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Isolation and identification of seven microcystins from a cultured *M*.TN-2 strain of *Microcystis aeruginosa*

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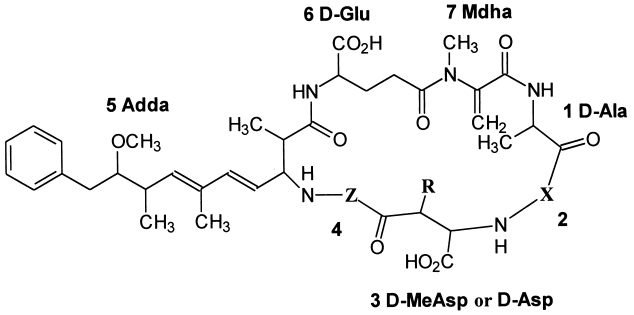
Abstract. Seven microcystins (MCYSTs), three major and four minor ones, were isolated from a cultured *Microcystis aeruginosa* strain *M*.TN-2 isolated from an eel pond in southern Taiwan in the summer of 1993. Microcystins were separated by a column separation of LH-20 gel filtration chromatography, a subsequent silica gel flash column chromatography, and then purified by a reversed phase high performance liquid chromatography. MCYST-LR, -RR, -RA, -FR, -WR, [D-Asp^3] MCYST-FR, and [D-Asp^3] MCYST-WR were characterized and determined by amino acid analysis, MALDI-TOF mass spectrometry, ^{1}H and DQF-COSY nuclear magnetic resonance spectroscopy and UV spectroscopy. Among them, [D-Asp^3] MCYST-FR and [D-Asp^3] MCYST-WR, two new compounds, were the desmethyl variants of MCYST-FR and MCYST-WR, respectively.

Keywords: Cyanobacteria; Hepatotoxin; Microcystin; *Microcystis aeruginosa*.

Introduction

Toxic cyanobacteria have caused fatal poisoning of mammals, birds, and fish (Codd and Poon, 1988; Carmichael, 1992; Kotak et al., 1996; Tencalla and Dietrich, 1997), and may also have exerted adverse effects on human health (Gorham and Carmichael, 1988; Carmichael, 1994, 1997). Microcystins, a vast group of cyanobacterial toxins, are known to be hepatotoxic cyclic heptapeptides produced by some species within the genera of *Microcystis* (Shirai et al., 1991; Namikoshi et al., 1992; Luukkainen et al., 1994; Lee et al., 1998), *Oscillotoria* (Meriluoto et al., 1989; Luukkainen et al., 1993), *Nostoc* (Namikoshi et al., 1990; Sivonen et al., 1990; Beattie et al., 1998), and *Anabaena* (Harada et al., 1991a; