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Detoxification and degradation of microcystin-LR and -RR by ozonation

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ABSTRACT

In the present study, two Microsystins (MCs) of Microcystin-LR and Microcystin-RR were degraded with different dosages of ozone (O_3). The possible degradation pathways were elucidated by analyzing their intermediates and end-products with liquid chromatography–mass spectrometry (LC–MS) method. The toxicity of the MCs ozonation products was also evaluated by assaying the protein phosphatase inhibition *in vitro* and acute toxicity *in vivo*. Results demonstrated that ozonation was a promising technology for removal and detoxification of the cyanotoxins. The MCs destruction was mainly involved in the attack of ozone on Adda side chain. First, the conjugated diene structure of Adda moiety was attacked by hydroxyl radical ($OH^{\cdot}$) to produce dihydroxylated products, then the hydroxylated 4–5 and/or 6–7 bond of Adda was cleaved into aldehyde or ketone peptide residues, and finally the residues were oxidized into the corresponding carboxylic acids. The fragmentation of the Mdha-Ala peptide bond of MCs also contributed positively to the oxidation process. Additionally, the attack on the benzene ring of Adda side chain was exclusively observed during MC-RR degradation. The toxicity evaluation of MCs ozonation products revealed that those end-products had no adverse effects *in vivo* and *in vitro* ozonation that could completely remove the MCs' toxicity.

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1. Introduction

Microcystins (MCs) are a group of monocyclic heptapeptide toxins produced by species of cyanobacteria including *Microcystis*, *Anabaena*, *Oscillatoria*, *Anabaenopsis*, *Hapalosipon*, *Nostoc* and *Planktothrix*, which are usually found in surface waters (Hoeger et al., 2005; Edwards et al., 2008; Chen et al., 2009). There are more than 75 microcystin variants, and toxic strains usually produce a mixture of different MCs (Li et al., 2009). MCs are composed of γ-linked D-glutamic acid (D-Glu), D-alanine (D-Ala), β-linked D-erythro-β-methylaspartic acid (D-Asp), *N*-methyldehydroalanine (Mdha), and a unique C_{20} β-amino acid, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid (Adda), and two variable L-amino acids. Amino acid variations at position 2 and 4 gives rise to the naming system. The congener with leucine (L) at position 2 and Arginine (R) at position 4 is known as MC-LR, while the congener with the Arginine (R) at both positions is MC-RR. Their toxicity relates with the Adda moiety, which can interact with eukaryotic serine/threonine protein phosphatase-1 (PP-1) and protein phosphatase-2A (PP-2A) and irreversibly inhibit their activities (Harada et al., 2004; Ott and Carmichael, 2006).

MCs exhibit acute and chronic effects on humans and wildlife by damaging liver, which is of increasing concern all over the world (Gupta et al., 2003; Li et al., 2008). Generally MCs are transported into liver cells (hepatocytes), causing pooling of blood in the liver and death of mammals due to hemorrhagic shock (Kondo et al., 1996). Furthermore, MCs are suspected of promoting primary liver cancer with long-term exposure to sub-lethal levels (Yan et al., 2006; Weng et al., 2007). MC-LR is usually regarded as one of the most acutely toxic cyanobacteria toxins with a median lethal dose (LD_{50}) ranging from 40 to 100 $\mu g\ kg^{-1}$ (i.p. mouse). The toxicity of MC-RR is lower compared with MC-LR, and its LD_{50} is 200–350 $\mu g\ kg^{-1}$ (Dawson, 1998; Tarczynska et al., 2001; Gupta et al., 2003; Chen et al., 2006). A recommended guideline value of 1.0 $\mu g\ L^{-1}$ for total MC-LR for safe drinking water has been established by The World Health Organization (WHO). It should be pointed out that MCs are stable against physicochemical and biological factors including temperature, sunlight and enzymes (Tsuji et al., 1995; Harada et al., 1996; Liu et al., 2009). The conventional water treatment processes have been proven to be unreliable for the removal of these toxins. Therefore, elimination of those compounds in water treatment processes merits investigation. Up to now, many methods including advanced oxidation processes (AOPs) have been conducted for the degradation of MCs and the

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results obtained by using ozone (O_3) (Rositano et al., 2001; Brooke et al., 2006; Rodriguez et al., 2007b), chlorine (Acero et al., 2005; Shi et al., 2005; Rodriguez et al., 2007b), permanganate (Rodriguez et al., 2007a) and photolysis in the presence of TiO_2 (Lawton et al., 1999, 2003; Liu et al., 2009) have revealed that MCs were readily oxidized to non-toxic degradation products under appropriate conditions.

Ozone is a strong oxidizing agent and has been used to control taste and odor, eliminate color and inhibit biological growth in water treatment plants (Ozaki et al., 2008). Ozone with its hydroxyl radical ($OH^{\cdot}$) has great potential for oxidation of double bonds and amine moieties in MCs (Onstad et al., 2007). Therefore ozonation may be useful in MCs degradation and detoxification by its high efficiency of direct molecular reaction and indirect radical reaction (Brooke et al., 2006). Moreover, ozonation has several following advantages in drinking water treatment: (1) it is the destructive process with less harmful byproducts formation (comparing to chlorine); (2) it can oxidize contaminants in a short period of time (comparing to UV/TiO_2); and (3) it will not lead to secondary pollution (comparing to permanganate). MCs are typically attacked by O_3 with the following processes of addition, electrophilic substitution, hydrogen abstraction and electron abstraction, which results in a series of byproducts (Rodriguez et al., 2007b).

To further validate the degradation process of MCs, liquid chromatography–mass spectrometry (LC–MS) is applied to analyze the intermediates and end-products from MC-LR and MC-RR ozonation process, and the mechanisms of MCs degradation were elucidated in this study. Protein phosphatase inhibition *in vitro* and mice model *in vivo* were also applied to evaluate the safety of the degraded products and explain the detoxification efficacy of the MCs ozonation process.

2. Materials and methods

2.1. Isolation and purification of MCs

MC-LR and MC-RR were extracted from the natural cyanobacteria of *Microcystis Aeruginosa* (*M. Aeruginosa*) in Tai Lake, China. MCs were purified using the modified method described by Song et al. (2005). Briefly, dried cyanobacteria was suspended in 50% methanol solutions and then sonicated five times for 30 s with an ultrasonic cell disrupter. After ultrasonic disruption, the solution was centrifuged at 9300*g* for 30 min at 4 °C, and the supernatant was collected. The above procedure was repeated for three times. Finally, the supernatants were combined, concentrated to 10 mL, and filtered through 0.45 μm cellulose acetate filter. The samples were referred to as crude microcystin extracts and stored at 4 °C.

A 50 mL of crude microcystin extracts was applied to a 1 g preconditioned Sep-Pak C_{18} cartridge at the flow rate of 5 mL min^{-1}. The loaded cartridge was washed successively with 10 mL of 10% methanol and 10 mL of 20% methanol, the toxin fraction was eluted using 5 mL of 80% methanol at 5 mL min^{-1} and finally evaporated to dryness at 40 °C.

The purifications of MC-LR and MC-RR were performed using high performance liquid chromatography (HPLC) system equipped with a Sepax GP-C_{18} semi-preparative reverse-phase (100 mm × 10 mm I.D.) column (Lawton and Edwards, 2001). The mobile phase was a mixture of 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN) (65%:35%) at a flow rate of 3 mL min^{-1}. The wavelength of the UV absorbance detector was 238 nm. The MC-LR with 96.4% purity and MC-RR with 97.2% purity were obtained after purification, and a 50 mg L^{-1} stock solution of each were prepared for experiments.

2.2. Ozonation experiments

An O_3 stock solution was obtained by bubbling O_3 gas through Milli-Q purified water maintained at 25 ± 1 °C to maximize O_3 dissolution. The concentration of O_3 in stock solution was determined by the indigo colorimetric method (Bader and Hoigné, 1981). Ozonation was started by injecting the O_3 stock solution through a Teflon septum into the MCs samples with a gas tight syringe, and shaking the reaction vessel vigorously for 60 s. The samples were taken for analysis when the reaction was quenched by aeration with nitrogen (N_2). Temperature of the reaction was kept at 25 ± 1 °C.

2.3. Analytical methods

The obtained samples were analyzed for MCs and reaction intermediates with LC–MS. The LC–MS system equipped with a Waters Alliance 2690 HPLC pump connected with a photodiode array detector (Waters 996), Sunfire C_{18} column (150 mm × 2.1 mm I.D.), and ZMD 4000 mass spectrometer with electrospray ionization source. The mobile phase was a mixture of 0.1% TFA and ACN (65%:35%) at a flow rate of 0.3 mL min^{-1}. Chromatograms were analyzed and integrated at 238 nm. The mass spectra data were obtained in the positive ion mode by full scanning from *m/z* 100 to 1200. Masslynx software workstation was used for the LC–MS instrument control, data acquisition and data processing.

2.4. Toxicity evaluation in vitro (protein phosphatase inhibition method)

Toxicity of samples *in vitro* was evaluated by inhibition assay of protein phosphatase activity with previously described colorimetric procedures (Heresztyn and Nicholson, 2001; Ortea et al., 2004). PP-1 was diluted with buffer containing 50 mM Tris–HCl, 1.0 g L^{-1} BSA, 1.0 mM $MnCl_2$ and 20 mM dithiothreitol, pH 7.4. p-Nitrophenyl phosphate (5 mM) was prepared in buffer containing 50 mM Tris–HCl, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5 g L^{-1} BSA, pH 8.1. All buffers were freshly prepared before use. The solution used for the PP-1 inhibition activity studies were diluted 1000 times with Milli-Q water from those by HPLC.

Ten microlitres of each diluted solution was added to a well on the 96 microplate. After gentle shaking for a few seconds, the plate was kept in room temperature for 5 min followed by addition of 200 μL p-nitrophenyl phosphate solution (substrate). The plate was incubated at 37 °C during the reaction. The rate of p-nitrophenyl production was measured at 4 min intervals for 60 min at 405 nm on a Spectramax microplate reader. A dose dependent kinetic activity for PP-1 against substrate was established to assess the enzyme activity prior to sample test. All enzyme assays were performed in triplicate.

2.5. Toxicity evaluation in vivo (mouse bioassay method)

Toxicity of untreated and ozone-treated MCs *in vivo* was determined using 25–30 g male Kunming (KM) mice, purchased from Slac Laboratory Animal Co., China. The dose administrated in this study were at 25 μg kg^{-1} of MC-LR and 140 μg kg^{-1} of MC-RR, which were about half the reported LD_{50} of each MCs, respectively. The equal volumes (1 mL) of samples were injected intraperitoneal (i.p.). All mice were sacrificed after 4 h post-injection. Blood were collected from retro-orbital plexus of mice at mean time to death (MTD). Serum harvested from each mouse were used to determine the indicators of hepatotoxicity including lactate dehydrogenase (LDH), alanine amino transferase (ALT) and aminotransaminase (AST) by using a automatic biochemical analyzer (Hitachi 7170, Japan) (Fawell et al., 1999; Rao et al., 2004).

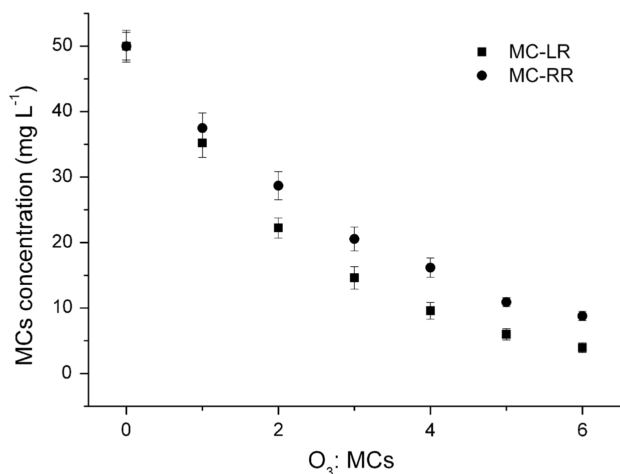

Fig. 1. Ozonation of microcystins (MC-LR and MC-RR) under different ozone dosages (temperature = 25 ± 1 °C, pH 7 and reaction time 60 s).

3. Results and discussion

3.1. Ozonation of MC-LR and MC-RR

Ozone has a standard oxidation potential of 2.07 V under acidic conditions, and is considered as one of the most powerful oxidants for water treatment (Shawwa and Smith, 2001). Herein the concentration of MC-LR or MC-RR solution for oxidation was 50 mg L^{-1}, and the molar ratio of O_3:MCs ranged from 1 to 6. Fig. 1 illustrated the degradation of MC-LR and MC-RR treated with different dosages of O_3 for 60 s. From Fig. 1, the dosage of O_3 influenced significantly the removal efficiency of MCs. The MC-LR removal rate increased from 29.5% to 92.1% with the increase of O_3 molar ratio from 1 to 6. Similarly, the MC-RR removal rate increased from 25.0% to 82.4% at the same conditions. However, under the same condition the degradation of MC-LR was slightly higher than that of MC-RR. Commonly, O_3 and its radical attack could be attributed to high reactivity towards unsaturated bonds (Sekusak et al., 1998; Ljubic and Sabljic, 2002). The conjugated double bond in the Adda group in MCs would be prone to such an attack (Brooke et al., 2006). Hence, MC-LR and MC-RR show similar susceptibility to ozonation at the Adda conjugated position since they both had the Adda moiety. The relative small differences of degradation rates between these two toxins can be contributed to their space steric effect. MC-RR with the larger Arg group hindered the O_3 attack leading to decrease of reactivity.

It should be pointed out, that the investigation about ozonation of MCs was conducted under much higher O_3 dosages in previous research (Al Momani et al., 2008). In the present study, we applied a much higher MCs concentrations and lower O_3 dosages during reaction for the better elucidation of reaction pathways. Rapid degradation of high concentrations of MC-LR and MC-RR were

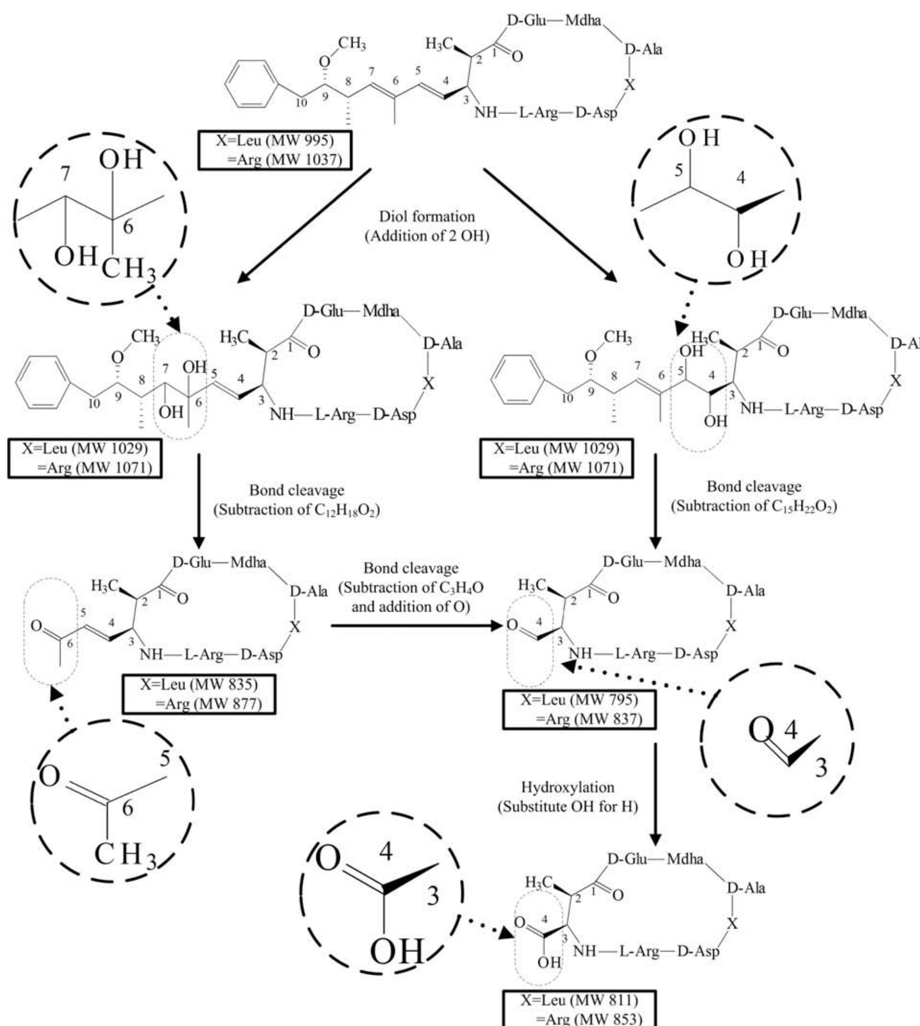

Fig. 2. Byproducts and proposed reaction pathways for MC-LR (X = Leu) and MC-RR (X = Arg) degradation by ozone on the Adda side chain.

achieved upon ozonation with 30–90% of the substrates being destroyed at the O_3:MCs molar ratio of 1–6 as showed in Fig. 1.

3.2. Analysis of ozonation intermediates from MCs

Ozonation with its direct and indirect reaction pathways could attack the organic compounds with high efficiency and bring out a series of byproducts. This process typically leads to hydroxyl radical and hydrolytic degradation with a series of mechanisms such as addition, electrophilic substitution, hydrogen abstraction and electron abstraction (Guesten et al., 1995; Sabljic and Peijnenburg, 2001). In the present study, several intermediate products of MCs during ozonation process were observed using LC–MS analysis. Their structural assignments were based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum because of the high sensitivity of the mass spectrum. For the purpose of this paper, we will refer to the products by molecular weight (MW). MC-LR with MW of 995 was detected as the mass spectrum showing a molecular $(M+H)^+$ ion at *m/z* 996, and MC-RR with MW of 1037 was detected as the mass spectrum showing a molecular $(M+2H)^{2+}$ ion at *m/z* 520.

3.2.1. Adda degradation pathway

Structure assignment of intermediate products of MC-LR in ozonation was based on the analysis of the LC–MS and correspondent mass spectrum of products. First, MC-LR in the positive ionization mode was easily substituted with two hydroxyl groups (MW $995 + 2 \times 17 = 1029$) in the Adda side chain. Products with MW of 1029 showing a molecular $(M+H)^+$ ion at *m/z* 1030 were due to the O_3 molecular and/or $OH^·$ attack at either the C_4–C_5 or C_6–C_7 double bond pairs on the conjugated diene structure system in the Adda side chain resulting in hydroxylation of MC-LR (1,2 addition). According to the photocatalytic oxidation results of MC-LR proposed by Liu et al. (2003), the products with MW of 1029 resulted from the hydroxylation of MC-LR by $OH^·$ attack. Second, dihydroxy-MC-LR was further oxidized with cleavage of the dihydroxylated bond at positions C_4 or C_6 by electrophilic attack. The process was verified by the detecting intermediates of ketone-MC-LR with $(M+H)^+$ ions at *m/z* 796 and 836. The formation of these products depended on the location of dihydroxy substitutes in the precursors. When the hydroxyl groups were located at C_4–C_5 positions, the resulting product would have an $(M+H)^+$ ion at *m/z* 796 (MW $1029 - 206 - 18 = 795$). Likewise if the hydroxyl groups were located at C_6–C_7 positions, the product with $(M+H)^+$ ion at *m/z* 836 (MW $1029 - 176 - 18 = 835$) would be obtained. The corresponding cleavage product with MW of 835 could subsequently transform to the product with MW of 795 by further dihydroxylation of the C_4–C_5 double bond, followed by bond cleavage at C_4–C_5 position ($835 - 56 + 16 = 795$). Finally, the observed $(M+H)^+$ ion at *m/z* 812 (MW $795 + 17 - 1 = 811$) was consistent with a carboxylic acid structure resulting from further oxidation of the product with MW of 795. The addition of 16 mass unit

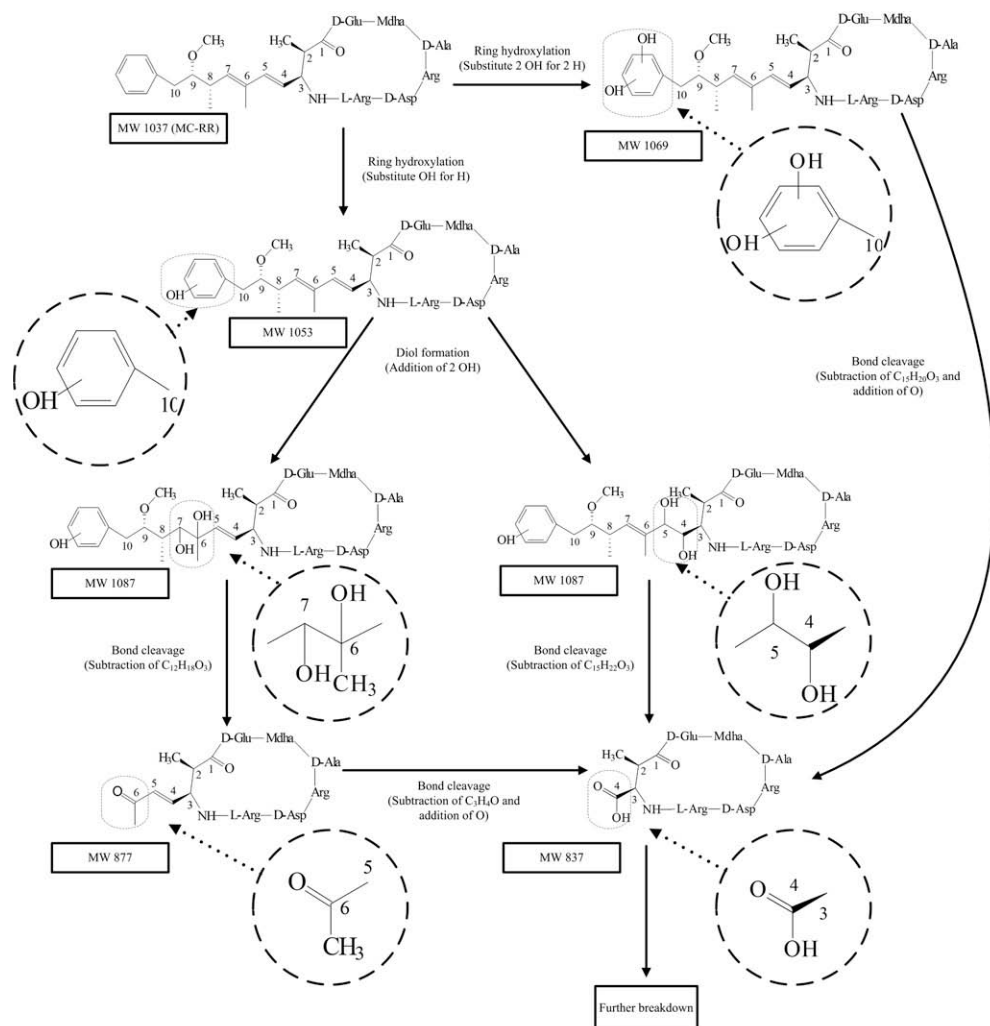

Fig. 3. Ring hydroxylation pathways for MC-RR degradation by ozone on the Adda side chain.

was consistent with hydroxylation of C=O bond at C_4 position. A summary of the proposed reaction pathways involving Adda was illustrated in Fig. 2.

The intermediate products of MC-RR were also monitored by LC–MS as a function of O_3 dosage. The reaction profile for MC-RR paralleled those observed for MC-LR, with diol formation, diene cleavage and carboxylic acid production. Products from hydroxylation and bond breakdown with MW of 1071 ($1037 + 2 \times 17 = 1071$), 877 ($1071 - 176 - 18 = 877$), and 837 ($1071 - 206 - 18 = 837$) were observed during ozonation, while the $(M+2H)^{2+}$ ions at *m/z* 536, 440, and 420. The end-product of Adda degradation pathway with MW of 853 ($837 + 17 - 1 = 853$) was evidenced by the $(M+2H)^{2+}$ ion at *m/z* 428. The MC-LR and MC-RR variants are proposed to have analogous product distribution and degradation pathway in Adda side chain under ozonation, as showed in Fig. 2.

Interestingly, the intermediate products from MC-RR degradation revealed that the ring hydroxylation led to phenol group formation (Fig. 3). Commonly, the necessary mechanistic steps for benzene ozonation include the addition of $OH^{\cdot}$ to one of the aromatic double bonds and formation of a carbon-centered radical, which rapidly reacts with oxygen, to form a peroxy radical. The release of a perhydroxyl radical results eventually in the substitution of the hydrogen with a hydroxyl group (Tahmasseb et al., 2002). The substitution of the hydroxyl group to the phenyl ring increases the electron rich character making it more reactive toward the addition of a second $OH^{\cdot}$. The intermediate products with the MW of 1053 ($1037 + 17 - 1 = 1053$) and 1069 ($1037 + 2 \times 17 - 2 = 1069$) were due to the addition of the 1 and 2 oxygen on the phenyl group to yield the phenol. The results were evidenced by the $(M+2H)^{2+}$ ions at *m/z* 528 and 536. The phenomenon was also observed when MCs were degraded with the ultrasonic process by Song et al. (2006). These intermediates were considered to be further oxidized to diol and breakdown according to Adda pathway shown in Fig. 3. However, the phenol formation was exclusively taking place in the degradation of MC-RR. The intermediates from MC-LR with high reaction activity like phenol-MC-LR could be quickly oxidized and further induced the bond cleavage, which resulted in fewer intermediate products detected by LC–MS.

3.2.2. Mdha degradation pathway

Reaction leading to opening of the MCs peptide ring has been proposed and analogous products observed during ozonation were summarized in Fig. 4. Hindering effects caused from the functional groups of the other amino acids made degradation of cyclic structure less, and the unsaturated bond of the Mdha amino acid was the only observed active site of ozonation in the cyclic ring of MCs in the present study. Intermediates with MW of 943 ($(M+Na)^+$ ion at *m/z* 966) might be due to peptide bond ($N1–N_2$) cleavage between Mdha and Ala on the MC-LR peptide ring structure ($995 - 54 + 2 = 943$), while 985 ($(M+H)^+$ ion at *m/z* 986) was the most probable analogous product from MC-RR ($1037 - 54 + 2 = 985$). In present study, product from the co-degradation of Adda and Mdha was also observed. Products with MW of 743 ($(M+H)^+$ ion at *m/z* 744) were responsible for the cleavage on both Adda side chain and Mdha of MC-LR, while 785 for MC-RR ($(M+H)^+$ ion at *m/z* 786). The pathway of opening peptide ring was reported as the subsidiary one in the MCs degradation (Lawton et al., 1999; Liu et al., 2003), hence the byproducts from this pathway were much less abundant than from Adda cleavage.

The ozonation products of MC-LR and MC-RR were semi-quantitatively monitored using LC–MS as a function of O_3 dosage. Since the products have similar structure, we assume that they have the similar response factors (peak intensity/molecular); so, the peak intensities are indicative of the relative yields. Results showed that degradation on Adda side chain of MCs would be the predominant pathway, illustrated in Fig. 5. The assumption is supported by the observation that the majority of the byproducts were those with MW of 795, 835 and 1029 in MC-LR ozonation. The products with MW of 837, 877, 1071 and 1053 are also the major byproducts

