injection time set to 100 ms with a total of three microscans, with the tray temperature set to 30 °C for the determination of relative reaction rates. 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 for $[M + H]^+$, an isolation width of 2.0, normalized collision energy of 50, activation Q of 0.250, and an activation time of 0.25 ms. Relative abundances were estimated from the areas of the respective $[M + H]^+$ peaks or, for reactions where some of the compounds displayed significant $[M+2H]^{2+}$ intensity, from the sum of the areas of the $[M + H]^+$ and $[M+2H]^{2+}$ peaks. For kinetic experiments, the temperature of the sample tray was set to 30 °C, and samples were equilibrated to tray-temperature prior to initiating the reaction. Atomic compositions and exact values for m/z of microcystins and their adducts were calculated with Toxin Mass Calculator version 15,[18] and data processing and calculation of Δ were performed with Xcalibur version 2.3 (Thermo Fisher Scientific Inc., San Jose, CA, USA).

LC/HRMS (Method B). Liquid chromatography with high-resolution MS (LC/HRMS) was as described for method A, except that a Waters Acquity UPLC pump and autosampler were used. A Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used as the detector, with a spray voltage of 3.5 kV, capillary temperature of 350 °C, probe heater of 300 °C, S-lens RF level of 50, and sheath and auxiliary gas at 35 and 10, respectively. The spectrometer was operated in the full-scan and 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).

RESULTS AND DISCUSSION

Deconjugation of thiol adducts of microcystins can be achieved by treatment of the conjugates with base,[2,11] but the rate of the reaction is only modest below pH 11.[2] Miles et al.[2] suggested three strategies for making the deconjugation reactions more effective: increasing the pH to increase the deconjugation rate; addition of a thiol-sequestering agent to prevent reconjugation of the microcystin by thiol; and adding a reagent to derivatize the reactive double bond of the microcystin, thereby trapping it in an unreactive form. These strategies have been successfully demonstrated on thiol-conjugated microcystins in vitro,[2,11] but the use of high pH can result in artifacts,[2] and derivatization reagents can result in the formation of isomers that can complicate subsequent chemical analysis.[2,11]

A fourth approach for favoring the deconjugation reaction in microcystins is to convert the sulfide group into a better leaving group, such as a sulfone, which would also be expected to make the hydrogen atom α-to the carbonyl group more acidic. The deconjugation reaction would thus become the well-known base-catalyzed β-sulfone elimination reaction.[19,20] For example, the rate of base-catalyzed cleavage of a thiol–maleimide conjugate was markedly increased after oxidation to the sulfone, and deconjugation occurred at a measurable rate even at

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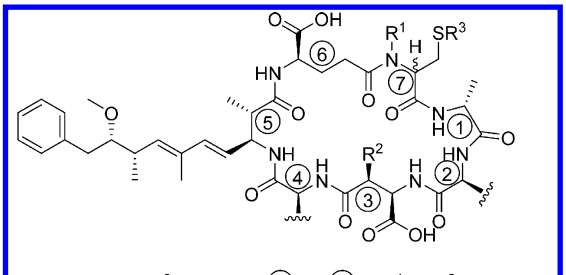

	R^3SH	②	④	R^1	R^2	*m/z*
1a	$HOCH_2CH_2SH$	Leu	Arg	Me	Me	1073.5700
1a(O)	$HOCH_2CH_2S(O)H$	Leu	Arg	Me	Me	1089.5649
1a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Arg	Me	Me	1105.5598
2a	$HOCH_2CH_2SH$	Tyr	Arg	Me	Me	1123.5492
2a(O)	$HOCH_2CH_2S(O)H$	Tyr	Arg	Me	Me	1139.5442
2a(O_2)	$HOCH_2CH_2S(O_2)H$	Tyr	Arg	Me	Me	1155.5391
3a	$HOCH_2CH_2SH$	Arg	Arg	Me	Me	1116.5870
3a(O)	$HOCH_2CH_2S(O)H$	Arg	Arg	Me	Me	1132.5819
3a(O_2)	$HOCH_2CH_2S(O_2)H$	Arg	Arg	Me	Me	1148.5769
4a	$HOCH_2CH_2SH$	Leu	Ala	Me	Me	988.5060
4a(O)	$HOCH_2CH_2S(O)H$	Leu	Ala	Me	Me	1004.5009
4a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Ala	Me	Me	1020.4958
5a	$HOCH_2CH_2SH$	Leu	Phe	Me	Me	1064.5373
5a(O)	$HOCH_2CH_2S(O)H$	Leu	Phe	Me	Me	1080.5322
5a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Phe	Me	Me	1096.5271
6a	$HOCH_2CH_2SH$	Leu	Tyr	Me	Me	1080.5322
6a(O)	$HOCH_2CH_2S(O)H$	Leu	Tyr	Me	Me	1096.5271
6a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Tyr	Me	Me	1112.5220
7a	$HOCH_2CH_2SH$	Leu	Trp	Me	Me	1103.5482
7a(O)	$HOCH_2CH_2S(O)H$	Leu	Trp	Me	Me	1119.5431
7a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Trp	Me	Me	1135.5380
8b	CH_3SH	Arg	Tyr	Me	H	1079.5230
8b(O)	$CH_3S(O)H$	Arg	Tyr	Me	H	1095.5179
8b(O_2)	$CH_3S(O_2)H$	Arg	Tyr	Me	H	1111.5129
9a	$HOCH_2CH_2SH$	Leu	Arg	H	Me	1059.5543
9a(O)	$HOCH_2CH_2S(O)H$	Leu	Arg	H	Me	1075.5492
9a(O_2)	$HOCH_2CH_2S(O_2)H$	Leu	Arg	H	Me	1091.5442
9c	Cys	Leu	Arg	H	Me	1102.5601
9c(O)	Cys-sulfoxide	Leu	Arg	H	Me	1118.5551
9c(O_2)	Cys-sulfone	Leu	Arg	H	Me	1134.5500
9d	GSH	Leu	Arg	H	Me	1288.6242
9d(O)	GSH-sulfoxide	Leu	Arg	H	Me	1304.6191
9d(O_2)	GSH-sulfone	Leu	Arg	H	Me	1320.6140
10a	$HOCH_2CH_2SH$	Arg	Tyr	Me	Me	1123.5492
10a(O)	$HOCH_2CH_2S(O)H$	Arg	Tyr	Me	Me	1139.5442
10a(O_2)	$HOCH_2CH_2S(O_2)H$	Arg	Tyr	Me	Me	1155.5391
11b	CH_3SH	Leu	Tyr	Me	H	1036.5060
11b(O)	$CH_3S(O)H$	Leu	Tyr	Me	H	1052.5009
11b(O_2)	$CH_3S(O_2)H$	Leu	Tyr	Me	H	1068.4958

