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A Photodetoxification Mechanism of the Cyanobacterial Hepatotoxin Microcystin-LR by Ultraviolet Irradiation

Kunimitsu Kaya* and Tomoharu Sano

Environmental Chemistry Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305, Japan

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When microcystin-LR was exposed to UV, three major nontoxic compounds were formed. These compounds were identified as [4(*E*),6(*Z*)-Adda[5]]- and [4(*Z*),6(*E*)-Adda[5]]microcystin-LR, which are geometrical isomers of the Adda [3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(*E*),6(*E*)-decadienoic acid] moiety of microcystin-LR, and a novel compound, tricyclo-Adda [(2*S*,3*S*,1′*R*,3′*S*,4′*S*,5′*R*,6′*R*,7′*R*)-3-amino-5-(4′,6′-dimethyl-3′-methoxytricyclo[5.4.0.0$^{1',5'}$]undeca-8′,10′-dien-6′-yl)-2-methyl-4(*E*)-pentenoic acid]-containing microcystin-LR ([tricyclo-Adda[5]]-microcystin-LR), which was formed by [2 + 2] addition between the benzene ring and the double bond at position 6-7 of the Adda moiety of the microcystin. The geometrical isomers were formed reversibly, and their equilibrium constants were almost the same. [Tricyclo-Adda[5]]-microcystin-LR was also formed reversibly and was decomposed under UV light. These results suggest that the breakdown of microcystin-LR by UV irradiation proceeds via [tricyclo-Adda[5]]-microcystin-LR.

Introduction

Toxic cyanobacterial waterblooms are occurring in drinking water reservoirs in the world. Toxic freshwater cyanobacteria such as *Microcystis*, *Oscillatoria*, and *Anabaena* produce cyclic hepatotoxic peptides called microcystins. Microcystins are the most common offenders worldwide in the case of drinking water-based disease (*1*). Microcystins have a dehydroamino acid, a characteristic amino acid [Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(*E*),6(*E*)-decadienoic acid], three D-amino acids, and two L-amino acids. General structure of the toxins is cyclo(-D-Ala[1]-L-X[2]-D-*erythro*-β-methylAsp[3]-L-Z[4]-Adda[5]-D-Glu[6]-*N*-methyl-dehydroAla[7]), where X is leucine (L), arginine (R), or tyrosine (Y) and Z is arginine (R), alanine (A), or methionine (M) (*2*, *3*). In the structure of microcystins, Adda plays an important role in their toxicity, since hydrogenation or ozonolysis of the diene system in Adda gives inactive products, and geometrical isomers at the Δ^6 double bond of the Adda unit are also inactive (*4*). With sunlight exposure, the nontoxic geometrical isomer [6(*Z*)-Adda[5]]microcystin is formed and microcystins are decomposed (*5*). Also, nontoxic [4(*Z*)-Adda[5]]- and [6(*Z*)-Adda[5]]microcystins are formed and decomposed by UV irradiation (*6*). Moreover, formation of an unknown compound (compound **X**) was observed (*6*).

* Address correspondence to this author. Tel: +81-298-50-2428. Fax: +81-298-50-2574. E-mail: kayakuni@nies.go.jp.

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Table 1. ^{1}H and ^{13}C NMR Data for [Tricyclo-Adda^5]microcystin-LR in D_2O[a]

