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Mechanistic Studies of the Photocatalytic Oxidation of Microcystin-LR: An Investigation of Byproducts of the Decomposition Process

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Microcystins (cyclic heptapeptides) are produced by a number of freshwater cyanobacteria and cause concern in potable water supplies due to their acute and chronic toxicity. The present study reports the structural characterization of the degradation products of the photocatalytic oxidation of microcystin-LR, so aiding the mechanistic understanding of this process. TiO_2 photocatalysis is a promising technology for removal of these toxins from drinking water. However, before it can be adopted in any practical application it is necessary to have a sufficient knowledge of degradation byproducts and their potential toxicity. Liquid chromatography−mass spectrometry analysis demonstrated that the major destruction pathway of microcystin appears to be initiated via three mechanisms: UV irradiation, hydroxyl radical attack, and oxidation. UV irradiation caused geometrical isomerization of microcystin converting the (4E), (6E) of the Adda configuration to (4E), 6(Z) or 4(Z), 6(E). Hydroxyl radical attack on the conjugated diene structure of Adda moiety produced dihyroxylated products. Further oxidation cleaved the hydroxylated 4−5 and/or 6−7 bond of Adda to form aldehyde or ketone peptide residues, which then were oxidized into the corresponding carboxylic acids. Photocatalysis also hydrolyzed the peptide bond on the ring structure of microcystin to form linear structures although this appeared to be a minor pathway.

Introduction

The production and release of cyanotoxins by cyanobacteria in freshwaters around the world has been well documented (*1*, *2*). Microcystins are the most common cyanotoxins found in water and are frequently responsible for poisoning animals and humans who come into contact with toxic blooms and contaminated water (*3*). Acute exposure results in hepatic injury, which in extreme cases can prove fatal (*4*). One such incident resulted in the death of over 50 dialysis patients due to the use of microcystin-contaminated water in their treatment (*4*). Chronic exposure due to the presence of microcystin in drinking water is thought to be a contributing factor in primary liver cancer (PLC) through the known tumor-promoting activities of these compounds (*5*).

Since cyanotoxins present in drinking water sources pose a considerable threat to human health, various treatments have been used for their removal. However, it is believed that conventional water treatment systems have proven unreliable for the elimination of these toxins from potable water (*6*−*8*).

Previous work has shown that TiO_2 photocatalysis effectively destroys microcystin-LR in aqueous solutions even at extremely high concentrations (*9*). A variety of byproducts were generated, however, these have not been fully characterized (*10*). Further studies facilitated the partial characterization of some of the breakdown products and the assessment of their toxicity using protein phosphatase inhibition assay and brine shrimp bioassay (*11*).

In this study, the decomposition products of the photocatalytic destruction of microcystin-LR (Figure 1) were analyzed by liquid chromatography−mass spectrometry (LC-MS) in order to elucidate possible mechanisms of degradation. This detailed investigation followed the reaction sequence over a period of 1-h characterizing products and intermediates at predetermined time intervals.

Experimental Section

Materials. Microcystin-LR was purified from a laboratory culture of *Microcystis aeruginosa* PCC7820 using the procedure previously detailed (*12*). Titanium dioxide (Degussa P-25) and hydrogen peroxide (Sigma, Pool, England) were used as received. All solutions were prepared in Milli-Q water, and all other reagents and solvents used were analytical or HPLC grade.

Photocatalysis. Aqueous solutions of microcystin-LR containing 1% (w/v) TiO_2 plus 42.8 mM H_2O_2 were illuminated in the presence of air with a 480 W xenon lamp (Uvalight Technology Ltd.; spectral output 330−450 nm). The reactions were carried out in glass bottles with constant stirring. The distance from the UV lamp to the surface of the test solution was 30 cm. Samples were taken and centrifuged to remove TiO_2 prior to analysis by LC-MS. The relative intensity of each of these compounds was followed at timed intervals over a 60-min irradiation period. Since pretest using 0.1% H_2O_2 without TiO_2 and UV did not show any significant destruction of microcystin-LR, no step was taken to remove excess H_2O_2 in the test. The initial concentration of microcystin-LR was 1 mg mL^{-1}, although this concentration is significantly higher than that found in the environment (in the range of ng mL^{-1}) it allows direct analysis by HPLC with out sample processing.

Analysis. The LC-MS system used in the study consisted of Waters Alliance 2690 HPLC Pump connected with Waters 996 PDA and Micromass ZQ Mass spectrometer with electrospray ionization source. HPLC column was Waters Symmetry 300TM C18 column (5 μm, 2.1 × 150 mm, Waters, U.S.A.). Treated samples were diluted 10-fold with Milli-Q water before analysis, and the injection volume was 10−50 μL. Mobile phase was gradient elution of water and acetonitrile, both containing 0.05% trifluoroacetic acids (TFA). Gradient elution was programmed as 15−25% of acetonitrile in 10 min followed by an increase to 40% by 20 min and 80% by 35 min. The mass data was obtained in the positive ion mode by full scanning from m/z 400−1200 at a Dwell time of 3 s and Select Ion Recording (SIR) acquisition at a Dwell time 0.75 s. Masslynx software workstation was used for the

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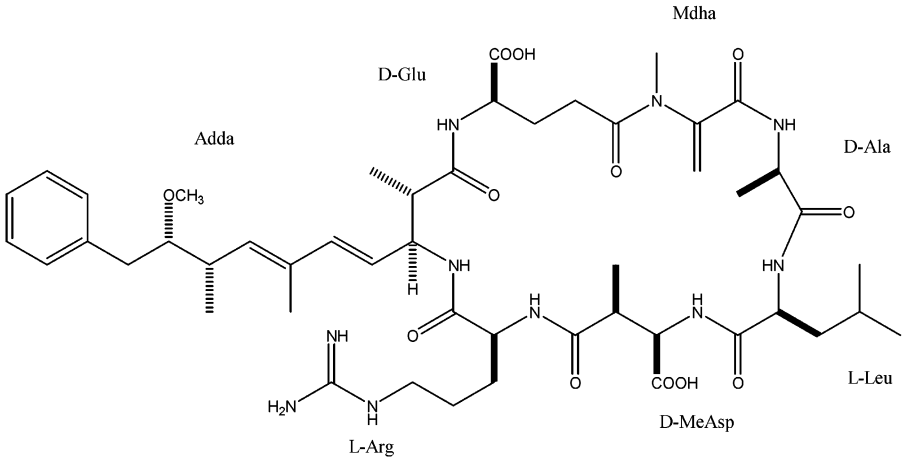


FIGURE 1. Structure of microcystin-LR where L-Leu is L-Leucine, D-Me-Asp is D-*erythro*-β-methylaspartic acid, L-Arg is L-arginine, Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid, D-Glu is D-glutamic acid, and Mdha is *N*-Me-dehydroalanine.

TABLE 1. Peak Area (Counts × 10^5) of Microcystin-LR Degradation Products Detected with LC-MS

	product name	1	2	3	4	5[a]	6	7	8	9	10	MC-LR
	retention time (min)	1.7	1.7−1.8	1.8−1.9	1.9−2.0	13.3−15.8	18.3−18.4	18.9−19.0	23.5−23.6	26.5−26.6	28.8−28.9	20.5−20.6
time (min) of photocatalysis	$[M + H]^+$ (m/z)	783	795	811	835	1029	759	999	965	617	651	995
0		0	0	0	0	0	0	0	0	0	0	3003.0
2		3.1	3.7	0.9	12.5	22.7	0	0	4.5	0	0	797.6
5		4.2	5.2	1.6	18.5	37.3	0.5	0	6.4	0	0	500.2
10		5.7	6.6	1.6	19.6	47.3	1.3	1.0	4.8	0	0	267.5
20		7.4	7.0	3.0	19.7	51.3	0.9	1.6	3.0	0.6	0	72.9
30		5.3	5.3	3.2	15.9	32.1	0.9	0.6	1.4	3.0	0.9	12.9
45		4.8	3.3	2.8	11.4	18.2	0.6	1.2	0.5	0.7	0.8	2.3
60		2.9	2.3	3.2	5.8	11.2	0.7	0.7	0.5	1.0	0.9	0.7

[a] Sum of products **5a**−**5d**.

LC-MS instrument control, data acquisition and data processing.

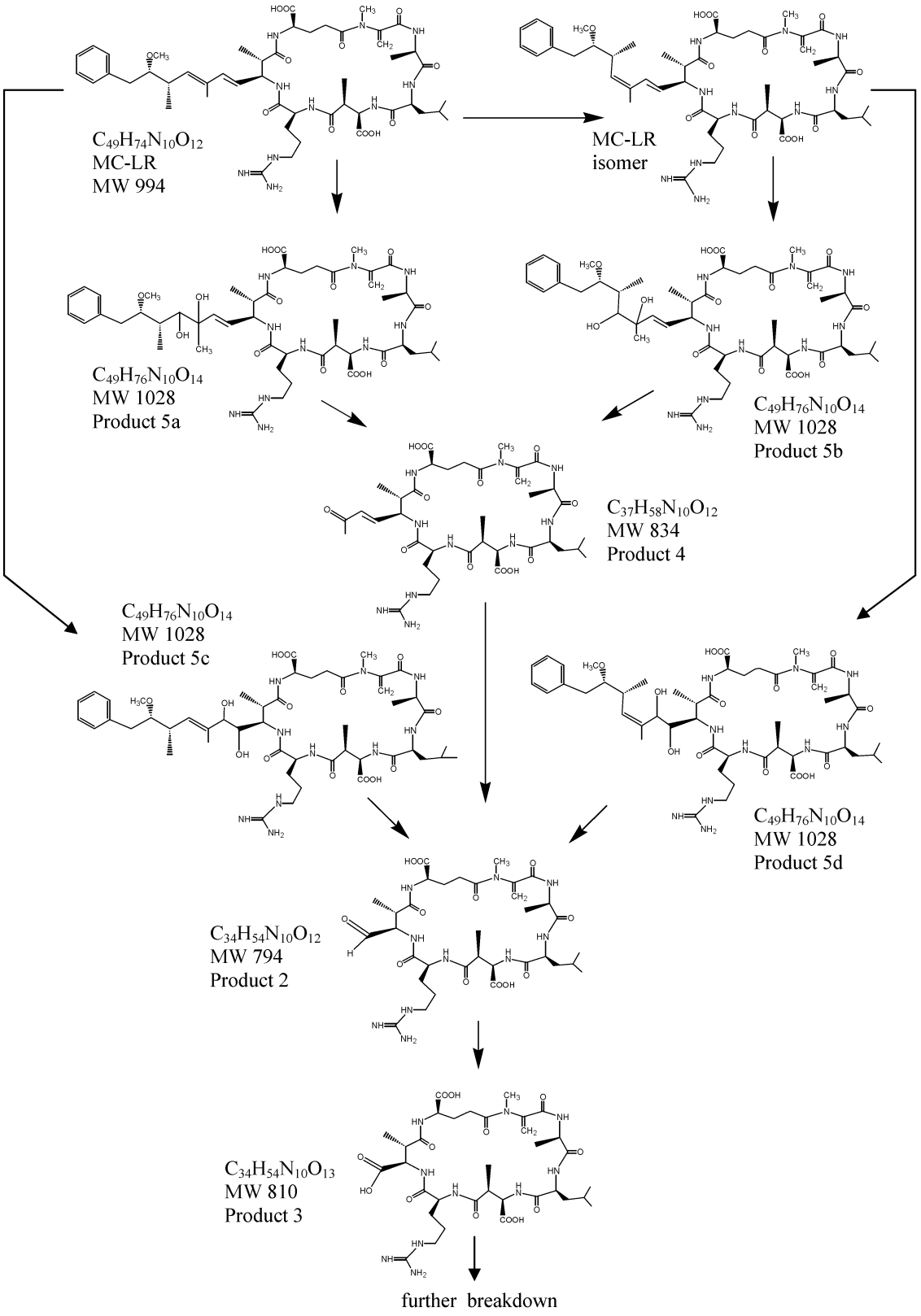
Results and Discussion

Previously we reported the destruction of microcystin-LR using TiO_2 photocatalysis in the presence of hydrogen peroxide and the removal of toxicity even at extremely high initial concentration of the toxin (*10*, *11*). The present study aims to characterize structurally the degradation products and to understand the degradation mechanism under photocatalysis. Following photocatalysis with TiO_2 and hydrogen peroxide, the reaction solution of microcystin-LR was analyzed with LC-MS to separate and characterize the degradation products of the parent compound.

LC-MS analysis of the photocatalytic degradation process revealed at least 10 decomposition byproducts detectable by MS (Table 1). Interestingly a significant number of the byproducts (products 1−5 and 8) were detectable after only 2 min photocatalysis time. Furthermore, the abundance of most of the byproducts initially increased but subsequently decomposed under prolonged photocatalysis. These findings may appear contrary to our previous study in which we observed only one of the seven UV-detectable products was degraded after 60 min photocatalysis (*10*). This previous study, however, was carried out in the absence of hydrogen peroxide. We have subsequently demonstrated that the addition of hydrogen peroxide significantly enhances the efficiency of the photocatalytic destruction of microcystin and all UV-detectable byproducts were eliminated (*13*).

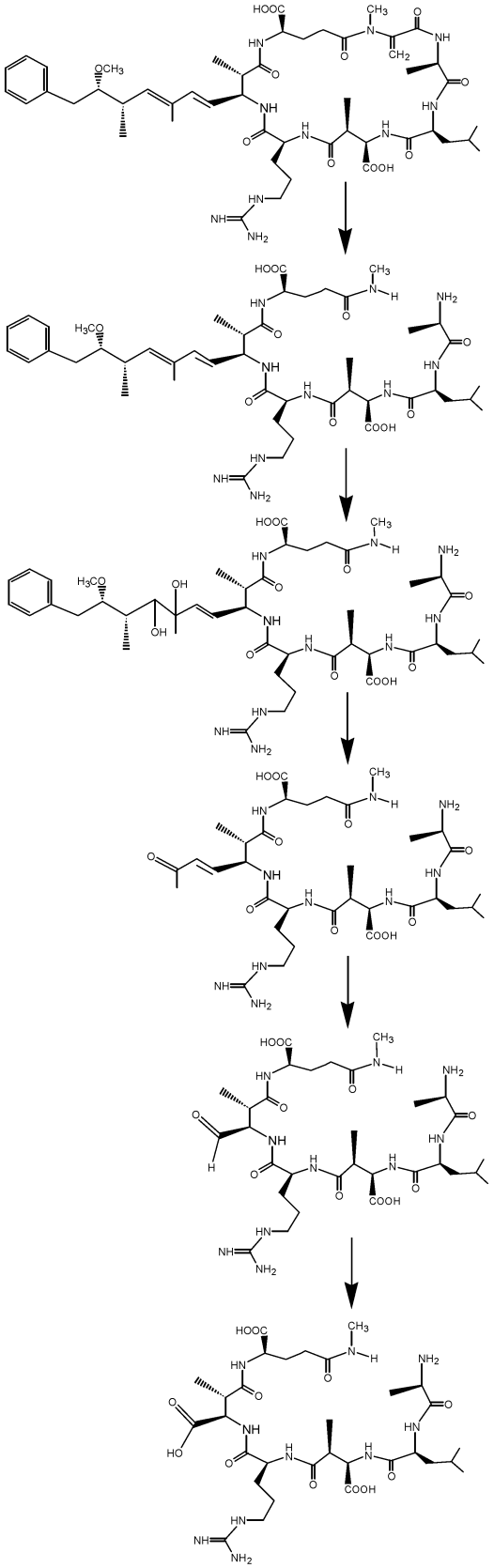
Structure assignment of breakdown products of microcystin-LR in photocatalysis was based on the analysis of the LC-Mass chromatogram and correspondent mass spectrum of products 1−10. The breakdown mechanisms could be interpreted in three schemes according to the elucidation of byproducts (Schemes 1−3). One of the most intense peaks observed in the analysis of the TIC corresponded to product 5a with a mass spectrum showing a molecular ion $(M + H)^+$ at m/z 1029 (Scheme 1). This ion corresponds to one of the structures previously reported as a major breakdown product of photocatalysis (*10*). This product is generated following hydroxyl radical attack at either the 4 or 6 double bond on the conjugated diene structure system in the Adda side chain resulting in hydroxylation of microcystin-LR (*10*). Interestingly, products 5b, 5c, and 5d had the same molecular ion $(M + H)^+$ at m/z 1029 (Scheme 1). This suggested that in addition to the dihydroxylated isomer of 5a (4 and 5 dihydroxy or 6 and 7 dihydroxy), the geometrical isomers of dihydroxy-microcystin-LR could be deduced, where the diene structure had either (4E), 6(Z) or 4(Z), 6(E) configuration. The geometrical isomers of dihydroxy-microcystin-LR could be produced from their corresponding precursors, i.e., (4E), 6(Z) or 4(Z), 6(E) Adda-microcystin-LR. This molecular assignment is supported by published observations of geometrical isomerization of microcystin-LR following UV irradiation (*14*, *15*). Dihydroxy-microcystin-LR underwent further oxidation with cleavage of the dihydroxylated bond at positions 4−5 or 6−7 on the Adda side chain. This is evidenced by the detection of cleavage products with $(M + H)^+$ ions at m/z 795 and 835 (products 2 and 4, respectively). The formation of products 2 or 4 depends on the location of the dihydroxy substitutes in the precursors (products 5a−d). Where the hydroxy groups are located at the 6 and 7 positions, the

SCHEME 1. Principal Pathway for Microcystin Degradation Following TiO_2 Photocatalysis



resulting product will have an ion $(M + H)^+$ at m/z 835 (product 4). Likewise if the hydroxy groups are located at 4 and 5 positions, the product with ion $(M+H)^+$ at 795 (product 2) will be obtained. Product 4 may subsequently transform to product 2 by further dihydroxylation of the 4−5 double bond, followed by bond cleavage. The observed ion $(M + H)^+$ at m/z 811 (product 3) is consistent with a carboxylic acid structure resulting from photocatalytic oxidation of product 2. The products discussed in Scheme 1 were observed at the first sampling point (2 min photocatalysis time). This would suggest that the unsaturated bonds on the Adda group are the most vulnerable to attack by oxidizing species generated by the TiO_2 photocatalyst. The vulnerability of this site to chemical oxidation has been exploited

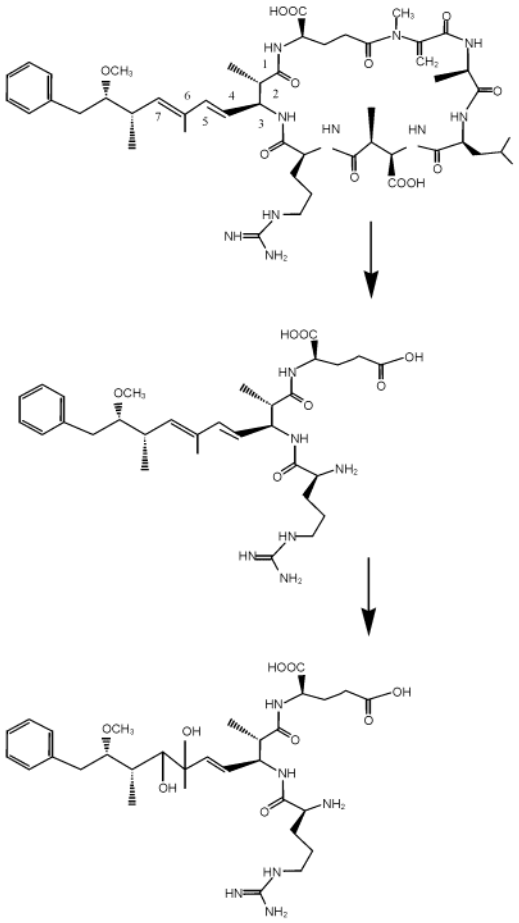
SCHEME 2. Secondary Pathway for Microcystin Degradation Following TiO_2 Photocatalysis



by other researchers as a means for microcystin quantification (*16*).