Figure 4. Structures of the microcystin thiol-derivatives (sulfides **1a**−**7a**, **8b**, **9a**, **9c**, **9d**, **10a**, and **11b**) of microcystins **1**−**11**, and their corresponding sulfoxides and sulfones (designated with suffixes "(O)" and "(O_2)", respectively) produced by oxidation of the sulfides, used in deconjugation experiments. For the thiol derivatives, the structure-number refers to the precursor microcystin (Figure 1), while the lower case letter denotes the adducted thiol shown in Figure 2. 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 $[M + H]^+$.[18] NB: The conjugate of Cys−Dha is known as lanthionine (Lan), i.e., **9c** is [Lan^7]MC-LR.

physiological pH.[21] Oxidation of sulfides to sulfoxides has been shown to facilitate thermal elimination of the thiol moiety to form didehydroamino acids and peptides,[22] but this approach does not appear to have been widely used for natural samples because the reaction conditions are not compatible with proteins nor with many natural products.[23]

In this study, a range of microcystins conjugated to mercaptoethanol or methanethiol were used as model substrates for the development of reaction conditions suitable for deconjugation, and then the reaction conditions were tested on Cys- and GSH-conjugates of [Dha^7]MC-LR. These semisynthetic sulfide-linked conjugates were produced as part of an earlier study, where they were thoroughly characterized and their structures verified by LC/MS^2, LC-HRMS, and NMR spectroscopy.[2] The sulfoxides and sulfones produced by oxidation of these sulfides were analyzed by LC-HRMS (Table 1) and LC/MS^2 (Supporting Information) to verify that their MS characteristics were consistent with the proposed S-oxidation products.

During MS fragmentation, the $[M + H]^+$ ions of both the sulfoxide and sulfone derivatives of the mercaptoethanol, methanethiol, and Cys adducts of microcystins gave the original deconjugated microcystin as the major product ion via β-elimination. The oxidized GSH-adducts also displayed prominent product ions from β-elimination, but product ions arising from peptide cleavages dominated the MS^2 spectra under the conditions used. The mass spectra of the product ions (MS^3 spectra) produced via β-elimination were identical to the MS^2 spectra of the underivatized parent microcystins, confirming the facile β-elimination from the sulfoxide and sulfone derivatives of the conjugated microcystins. This behavior is different from that of Met-containing microcystins in which the Met residue has been oxidized. In this case, the Met-sulfoxide fragmentation is dominated by elimination, but the Met-sulfone derivatives fragment in a manner analogous to their unoxidized Met-containing congeners.[24]

Sulfone-Mediated Deconjugation. In a preliminary experiment (experiment A), the mercaptoethanol conjugate of MC-RY (**10a**) was used as a model compound to study the feasibility of the oxidative deconjugation approach for microcystins. Treatment of **10a** in methanolic carbonate buffer with Oxone led to rapid and complete conversion to MC-RY (**10**), apparently via the corresponding sulfone (**10a(O_2)**), and there was no detectable thiol conjugate remaining at 26 min (Figure 5). However, upon standing overnight, the intensity of the peak for **10** decreased to less than half its initial intensity, in accord with the reported instability of microcystins toward Oxone.[24] A second attempt, where **10a** was treated with Oxone to generate sulfone **10a(O_2)** prior to the addition of carbonate buffer, gave similar results (Figure S1). The latter experiment revealed that **10a** was rapidly oxidized to sulfoxide **10a(O)** ($t_{1/2}$ < 5 min), which in turn was oxidized much more slowly to sulfone **10a(O_2)** ($t_{1/2}$ = 69 min), by Oxone. Treatment of [Dha^7]MC-LR (**9**) in MeOH−H_2O with Oxone resulted in a slow but steady decrease in the intensity of the peak for **9** in LC/MS^2, with the corresponding appearance of numerous earlier-eluting peaks consistent with oxidized derivatives of **9** (Figure S2). These results confirm the long-term instability of microcystins toward Oxone. While it might be possible to circumvent this stability problem by quenching the reaction after the formation of the sulfones[24] or by extracting the products by SPE, to remove excess oxidant, these options were not investigated further.

Sulfoxide-Mediated Deconjugation. In a previous study (experiment B), although the base-catalyzed deconjugation of **10a** followed first-order kinetics at all pH values tested,[2] anomalously rapid formation of **10** was observed in the initial stages of the reactions. This suggested rapid production of **10** by a minor component in the reaction mixture. Careful examination of the original LC/MS chromatograms revealed the presence of an earlier-eluting peak with *m/z* 1139.1,

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Table 1. Accurate Masses of New Microcystin Thiol Derivatives Obtained from LC-HRMS Analysis (Method B)[a]

compound	t_R (min)	ion	m/z found	formula	Δ (ppm)
1a-sulfoxide (**1a(O)**)	4.08	$[M + H]^+$	1089.5676	$C_{51}H_{81}O_{14}N_{10}S^+$	2.5
2a-sulfoxide (**2a(O)**)	4.13	$[M + H]^+$	1139.5470	$C_{54}H_{79}O_{15}N_{10}S^+$	2.5
3a-sulfoxide (**3a(O)**)	3.00	$[M+2H]^{2+}$	566.7946	$C_{51}H_{83}O_{14}N_{13}S^{2+}$	0.0
4a-sulfoxide (**4a(O)**)	7.00	$[M + H]^+$	1004.5034	$C_{48}H_{74}O_{14}N_7S^+$	2.5
4a-sulfone[b] (**4a(O_2)**)	7.67	$[M + H]^+$	1020.5005	$C_{48}H_{74}O_{15}N_7S^+$	4.6
5a-sulfoxide (**5a(O)**)	8.51	$[M + H]^+$	1080.5325	$C_{54}H_{78}O_{14}N_7S^+$	0.3
6a-sulfoxide (**6a(O)**)	7.41	$[M + H]^+$	1096.5288	$C_{54}H_{78}O_{15}N_7S^+$	1.5
7a-sulfoxide (**7a(O)**)	8.39	$[M + H]^+$	1119.5431	$C_{56}H_{79}O_{14}N_8S^+$	0.0
8b-sulfoxide (**8b(O)**)	4.70	$[M + H]^+$	1095.5220	$C_{52}H_{75}O_{14}N_{10}S^+$	3.7
8b-sulfone (**8b(O_2)**)	5.04	$[M + H]^+$	1111.5163	$C_{52}H_{75}O_{15}N_{10}S^+$	3.1
9a-sulfoxide (**9a(O)**)	4.16	$[M + H]^+$	1075.5518	$C_{50}H_{79}O_{14}N_{10}S^+$	2.4
9a-sulfone[b] (**9a(O_2)**)	4.29	$[M + H]^+$	1091.5468	$C_{50}H_{79}O_{15}N_{10}S^+$	2.4
9c-sulfoxide[c] (**9c(O)**)	3.97	$[M + H]^+$	1118.5556	$C_{51}H_{80}O_{15}N_{11}S^+$	0.5
9c-sulfoxide[c] (**9c(O)**)	3.97	$[M+2H]^{2+}$	559.7825	$C_{51}H_{81}O_{15}N_{11}S^{2+}$	2.4
9c-sulfone[c] (**9c(O_2)**)	4.01	$[M + H]^+$	1134.5500	$C_{51}H_{80}O_{16}N_{11}S^+$	0.0
9c-sulfone[c] (**9c(O_2)**)	4.01	$[M+2H]^{2+}$	567.7775	$C_{51}H_{81}O_{16}N_{11}S^{2+}$	−2.0
9d-sulfoxide[c] (**9d(O)**)	3.91	$[M + H]^+$	1304.6191	$C_{58}H_{90}O_{19}N_{13}S^+$	0.0
9d-sulfoxide[c] (**9d(O)**)	3.91	$[M+2H]^{2+}$	652.8151	$C_{58}H_{91}O_{19}N_{13}S^{2+}$	2.9
9d-sulfone[c] (**9d(O_2)**)	3.95	$[M + H]^+$	1320.6150	$C_{58}H_{90}O_{20}N_{13}S^+$	0.7
9d-sulfone[c] (**9d(O_2)**)	3.95	$[M+2H]^{2+}$	660.8101	$C_{58}H_{91}O_{20}N_{13}S^{2+}$	−0.8
11b[d]	8.30	$[M + H]^+$	1036.5080	$C_{52}H_{74}O_{13}N_7S^+$	2.0
11b-sulfoxide[d] (**11b(O)**)	7.01	$[M + H]^+$	1052.5036	$C_{52}H_{74}O_{14}N_7S^+$	2.6
11b-sulfone[b,d] (**11b(O_2)**)	7.89	$[M + H]^+$	1068.4975	$C_{52}H_{74}O_{15}N_7S^+$	1.6