position		^{1}H, *J* (Hz)	^{13}C	position		^{1}H, *J* (Hz)	^{13}C
Ala	1		176.1	tricyclo-Adda	6	0.84 (d, 7.0)	15.7
	2	4.33 (q, 7.9)	50.5		1′		41.4
	3	1.25 (d, 7.9)	16.9		2′	1.67 (m)	46.3
Leu	1		175.9		3′	3.44 (m)	91.7
	2	4.17 (m)	54.5		4′	2.18 (m)	41.7
	3	1.82 (m)	40.1		5′	2.14 (d, 4.6)	66.5
		1.50 (m)			6′		47.8
	4	1.54 (m)	25.2		7′	2.86 (d, 5.5)	50.4
	5	0.73 (d, 6.1)	20.8		8′	5.36 (m)	125.4
	5′	0.77 (d, 6.4)	23.2		9′	5.67 (m)	123.9
Me-Asp	1		176.9		10′	5.55 (m)	121.8
	2	4.29 (d, 4.3)	57.3		11′	5.41 (d, 9.7)	134.0
	3	3.1 (m)	42.1		12′	3.24 (s)	57.2
	4		178.7		13′	0.82 (d, 7.0)	19.6
	5	0.90 (d, 6.7)	15.0		14′	1.05 (s)	22.1
Arg	1		172.1	Glu	1		179.8
	2	4.10 (m)	52.6		2	3.77 (dd, 6.4, 8.5)	56.6
	3	1.85 (m)	28.4		3	1.90 (m)	27.3
		1.40 (m)				1.72 (m)	
	4	1.41 (m)	25.4		4	2.65 (m)	32.6
	5	3.02 (m)	41.4			2.42 (m)	
	6		157.6		5		177.8
tricyclo-Adda	1		177.8	MDha	1		167.0
	2	2.80 (m)	44.1		2		143.4
	3	4.24 (t, 8.2)	56.8		3	5.80 (s)	116.7
	4	5.19 (dd, 9.0, 15.5)	124.2			5.43 (s)	
	5	6.15 (d, 15.5)	143.4		N-Me	3.26 (s)	38.8

[a] ^{1}H, 500 MHz; ^{13}C, 125 MHz; s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet.

In order to clarify the breakdown mechanism of microcystins by UV irradiation, we focused on compound **X**, since compound **X** may be an intermediate for the breakdown. We now report the chemical structure of a novel nontoxic tricyclo-Adda-containing microcystin-LR ([tricyclo-Adda^5]microcystin-LR) from the UV irradiation products and propose a breakdown mechanism of microcystins by UV irradiation.

Experimental Procedures

Preparation of Microcystin-LR. *Microcystis aeruginosa* (NIES-88) was cultured in MA medium. The cells were grown isothermally at 25 °C (light intensity < 250 μmol of photons/m^2/s; aeration rate 1.5 L/min). Microcystin-LR was isolated from cultured cells and was purified using HPLC and TLC (*7*). The purity of microcystin-LR was confirmed by ^{1}H NMR.

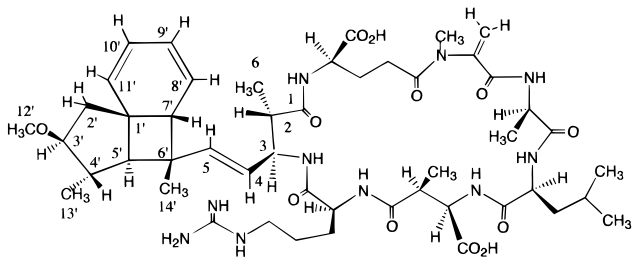
Photoreaction. Microcystin-LR was dissolved with 10 mM phosphate buffer (pH 7.0) at a concentration of 10 mg/100 mL. The solution (10 mL) was transferred to a Petri dish (9.5 cm in diameter) without cover. To remove ozone, the surface of the dish was covered with fresh air streams. The reaction was performed at 0 °C under UV light (130 μW at 250 nm) and was monitored at intervals of 15 min.

Isolation of Reaction Products. The photoreaction products were separated by HPLC using a Mightysil RP-18 column [20 mm × 25 cm; 60% methanol, 50 mM phosphate buffer (pH 3.0), flow rate at 10 mL/min]. Three major compounds and unreacted microcystin-LR were isolated by HPLC. Unreacted microcystin-LR was eluted at 24.9 min. Compounds **1**, **2**, and **3** were eluted at 21.2, 26.1, and 30.0 min, respectively. These compounds were further purified by HPTLC using chloroform/methanol/water (60/40/10, v/v) as a solvent.

Identification of Reaction Products. The structures of reaction products were identified by HRFAB/MS (JEOL, model JMS-700) and ^{1}H and ^{13}C NMR (JEOL, model JNM A-500; ^{1}H, 500 MHz; ^{13}C, 125 MHz) analyses (*7*).

Toxicity Test. Toxicity was measured by intraperitoneal injection of samples into inbred Swiss white BALB/c mice. Three mice per dose level were used. The injected mice were observed closely over a period of 7 h. Survival times of less than 5 h were considered to result from the samples.

Chart 1. Structure of [Tricyclo-Adda^5]microcystin-LR[a]



[a] Tricyclo-Adda, (2*S*,3*S*,1′*R*,3′*S*,4′*S*,5′*R*,6′*R*,7′*R*)-3-amino-5-(4′,6′-dimethyl-3′-methoxytricyclo[5.4.0.0$^{1',5'}$]undeca-8′,10′-dien-6′-yl)-2-methyl-4(*E*)-pentenoic acid.

Results

When microcystin-LR was exposed to UV, three major compounds and unreacted microcystin-LR were detected. Compound **1** was a colorless, amorphous solid and has no λ_{max} above 220 nm. The ratio of $\lambda_{240nm}/\lambda_{280nm}$ was 2.92. In the positive HRFAB/MS using glycerol as the matrix, the $[M + H]^+$ was observed at *m*/*z* 995.5628. From the results, the molecular formula of this compound was established to be $C_{49}H_{74}O_{12}N_{10}$ (calcd for $C_{49}H_{75}O_{12}N_{10}$: 995.5565, Δ +6.2 mmu). This molecular formula was the same as that of microcystin-LR. As shown in Table 1, ^{1}H and ^{13}C NMR spectra data of the Adda moiety in this compound were quite different from those of Adda in microcystin-LR, but the data of all of the other moieties in compound **1** agreed well with those of the corresponding moieties of microcystin-LR. The signals of protons (H-12) of a methyl group combined with C-6 and H-7 of Adda were observed at 1.59 and 5.41 ppm in the ^{1}H NMR spectra of microcystin-LR, whereas the signals of the same protons (H-14′ and H-5′) in compound **1** were observed at 1.05 and 2.14 ppm, respectively. Furthermore, the signal of H-7′ of compound **1** was observed at 2.89 ppm. This proton corresponded with the ortho proton (H-16 or H-20) of the phenyl group of Adda. The

Table 2. ^{1}H and ^{13}C NMR Data for Microcystin-LR, [4(*Z*)-Adda^5]microcystin-LR, and [6(*Z*)-Adda^5]microcystin-LR in D_2O[a]

		microcystin-LR		[4(*Z*)-Adda^5]microcystin-LR		[6(*Z*)-Adda^5]microcystin-LR	
position		^{1}H, *J* (Hz)	^{13}C	^{1}H, *J* (Hz)	^{13}C	^{1}H, *J* (Hz)	^{13}C
Ala	1		176.1		176.2		176.1
	2	4.36 (m)	50.5	4.31 (q, 7.5)	50.5	4.34 (q, 7.5)	50.4
	3	1.26 (d, 7.4)	16.9	1.26 (d, 7.5)	16.9	1.25 (d, 7.5)	16.8
Leu	1		175.8		175.8		175.9
	2	4.20 (dd, 3.9, 11.9)	52.5	4.18 (dd, 3.4, 8.2)	54.5	4.19 (dd, 3.2, 11.7)	54.5
	3	1.86 (m)	40.1	1.87 (m)	40.1	1.87 (m)	40.1
		1.52 (m)		1.50 (m)		1.52 (m)	
	4	1.53 (m)	25.2	1.55 (m)	25.2	1.57 (m)	25.2
	5	0.74 (d, 6.0)	20.8	0.73 (d, 6.1)	20.7	0.74 (d, 6.1)	20.8
	5′	0.78 (d, 6.0)	23.2	0.78 (d, 6.4)	23.2	0.78 (d, 6.4)	23.2
Me-Asp	1		182.6		180.5		180.3
	2	4.32 (d, 3.7)	57.3	4.26 (d, 4.3)	57.3	4.31 (d, 4.3)	57.3
	3	3.20 (m)	42.1	3.09 (m)	42.1	3.08 (m)	42.1
	4		178.6		178.5		178.7
	5	0.93 (d, 7.3)	14.9	0.90 (d, 6.7)	15.0	0.90 (d, 7.3)	14.9
Arg	1		172.4		171.6		172.1
	2	4.22 (dd, 3.1, 11.9)	54.5	4.12 (dd, 3.2, 11.9)	52.5	4.14 (dd, 3.1, 6.4)	52.5
	3	1.91 (m)	28.2	1.39 (m)	25.4	1.87 (m)	28.3
		1.74 (m)		1.37 (m)		1.37 (m)	
	4	1.41 (m)	25.4	1.88 (m)	28.4	1.38 (m)	25.3
				1.38 (m)			
	5	3.01 (m)	41.3	2.96 (m)	41.3	2.92 (m)	41.3
	6		157.6		157.5		157.5
Adda	1		177.7		177.3		177.5
	2	2.91 (m)	44.7	2.80 (m)	45.1	2.91 (m)	44.4
	3	4.36 (m)	56.2	5.03 (t, 10.7)	51.1	4.36 (m)	56.8
	4	5.47 (m)	125.4	5.14 (dd, 10.7, 11.9)	127.0	5.52 (dd, 9.5, 15.3)	128.5
	5	6.21 (d, 15.5)	138.6	6.01 (d, 11.9)	136.9	6.46 (d, 15.3)	131.5
	6		134.2		133.1		132.8
	7	5.41 (d, 9.8)	136.8	5.26 (d, 9.5)	135.4	5.28 (d, 9.8)	135.1
	8	2.60 (m)	36.5	2.50 (m)	36.7	2.80 (m)	35.4
	9	3.35 (m)	87.6	3.33 (m)	87.6	3.32 (m)	87.8
	10	2.79 (dd, 10.0, 14.0)	37.7	2.85 (dd, 4.0, 14.3)	37.7	2.75 (dd, 4.0, 14.3)	37.7
		2.62 (m)		2.58 (dd, 8.0, 14.3)		2.54 (dd, 8.3, 14.3)	
	11	0.94 (d, 7.2)	16.6	0.94 (d, 7.0)	14.9	0.87 (d, 7.0)	15.2
	12	1.59 (s)	12.6	1.64 (s)	17.2	1.73 (s)	20.6
	13	0.90 (d, 7.2)	15.2	0.92 (d, 4.9)	16.9	0.91 (d, 6.7)	16.8
	14	3.15 (s)	58.0	3.14 (s)	58.0	3.09 (s)	58.0
	15		140.2		140.2		140.4
	16, 20	7.15 (m)	130.4	7.17 (d, 7.3)	130.5	7.14 (d, 7.6)	130.3
	17, 19	7.23 (m)	129.3	7.23 (t, 7.3)	129.2	7.23 (t, 7.6)	129.4
	18	7.15 (m)	127.1	7.14 (t, 7.3)	127.0	7.15 (t, 7.6)	127.1
Glu	1		179.7		179.9		179.7
	2	3.79 (dd, 6.4, 9.0)	56.6	3.74 (dd, 6.1, 9.5)	56.8	3.80 (dd, 6.4, 8.9)	56.6
	3	1.94 (m)	27.3	1.92 (m)	27.2	1.93 (m)	27.4
		1.75 (m)		1.73 (m)		1.74 (m)	
	4	2.68 (m)	32.5	2.69 (m)	32.5	2.67 (m)	32.6
		2.45 (m)		2.43 (m)		2.44 (m)	
	5		177.6		177.7		177.7
MDha	1		167.0		167.0		167.0
	2		144.3		144.3		144.3
	3	5.83 (s)	116.8	5.81 (s)	116.7	5.81 (s)	116.7
		5.45 (s)		5.43 (s)		5.44 (s)	
	N-Me	3.28 (s)	38.8	3.27 (s)	3.88	3.27 (s)	38.8

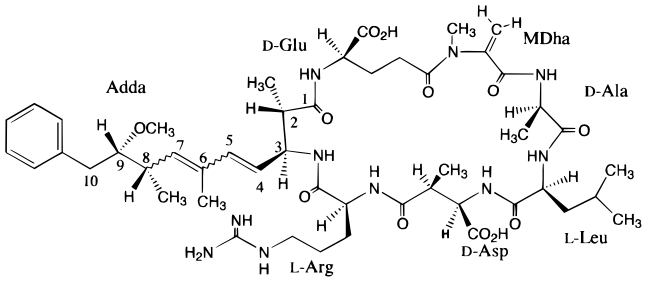
[a] ^{1}H, 500 MHz; ^{13}C, 125 MHz; s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet.