The observation of a product ion $(M + Na)^+$ at m/z 965 (product 8, Scheme 2) is consistent with a linearization of microcystin-LR. This may occur due to peptide bond cleavage between Mdha and Ala on the microcystin structure (Figure 1). This cleavage would be initiated by hydroxyl radical attack on the unsaturated bond of Mdha. Product 6 could be generated following dihydroxylation of the Adda 6 and 7 bonds of product 8, indicated by a molecular ion $(M + Na)^+$

SCHEME 3. Minor Pathway for Microcystin Degradation Following TiO_2 Photocatalysis



$C_{49}H_{74}N_{10}O_{12}$
MC-LR
MW 994

$C_{31}H_{48}N_6O_7$
Product 9
MW 616

$C_{31}H_{50}N_6O_9$
Product 10
MW 650

observed at m/z 999 in its mass spectrum. The LC-MS chromatogram revealed a shorter retention time for product 6 (18.3 min) than for product 8 (23.5 min) suggesting product 6 was more polar than product 8. This observation is in agreement with the structural elucidation of the two products since product 6 was dihydroxy substituted while product 8 was not. Following the same cleavage reaction as presented in Scheme 1, the dihydroxy-Adda 6 and 7 bond of product 6 cleaved forming product 1 (ion $(M + H)^+$ at m/z 783). Although product 6 is observed in the 5 min sample while product 1 appears in the 2 min sample, it might be concluded that product 1 is generated before product 6. Product 6, however is a dihydroxylated product with neighboring hydroxyl substitute and is a less stable structure than the ketone structure of product 1. Product 6 would, therefore, have a tendency to rapidly degrade to product 1. Further oxidation of product 1 to a carboxylic acid (product 7) resulted in the observed ion $(M + H)^+$ at m/z 759.

The formation of products 9 and 10 does not occur until 20 and 30 min photocatalysis time, respectively (Table 1 and Scheme 3). Product 9 is potentially a linearized residue of the parent microcystin-LR, which has undergone multiple cleavage of the peptide ring (ion $(M + H)^+$ at m/z 617). It is proposed that the Glu-Mdha and the Masp-Arg peptide chains of microcystin-LR have been hydrolyzed and subsequently cleaved with the loss of Mdha-Ala-Leu-Masp peptides to form this linearized product. The linearized residue with Adda side chain may then have been attacked by hydroxyl radicals forming a dihydroxy product based on the ion observed at m/z 651 $(M + H)^+$ (product 10). Product 10 may undergo cleavage of the hydroxylated Adda bond resulting in the formation of product 11 (not shown).

Quantitative analysis of product abundance indicated that 5a to 5d were the dominant products over all sampling time points. They were detected as early as the first sampling time (2 min) and their concentration rose sharply, reaching a maximum at 20 min. The breakdown product of 5a and b (product 4) was the second most abundant product. All other products, with the exception of product 8, increased and reached maximum from 2 to 20 min and then gradually decreased after 30 min photocatalysis time. In contrast, product 8 was initially detected at 2 min, reached a maximum at 5 min, and then immediately started to decrease. Kinetic analysis of the formation and disappearance of product 8 suggest this compound is a different component of an alternative decomposition pathway (Scheme 2). The appearance of the major breakdown products was closely related with the disappearance of the parent microcystin (Table 1). This observation provided direct evidence that the detected products resulted from the degradation of the toxin by TiO_2 photocatalysis.

It would appear that Scheme 1 is the predominant pathway for the photocatalytic destruction of microcystin-LR. This premise is supported by the observation that the majority of the breakdown products were product 5a (25−30%), product 4 (18−26%), product 5b (16−19%), product 5c (12−15%), and product 5d (9−10%), respectively. Products 2 and 8 represented less than 10% of the total breakdown products. As discussed previously, 5a−5d shared the same molecular ion at m/z 1029 and were assigned as the isomers of the products resulting from the corresponding isomerized precursors. Products 5a−5d accounted for as much as 73% of the total breakdown products. In Scheme 1, the majority of microcystin-LR was initially isomerized

and then dihydroxylated and cleaved at the Adda double bond, followed by further decomposition.