[a]Accurate masses of the corresponding unoxidized thiol derivatives of **1**−**10** are reported elsewhere.[2] [b]From a minor contaminant in sulfoxide-containing samples produced by oxidation with H_2O_2. [c]From purified samples of isomer-2.[2] [d]From a minor contaminant in **8b**.

consistent with **10a**-sulfoxide (**10a(O)**), that disappeared in a pH-dependent manner following first-order kinetics, suggesting that this might be the source of the rapid production of **10** (Figure S3). Consistent with this, kinetic analysis by fitting the concentration of **10** at pH 10.7 for the first 4 h to a 5-parameter exponential curve gave an excellent fit to the data (Figure 6). Furthermore, this analysis yielded two rate constants for the production of **10**, one of which closely matched that for the deconjugation of **10a** and the other of which closely matched the rate for deconjugation of the putative sulfoxide (**10a(O)**). Half-lives for **10a** and **10a(O)** at the four pH values tested are given in Table 2. These results indicated base-catalyzed deconjugation of the sulfoxide form (**10a(O)**) to be about 2 orders of magnitude faster than that for the corresponding sulfide form (**10a**) for the mercaptoethanol conjugate of **10**. The presence of the small amount of sulfoxide (ca. 10%) in the reaction mixture is attributable to autoxidation of sulfide-conjugate **10a** during storage, as other sulfide-containing microcystins such as MC-MR have been shown to undergo slow autoxidation to their sulfoxides (in the case of MC-MR, to MC-M(O)R).[24]

Because this reaction could form the basis of a rapid and efficient deconjugation procedure, it was desirable to produce authenticated sulfoxides of a wider range of microcystin−thiol conjugates in a controlled reaction to verify the potential utility of the reaction. As H_2O_2 is known to oxidize Met residues in microcystins to their sulfoxides with minimal side reactions,[24] it was used to oxidize a sample of methanethiol conjugate **8b**, and a mixed sample of microcystin−mercaptoethanol conjugates (**1a**−**7a** and **9a**) (experiment C). In each case, the oxidized conjugates were then separated by SPE from excess oxidant to preclude the possibility of base-promoted attack by peroxide ion after the addition of base. The samples were then treated with pH 10.7 carbonate buffer. Sulfoxide (**8b(O)**) was rapidly deconjugated to **8** with first-order kinetics (Table 3) without apparent side-reactions, as was a trace contaminant (the sulfoxide of the methane-thiol-conjugate of [Asp^3]MC-LY (**11b**)), albeit more slowly than **10b(O)** (Figure S5). Similar results were obtained for the sulfoxides of the mixture of mercaptoethanol conjugates of microcystins **1**−**7** and **9** (Table 3 and Figure S6). However, partial oxidation was observed for the mercaptoethanol derivative of MC-LW (**7a**), yielding the corresponding mercaptoethanol sulfoxide derivatives of MC-LOia, MC-LKyn, and MC-LNfk. Oxidation of Trp to Oia, Kyn, and Nfk in microcystins by H_2O_2 has been reported previously.[25] The sulfoxides of the oxidation artifacts from **7a** were also deconjugated upon addition of carbonate buffer, just like the other microcystins (Figures S6 and S7). In addition to the expected sulfoxides, small amounts of the corresponding sulfones were detected by LC/MS^2 for several of the conjugates in the mixture, due to overoxidation. Results from the kinetic analysis of the LC/MS data for these sulfoxides and sulfones, presented in Table 3, suggest that the sulfones are deconjugated at about twice the rate of their corresponding sulfoxides and that sulfoxides are deconjugated more than 100-fold faster than their corresponding sulfides. These results show that conversion to sulfoxides or sulfones could be a viable route for base-catalyzed deconjugation of thiol-conjugated microcystins, but the known instability of Trp-containing microcystins toward prolonged exposure to hydrogen peroxide[25] makes this oxidant potentially problematic.

It was therefore desirable to find an alternative procedure for oxidizing thiol conjugates of microcystins that was less subject to undesirable side reactions. During the studies on the oxidation of the conjugated microcystins to their sulfones with Oxone (experiment A), it was noted that oxidation to the sulfoxides occurred much more rapidly than the subsequent oxidation to the sulfones. Furthermore, the studies here as well as elsewhere[24] indicate that degradation of microcystins with

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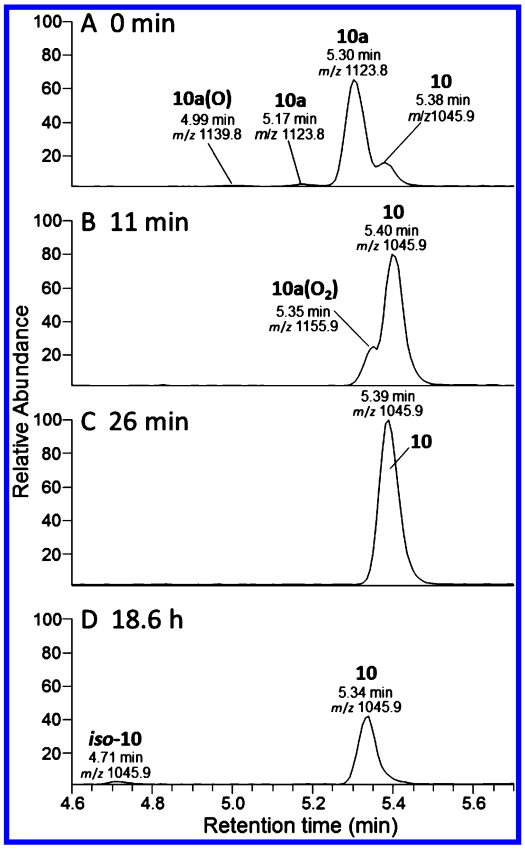