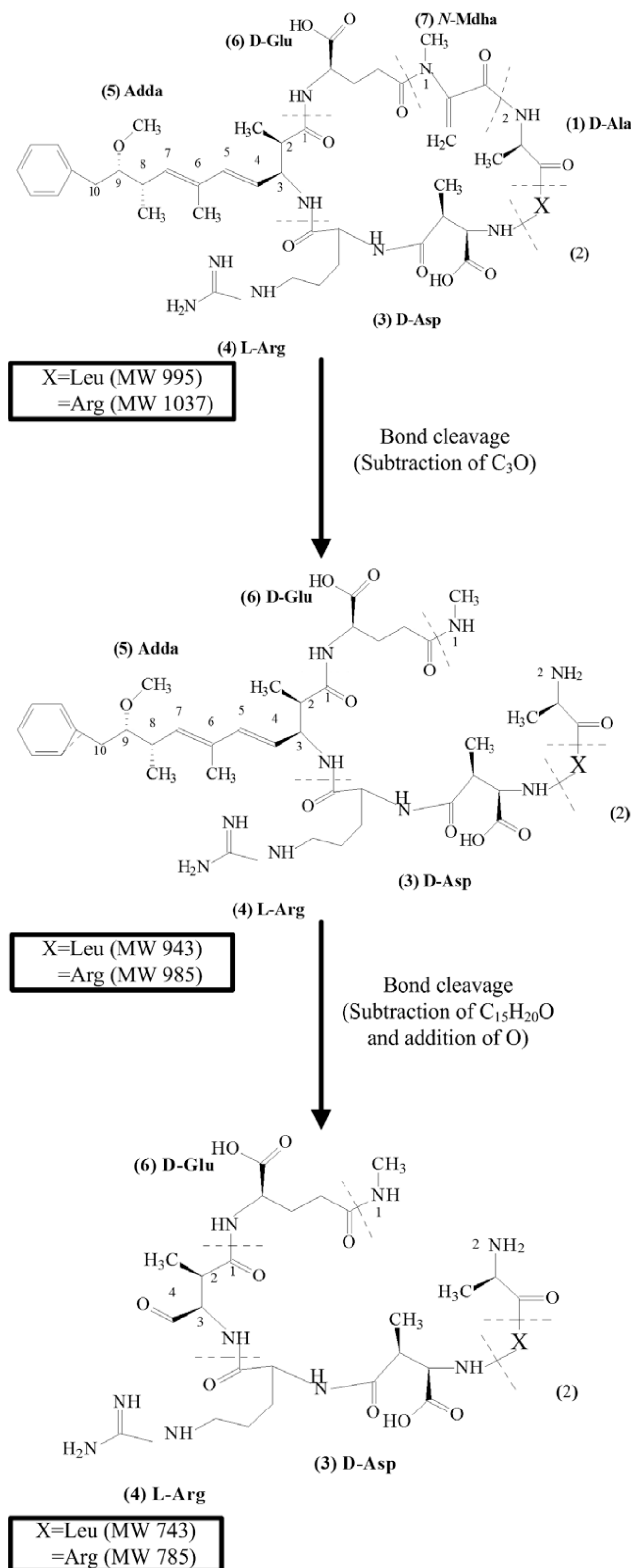

Fig. 4. Byproducts from the MC-LR (X = Leu) and MC-RR (X = Arg) degradation by ozone on peptide ring.

from MC-RR ozonation. Most of the products reached a maximum concentration when O_3:MCs molar ratio was 4, and these products are readily degraded upon continued ozonation.

3.3. Toxicity evaluation of degraded products from MCs in vitro and in vivo

The ozonation process was demonstrated to degrade the MCs in the solution and result in a series of intermediates. These products could be classified in three structural groups: (1) ring peptides with an Adda moiety form hydroxylation of MCs in Adda side chain; (2) linearized peptides with an Adda group from the cleavage between Mdha and Ala in MCs; and (3) rings or linearized peptides without the Adda moiety. The conjugated diene structure Adda in MCs has been reported to be essential for inhibition of PP-1 and PP-2A. Hence the peptide residues without an Adda moiety produced from MCs possibly had no function of protein phosphatase inhibition (Gulledge et al., 2003). Although the MCs degraded products had the Adda side chain after the modification such as hydroxylation, they were proved to eliminate toxicity (Taylor et al., 1996; Merel et al., 2009). Similar results were also obtained in our toxicity evaluation of MCs degraded products *in vitro*. Fig. 6 showed the comparative detection of MCs by HPLC and PP-1 methods under different O_3 dosages. In the present study, the toxicity of ozonation byproducts could be calculate as the sum of PP-1 inhibition subtracting that of HPLC, which was less than 5.4% of MCs in the equivalent volume. According to the MCs concentrations and taking into account the error band the data from PP-1 inhibition were almost equivalent to those from HPLC. Therefore, the results indicated that the byproducts did not exhibit any appreciable inhibition of PP-1.

The hepatotoxicity of MCs and their degraded products (at ozone dosage of 6) was further investigated *in vivo*. Serum hepatic marker enzymes like ALT, AST and LDH were consider indicative of hepatocellular damage previously observed in acute and chronic toxicity studies. As could be seen in Table 1, compared to control groups, MC-LR induced an increase of 16.1 times in ALT, 3.24 times in AST and 3.96 times in LDH, respectively. MC-RR had a relative small increase of 4.18 times in ALT, 2.11 times in AST and 1.73 times in LDH, respectively. The serum hepatic enzymes levels by degraded products were much lower than those in MCs group, but a little higher than those in the control group. The toxicity might come from the residual MCs which were not completely de-

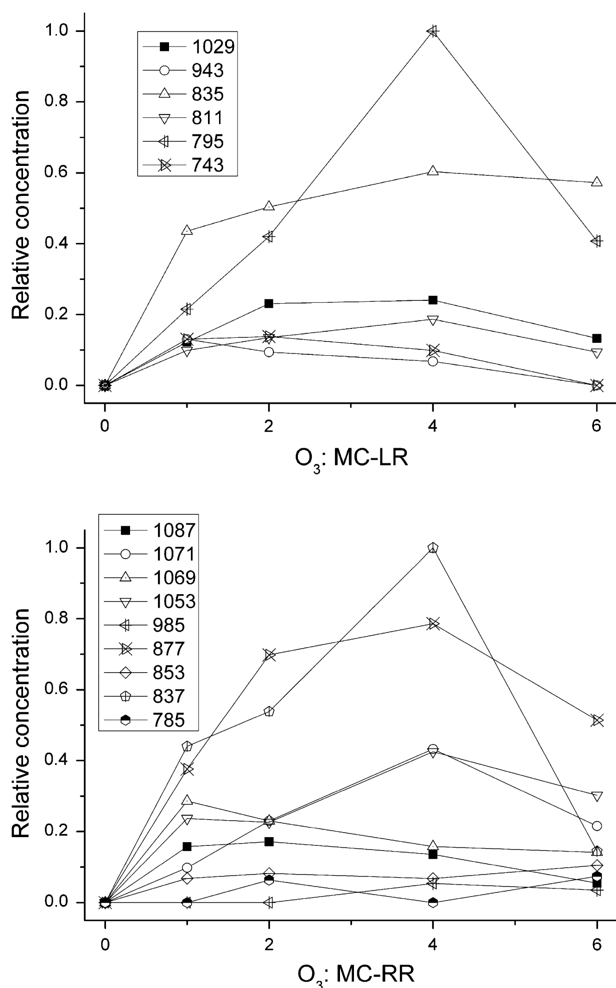

Fig. 5. Reaction profile of byproducts (list by MW) from degradation of MC-LR and MC-RR by ozone.

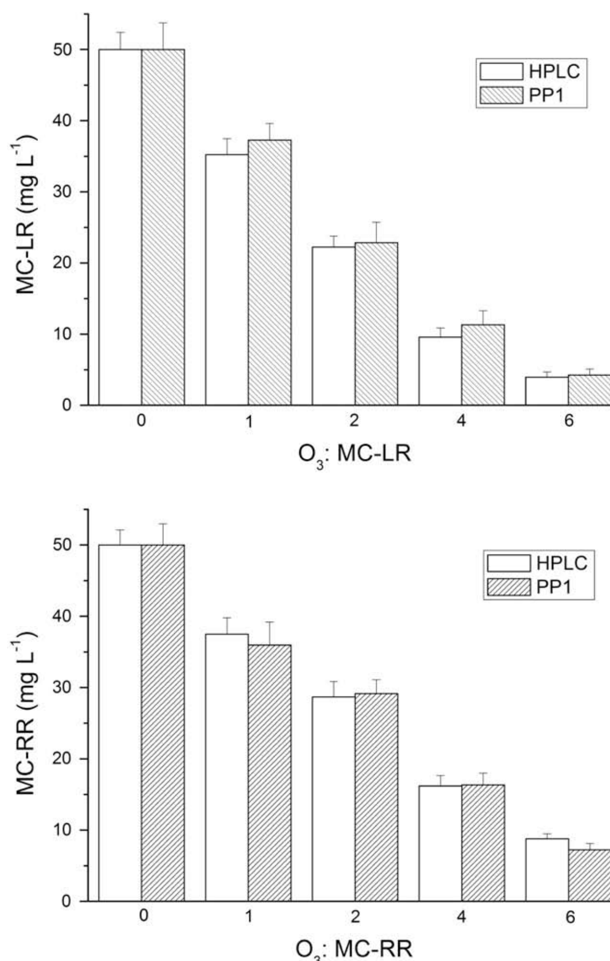

Fig. 6. Comparative results from degradation of MC-LR and MC-RR by using HPLC and PP-1 bioassay.

Table 1
Effect of ozonation on the toxicity of MC-LR and -RR, determined by mouse bioassay.

Serum enzymes ($UI\ L^{-1}$)	Control	MC-LR		MC-RR	
		Untreated	Treated	Untreated	Treated
ALT	32.2 ± 5.9	529 ± 26	56.2 ± 4.7	235 ± 9.7	50.8 ± 6. 2
AST	75.0 ± 5.2	243 ± 17	137 ± 12	158 ± 7.5	119 ± 20
LDH	402 ± 31	1590 ± 52	495 ± 15	696 ± 45	525 ± 63

graded at ozone dosage of 6 in the solution. Hence, the results revealed that ozonation treatment of MCs could efficiently reduce hepatotoxicity in mice.

4. Conclusions

The decomposition of MCs was observed under different O_3 dosages. Results showed that ozonation effectively degraded MC-LR and MC-RR. The process involved with substitution and cleavage of the Adda conjugated diene structure, bond breaking of the peptide between Mdha and Ala, and attack at the Adda benzene ring. Adda degradation seemed to be the major destruction pathway of both MC-LR and MC-RR. The degradation rate of MC-RR was somewhat lower than that of MC-LR, which gave more byproducts from Adda side chain cleavage. A molar ratio of O_3:MCs 6 was recommended, which led to MC-LR degradation rate of 92.1% and MC-RR degradation rate of 82.4%, respectively.

Toxicity evaluation results of the end-products revealed that the hepatotoxicity of MCs expressed as inhibition of PP-1 and damage in mouse liver was greatly reduced or eliminated by the ozonation process. Our results provided evidence that ozonation in the aqueous environment could effectively reduce of MCs' toxicity and could be regarded as feasible option for removal of dissolved MCs.

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