HMBC correlations from H-5′ and H-14′ to C-7′ and from H-2′ to C-5′ were observed. From these results, the structure of Adda moiety of compound **1** was confirmed as 3-amino-5-(4′,6′-dimethyl-3′-methoxytricyclo[5.4.0.0$^{1',5'}$]-undeca-8′,10′-dien-6′-yl)-2-methyl-4(*E*)-pentenoic acid. Furthermore, H-14′ correlated with H-4′, H-7′, and H-4, H-2′ also correlated with H-7′ in the ROESY spectrum. These results showed that the configuration of the Adda moiety of compound **1** was 2*S*,3*S*,1′*R*,3′*S*,4′*S*,5′*R*,6′*R*,7′*R*, since the configuration of the original Adda has been determined as 2*S*,3*S*,8*S*,9*S*. From these results, compound **1** was identified as [tricyclo-Adda^5]microcystin-LR (Chart 1).

The $[M + H]^+$ ion of compound **2** by positive FAB/MS spectra was observed at *m*/*z* 995. This was the same as that of microcystin-LR. The molecular formula of the compound obtained by HRFAB/MS agreed well with that of microcystin-LR. In ^{1}H NMR spectra of compound **2**, the coupling constant (11.9 Hz) of H-4 and H-5 of Adda was smaller than that (15.5 Hz) of Adda in microcystin-LR. The ^{1}H and ^{13}C NMR signals of the other moieties of compound **2** were the same as those of microcystin-LR (Table 2). From these facts, the structure of compound **2** was identified as [4(*Z*)-Adda^5]microcystin-LR (*6*) (Chart 2).

Compound **3** was identified by NMR and positive FAB/MS. These data completely agreed with those of [6(*Z*)-Adda^5]microcystin-LR (*4*, *8*). From the results, compound **3** was identified as [6(*Z*)-Adda^5]microcystin-LR (Chart 2).

Chart 2. Structures of Microcystin-LR, [4(*Z*)-Adda[5]]microcstin-LR, and [6(*Z*)-Adda[5]]microcystin-LR[a]



[a] The conjugated dienes in the Adda moieties of microcystin-LR, [4(*Z*)-Adda[5]]microcystin-LR, and [6(*Z*)-Adda[5]]microcystin-LR are 4(*E*),6(*E*), 4(*Z*),6(*E*), and 4(*E*),6(*Z*), respectively.

Table 3. Recovery and Composition of Photoreaction Products (Reaction conditions described in the text)[a]

reaction time (min)	recovery (%)	composition (%) of recovered products				ratio	
		LR	4(*Z*)	6(*Z*)	tricyclo	4(*Z*)/LR	6(*Z*)/LR
0	100	100	0	0	0		
15	71.5	67.4	15.0	14.9	2.7	0.22	0.22
30	64.6	64.9	15.1	15.3	4.7	0.23	0.24
45	58.9	64.5	14.9	14.9	5.7	0.23	0.23
60	53.0	64.4	14.8	14.4	6.4	0.23	0.22
75	50.2	63.9	14.4	14.6	7.1	0.23	0.23

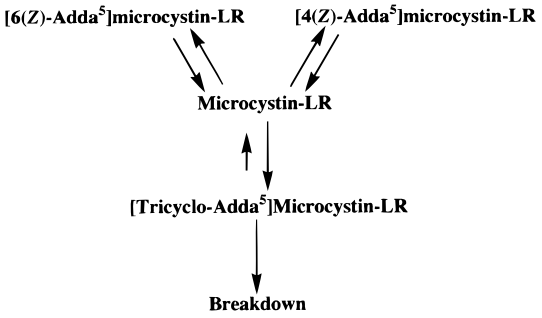
[a] LR, microcystin-LR; 4(*Z*), [4(*Z*)-Adda[5]]microcystin-LR; 6(*Z*), [6(*Z*)-Adda[5]]microcystin-LR; tricyclo, [tricyclo-Adda[5]]microcystin-LR.

In order to determine the LD_{50} of the isomers of microcystin-LR in mice, purified [4(*Z*)-Adda[5]]-, [6(*Z*)-Adda[5]]-, and [tricyclo-Adda[5]]microcystin-LR and microcystin-LR were injected into the peritoneum of the mice. When 100 μg/kg of mouse of microcystin-LR was injected, all mice in the group died. However, no mouse in each group died when 1500 μg/kg of mouse of [4(*Z*)-Adda[5]]-, [6(*Z*)-Adda[5]]-, or [tricyclo-Adda[5]]microcystin-LR was injected. These results suggest that 4(*E*),6(*E*)-Adda is the essential structure for hepatotoxicity.