Figure 5. LC/MS^2 analysis of the deconjugation of MC-RY–mercaptoethanol conjugate **10a** in methanolic carbonate buffer, by oxidation to the corresponding sulfone (**10a**(O_2)) with Oxone (experiment A). A, before the addition of Oxone; B, 11 min after the addition of Oxone; C, 26 min after the addition of Oxone, with only MC-RY (**10**) detectable; D, 18.6 h after the addition of Oxone. All chromatograms are shown using the same vertical scale, corrected for dilution by the addition of reagents. Each chromatogram shows the sum of m/z 1045.5, 1123.8, 1139.8, and 1155.8, and peaks are marked with their retention times and m/z for $[M + H]^+$.

Oxone occurs relatively slowly, and Miles et al.[24] suggested that adding a sacrificial sulfoxide such as Me_2SO might consume excess Oxone and thus protect microcystins from overoxidation.

Because the oxidation of sulfide conjugates to their sulfoxides was so much faster than the oxidation of the sulfoxides to their sulfones and the oxidative degradation of microcystins, a one-step oxidation procedure was developed where Me_2SO was added to conjugated microcystins, followed by the addition of Oxone, and the reactions were followed by LC/MS^2. After 1–2 h, to allow oxidation of the thiol-conjugates and consumption of excess oxidant by the Me_2SO, carbonate buffer was added to initiate deconjugation. Figure 7 shows this procedure being applied to mercaptoethanol conjugates of a mixture of microcystins (**1a**–**7a** and **9a**) in a one-pot reaction in an LC-vial (experiment D). Analysis showed that oxidation was nearly complete 2 h after the addition of Oxone and that deconjugation was complete within 5 h of the addition of carbonate buffer with no signs of over oxidation, even for derivatives of MC-LW. The chromatograms suggest that microcystins containing both an Arg^2 and an Arg^4 residue are deconjugated more quickly than those containing either an Arg^2 or an Arg^4 residue, which react more quickly than non-Arg-containing microcystins, in accord with the data in Table 3. A trace contaminant of underivatized **8** was visible in all the chromatograms,

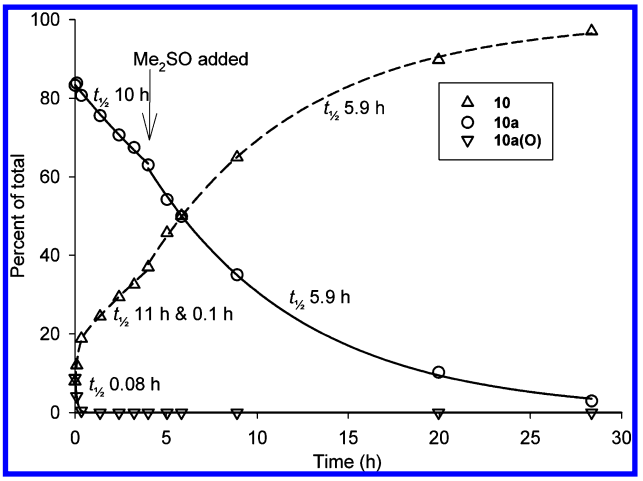

Figure 6. Deconjugation reaction for **10a** at pH 10.7 (LC/MS data from Miles et al.[2] but replotted to include its sulfoxide, **10a**(**O**)) (experiment B). Concentrations of **10a** (before and after the addition of Me_2SO) and **10a**(**O**) are fitted to 2-parameter exponential decay curves. The concentration of **10** is fitted to a 5-parameter exponential increase to a maximum before the addition of Me_2SO (resulting in two rate constants) and to a 2-parameter exponential rise to a maximum after the addition of Me_2SO. The calculated half-lives from the curve fitting are shown on the graph, and the half-lives for **10a** and **10a**(**O**) prior to the addition of Me_2SO are shown in Table 2.

Table 2. Effect of pH on Half-Lives (Min) of 10a and 10a-Sulfoxide[a] (Experiment B)

	pH 9.2[b]	pH 9.7	pH 10.2	pH 10.7
10a	7700	2300	1300	610
10a-sulfoxide	110	32	13	5

[a]Experiment from Miles et al.,[2] but LC/MS data were reanalyzed to include **10a**-sulfoxide. [b]Includes Me_2SO, which increases the deconjugation rate of **10a**.[2]

indicating that unconjugated microcystins are unaffected by the reaction conditions used for deconjugation.