A comparative quantitative analysis of the relative amounts of the decomposition products compared to the parent toxin indicates that after 60 min photocatalysis the total peak area of degradation products (27×10^5) represents less than 1% of the starting material (3003×10^5). This could be interpreted to suggest that almost total mineralization had occurred; however, our previously reported findings (*13*) indicated that less than 20% mineralization results under these conditions. The probable explanation of this apparent discrepancy is that products with mass below 400 Dalton were difficult to detect because of overwhelming noise signals. Therefore further breakdown products with ion $(M + H)^+$ below 400 were not identified.

Our previous study showed that photocatalytic degradation of microcystin-LR resulted in elimination of protein phosphatase inhibition and toxicity against brine shrimp. The present study showed that the major decomposition products were peptide residues of microcystin-LR. These peptide residues can be classified into three structural groups: group 1, ring peptides with an Adda moiety (products 5a−d); group 2, linearized peptides with an Adda moiety (products 6, 8, 9, and 10); and group 3, ring or linearized peptides without the Adda moiety (products 1−4 and 7). Since the conjugated diene structure of Adda in microcystin-LR have been reported to be essential for inhibition of protein phosphatase 1 and 2A (*17*−*19*), the peptide residues without an Adda moiety produced from microcystin-LR appear unlikely to have the above-mentioned toxicity (i.e. products 1−4 and 7). While the Adda side chain is still present in the remaining products (products 5, 6, and 8−10) it has undergone modifications such as dihydroxylation or photoisomerization, which have been shown to eliminate toxicity (*17*−*20*). This observation could provide the structural evidence to explain the nontoxicity of breakdown products against protein phosphatase 1A, in which solutions containing high concentrations of microcystin-LR degradation products failed to show significant inhibition against this enzyme (*11*). It is presumed that photocatalysis appeared to have destroyed all conjugated diene systems whether intact on the microcystin structure or in a fragment with the Adda moiety. Schemes 2 and 3 showed that in the minor pathways, microcystin-LR was degraded into linearized products with conjugated diene structure in their Adda side chains. All toxicological studies on the toxicity of the linearized forms of microcystin indicate loss of toxicity (*21*). All products characterized in this study indicate that the toxicity associated with the parent microcystin has been eliminated. Therefore, it could be reasonably predicted that TiO_2 photocatalysis may be also effective for the destruction of other cyanotoxins with Adda conjugated diene systems in their structure i.e., other microcystins and nodularins.

It is clear from this study that the major destruction pathway of microcystin-LR (Scheme 1) appears to be initiated via three mechanisms, UV irradiation, hydroxyl radical attack, and oxidation (*10*, *22*). UV irradiation caused geometrical isomerization of microcystin on the Adda conjugated diene structure to form (4E), 6(Z) or 4(Z), 6(E) Adda configuration of microcystin. This process could lead to the reduction or partial loss of toxicity of the isomers (*14*). Hydroxyl radical attack destroyed the conjugated diene structure to form the dihyroxylated products. Further oxidative cleavage of the hydroxylated 4−5 and/or 6−7 bond of Adda may occur to form aldehyde or ketone peptide residues. These were subsequently oxidized into the corresponding carboxylic acids. Photocatalysis also hydrolyzed the peptide bond on the ring structure of microcystin-LR to form a linearized structure although it appeared to be a minor pathway (Schemes 2 and 3). Further reaction of the linearized structure underwent the dihydroxylation of conjugated diene followed by corresponding bond cleavage.

In conclusion, the major mechanism of photocatalysis is to isomerize, substitute, and cleave the Adda conjugated diene structure in either microcystin-LR or its resulting derivatives and consequently eliminate not only the toxicity of microcystin itself but also its breakdown products.

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