The results of recoveries and compositions of the products during the reaction are summarized in Table 3. The recoveries of the three major compounds and unreacted microcystin-LR decreased gradually during the reaction. About 50% of microcystin-LR was decomposed by UV irradiation for 75 min (0.585 J). In this reaction condition, [4(*Z*)-Adda[5]]- and [6(*Z*)-Adda[5]]microcystin-LR were given in about equal amount, and the ratios of [4(*Z*)-Adda[5]]microcystin-LR/microcystin-LR and [6(*Z*)-Adda[5]]-microcystin-LR/microcystin-LR were constant during the reaction. These results demonstrate that the reaction from microcystin-LR to [4(*Z*)-Adda[5]]microcystin-LR and [6(*Z*)-Adda[5]]microcystin-LR was reversible and in equilibrium. On the other hand, the relative content of [tricyclo-Adda[5]]microcystin-LR in the recovered products increased gradually.

When the aqueous solution of [tricyclo-Adda[5]]microcystin-LR (0.1 mg/10 mL) was placed under UV light for 15 min, 68.3% of [tricyclo-Adda[5]]microcystin-LR decomposed, and the remaining compounds were [4(*Z*)-Adda[5]]microcystin-LR (3.1%), [6(*Z*)-Adda[5]]microcystin-LR (3.1%), microcystin-LR (14.3%), and [tricyclo-Adda[5]]-microcystin-LR (11.2%). The ratios of [4(*Z*)-Adda[5]]microcystin-LR/microcystin-LR and [4(*Z*)-Adda[5]]microcystin-LR/microcystin-LR agreed well with those of the geometrical isomers from microcystin-LR by UV irradiation. The decomposed compounds eluted near the solvent front by HPLC.

Scheme 1. Possible Outline for Breakdown of Microcystin-LR by UV Irradiation



In the case of [6(*Z*)-Adda[5]]microcystin-LR (0.1 mg/10 mL), only 28.3% of the original was lost by UV light for 15 min, and the isomerized compounds from [6(*Z*)Adda[5]]-microcystin-LR were [4(*Z*)-Adda[5]]microcystin-LR (15.0%), [6(*Z*)-Adda[5]]microcystin-LR (15.1%), microcystin-LR (68.6%), and [tricyclo-Adda[5]]microcystin-LR (1.3%). These results were almost the same as those of microcystin-LR and [4(*Z*)-Adda[5]]microcystin-LR.

These results and the configuration of [tricyclo-Adda[5]]-microcystin-LR show that [tricyclo-Adda[5]]microcystin-LR was formed from the original microcystin-LR reversibly and was unstable under UV.

Discussion

Formation of [6(*Z*)-Adda[5]]- and [4(*Z*)-Adda[5]]microcystin-LR as geometrical isomers of the Adda moiety of microcystin-LR was reversible. The equilibrium constants of the two isomer formations were almost the same.

The formation of [tricyclo-Adda[5]]microcystin-LR suggested that the [2 + 2] addition between the double bond at position 6−7 and the double bond at the quaternary carbon−ortho position of the phenyl group was performed under UV light. Previously, a three-dimensional structure of Adda in microcystin-LR has been examined using computer modeling by Lanaras et al. (*9*). They showed that the Adda formed a "U-shape" and the phenyl group located around the conjugated diene. In order to confirm the three-dimensional structure of Adda of microcystin-LR in the aqueous solution, ROE (rotating Overhauser effect) was examined in D_2O. In the experiments, ROE between H-5 at the conjugated diene and H-17 as a proton at the meta position of the phenyl group was observed. These results support that the Adda moiety of microcystin-LR formed a "U-shape" in aqueous solutions. The U-shape of the Adda moiety in aqueous solutions is suitable to form [tricyclo-Adda[5]]microcystin-LR.

[Tricyclo-Adda[5]]microcystin-LR was formed from microcystin-LR reversibly and was decomposed under UV. These facts suggest that the breakdown of microcystin-LR proceeds via [tricyclo-Adda[5]]microcystin-LR (Scheme 1), the cyclization velocity is faster than that of the breakdown, and the ring-opening velocity from [tricyclo-Adda[5]]microcystin-LR to microcystin-LR is slower than that of the cyclization or the breakdown.

Our results suggest that detoxification of microcystins can be performed by UV irradiation at 239 nm as the λ_{max} of microcystins due to the conjugated diene in Adda.

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