The method was then tested on samples containing [Dha^7]-MC-LR conjugated to Cys and GSH, representative of the types of conjugate that might be encountered in natural samples. Application of this approach to a mixture of Cys-conjugate **9c** and MeSH conjugate **8b** (experiment E) yielded a completely deconjugated mixture of **8** and **9** in less than 3 h (Figure S8). The procedure was also effective on a mixture of GSH-conjugate **9d** and **8b** (experiment F), with complete deconjugation to **8** and **9** in a little over an hour (Figure 8). Furthermore, only minor changes were seen when the reaction mixture was allowed to stand for 3 d in the autosampler at 30 °C, with small amounts of *iso*-**8** (thought to be due to the attack of the guanidinium group of Arg^2 on the Mdha^7 group) and the MeOH-adduct at the Dha^7-group of **9** induced by slow reactions during storage under the basic reaction conditions.[2] Possibly, these side-reactions could be avoided by neutralization once deconjugation is complete, but this was not investigated further.

Methionine-containing microcystins are readily oxidized by a range of oxidants,[24] and conversion of Met to Met(O) or Met(O_2) will inevitably occur during oxidative deconjugation of thiol-conjugated microcystins, so interpretation of the toxin profile obtained after LC/MS analysis needs to take this into account. Trp-containing microcystins can be partially oxidized

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Table 3. Half-Lives (Min) at 30 °C for the Deconjugation of Sulfide,[a] Sulfoxide, and Sulfone Forms of Microcystin−Mercaptoethanol Conjugates 1a−7a and 9a in a Mixture, and 8b, at pH 10.7[b] (Experiment C)

	1a	2a	3a	4a	5a	6a	7a	9a	8b
sulfide[a]	2300	3100	1700	3200	2700	2600	2900	9400	1300
sulfoxide	8	8	5	14	13	14	15[c]	6.0	10
sulfone				7	3	3			5

[a]Data for the sulfide conjugates are from Table 2 of Miles et al.[2] and were obtained under similar reaction conditions to those used for the sulfoxides and sulfones in the present study. [b]Sulfoxides were produced by oxidation of the sulfides with H_2O_2 and contained sulfones as minor contaminants. Half-lives of the sulfones are approximate due to their rapid deconjugation and low abundance (typically less than 10% of the analogous sulfoxide, where observed). [c]Extensive oxidation of the tryptophan moiety in the mercaptoethanol derivative of MC-LW (**7a**) occurred to give the corresponding MC-LOia, MC-LKyn, and MC-LNfk derivatives of **7a**, each of which also deconjugated cleanly (Figures S6 and S7).

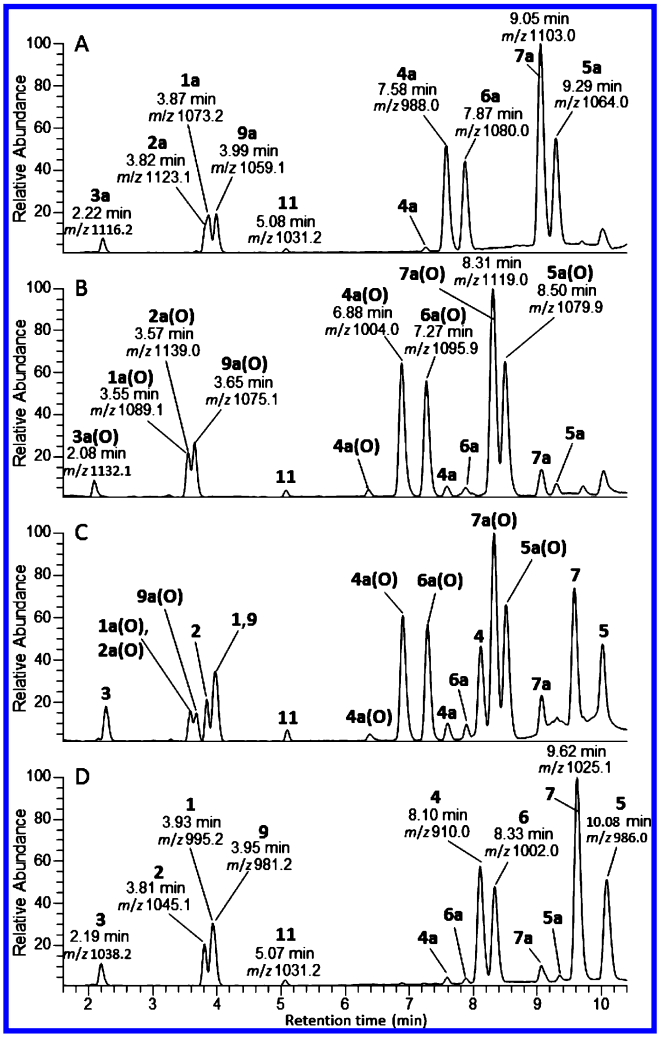

Figure 7. LC/MS^2 analyses (method A) of experiment D: (A) a mixture of mercaptoethanol derivatives **1a**−**7a** and **9a** contaminated with a small amount of **11**, in MeOH−H_2O; (B) 45 min after the addition of Me_2SO and Oxone; (C) 17 min after the addition of carbonate buffer (pH 10.7); and (D) 5.5 h after the addition of the carbonate buffer. Chromatograms are for the sum of m/z 515−570 and 900−1150, and the peaks are labeled with the m/z for $[M + H]^+$and are scaled to the highest peak in each chromatogram.

to the corresponding Oia-, Kyn-, and Nfk-congeners by some oxidants, including H_2O_2,[25] and this was observed in the present study when H_2O_2 was used on a mixture containing MC-LW (Figure S6). However, no such problem was observed when Oxone and Me_2SO were used to oxidize microcystin-sulfides to their sulfoxides (Figure 7). Although oxidation of the microcystin mixture with Oxone without Me_2SO was not

Table 4. Half-Lives (Min) at 30 °C for the Deconjugation of Sulfide[a] and Sulfoxide Forms of Mixtures of Microcystin Conjugates 8b and Isomer-2 of 9c (Experiment E), and of 8b and Isomer-2 of 9d (Experiment F), at pH 10.7[b]

	8b	9c	8b	9d
sulfide[a]			1600	1700
sulfoxide	6	18	8	6

[a]Data for the sulfide forms are from Table 2 of Miles et al.[2] and was obtained under similar reaction conditions to those used for the sulfoxides in the present study. [b]Sulfoxides were produced by oxidation of the sulfides with Oxone in the presence of Me_2SO.

tested, results with other microcystins in this (Figure 5) and other studies[24] suggest that all microcystins are slowly oxidized by Oxone. However, the results obtained here verify the suggestion[24] that oxidative degradation of microcystins by Oxone could be prevented by the addition of Me_2SO to consume excess oxidant. This finding also opens the way for application of the sulfide-oxidation method for identifying Met- and Met(O)-containing peptides by LC/MS, which was previously hampered by slow oxidative degradation of the reaction products by Oxone.[24]

It should also be possible to use other analytical methods, such as HPLC-UV, ELISA, and PP2A-inhibition assays for analysis of the microcystins released by oxidative deconjugation, but these methods will not usually provide the same level of information on the toxin profile as LC/MS.

The oxidative deconjugation procedure has not yet been tested on microcystins conjugated to proteins in solution or in tissue samples. However, provided the oxidant and base are able to access the sulfide linkage, there is no reason to expect the reaction not to work, although optimization of the reaction conditions may be required, and this might need to include partial enzymatic digestion of tissues. Nevertheless, the results reported here appear to provide the basis for a rapid, reliable, general, and efficient method for releasing thiol-conjugated microcystins from tissues and other sample types. It should be possible to modify the methodology reported here for deconjugation of other thia-Michael adducts, such as those formed from deoxynivalenol,[26−28] acrylamide,[29] and other Michael-acceptors.[30,31]

Toxicological Implications. As mentioned, nonconjugated sulfide-containing microcystins such as MC-MR undergo slow autoxidation to their sulfoxides (MC-M(O)R) in the presence of air, even with low temperature storage.[24] In this study, we observed the same type of reactivity in the sulfide linkages of thiol-conjugated forms of microcystins, such that many stored samples contained detectable amounts of the sulfoxides (Figures 5 and 8) that noticeably perturbed the deconjugation kinetics (Figure 6). Sulfide-linked microcystin conjugates are also likely to undergo slow oxidation in vivo, resulting in

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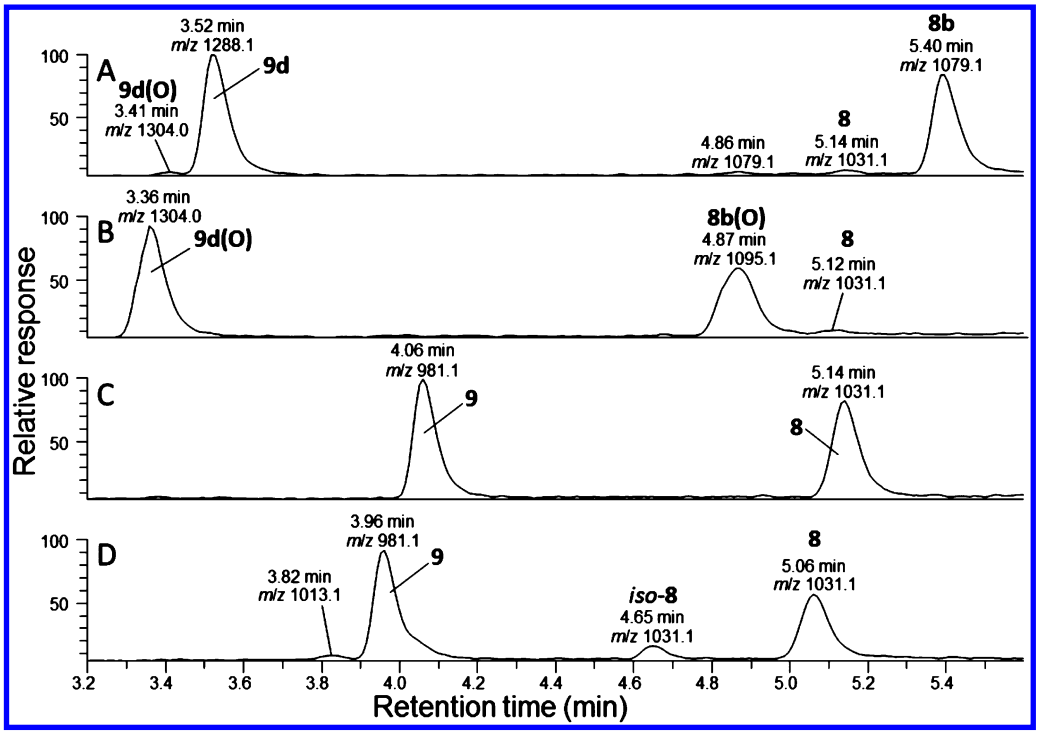

Figure 8. LC/MS chromatograms from a one-pot deconjugation of **8b** and isomer-2 of **9d** via oxidation to their sulfoxides (experiment F). A, mixture of **8b** and **9d** in MeOH−H_2O containing Me_2SO; B, the mixture 10 min after the addition of Oxone; C, the oxidized mixture 49 min after the addition of carbonate buffer; D, the reaction mixture after standing for 68 h. All of the chromatograms are extracted from full scan LC/MS for the sum of all $[M + H]^+ + [M+2H]^{2+}$ for all compounds shown, and peaks are labeled with the m/z for $[M + H]^+$. The same absolute intensity scale is used for all of the chromatograms, after correction for the dilution of the mixture with Oxone and carbonate buffer. Note the stability of the products, with the exception of the slow formation of *iso*-**8** (4.65 min) and [Ser^7]MC-LR (3.82 min) as described previously,[2] visible in chromatogram D.

the production of sulfoxide-linked conjugates, and oxidation is likely to be more rapid in tissues under oxidative stress, where higher concentrations of reactive oxygen species may be present.

Kinetic analysis of the deconjugation of microcystins indicates that deconjugation of the sulfoxides is about 2 orders of magnitude faster than that for the corresponding sulfide forms. This is equivalent to the increase in deconjugation rate for a sulfide conjugate that would result from an increase of two pH 2 units. Thus, even at physiological pH, sulfoxide-linked microcystin conjugates could be deconjugated at a significant rate. For example, the half-life for the glutathione-sulfoxide conjugate **9d(O)** was ca. 6 min at pH 10.7 and 30 °C (Figure 8, Table 4), so its half-life under physiological conditions (pH 7.6, 37 °C) could be expected to be around 2−3 d but might be shorter in tissues at higher pH. Formation of sulfoxides in vivo may have contributed to the very rapid partial deconjugation of the GSH-conjugate of MC-RR and the slower partial deconjugation of the Cys-conjugate administered to bighead carp.[12] It should be noted that the samples from that experiment were treated with 15% aqueous ammonia during sample preparation,[12] that MC-RR thiol-conjugates and their sulfoxides are more rapidly deconjugated by a base than the corresponding conjugates of other microcystins (Table 3 and Miles et al.[2]), and that base-catalyzed deconjugation of GSH conjugates and their sulfoxides appears to be faster than that for the corresponding Cys conjugates, which is still faster than that for the corresponding thioalkane conjugates of a given microcystin (Table 4 and Miles et al.[2]).

A number of natural toxins, drugs, metabolites, environmental contaminants, and cellular signaling molecules contain Michael acceptor systems,[30−35] including the Type-B trichothecene mycotoxins such as deoxynivalenol (DON),[36] the food contaminant acrylamide,[29] acrolein,[37] and the marine algal toxin brevetoxin-2,[38] and therefore have the potential to undergo thia-Michael addition. In the case of DON, the thiol conjugation has been shown to be reversible,[28] and Cys-conjugates of brevetoxins have been shown to undergo oxidation to their sulfoxides both in vivo and in storage.[38] Therefore, the observations of base-catalyzed release of thiol-conjugates of microcystins and DON[2,28] and the much faster deconjugation of the corresponding sulfoxides demonstrated here for microcystins are likely to be a general feature of thia-Michael adducts. There is also a possibility that some Michael acceptors could catalytically deplete GSH in vivo via conjugation with GSH, followed by oxidation to the sulfoxide and deconjugation with the release of GSH-sulfenate and the Michael acceptor.

Other Applications. Thia-Michael adducts are also used in biotechnological applications such as the controlled release of drugs, linking small molecules to carrier proteins, nanomedicine, and materials science such as polymers.[21,30,31,39−45] The ability to simply and conveniently cleave thia-Michael linkages or to control their stability, via oxidation to sulfoxides or sulfones, constitutes a useful tool for bioconjugates or polymers that utilize thia-Michael adducts and could potentially be used to direct release of bioconjugated forms in tissues with higher oxidation potential or pH.

■ CONCLUSION

Oxidation of sulfide-linked thiol-conjugates of microcystins to their sulfoxide- or sulfone-derivatives makes them much more

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susceptible to base-catalyzed deconjugation. This approach was found to be suitable for analysis of soluble thiol conjugates of microcystins and should, with minor modifications, be applicable to protein conjugates of microcystins in tissues and biological samples. An understanding of the factors affecting conjugation and deconjugation of microcystins is essential to understanding their toxicology, trophic transfer, and environmental effects. Similar considerations apply also to other biologically active or environmentally harmful molecules that undergo thia-Michael additions. Applications where thia-Michael reactions are used in biotechnology and materials science can also be envisaged.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.7b00121.

Selected LC/MS chromatograms, kinetic analyses, and mass spectra from LC/MS^2 and LC/MS^3 analyses (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; Kyn, kynurenine; Lan, lanthionine; MC, microcystin; Mdha, *N*-methyldehydroalanine; Mlan, *N*-methyllanthionine; Mser, *N*-methylserine; Nfk, *N*-formylkynurenine; Oia, oxindolylalanine; SPE, solid-phase extraction

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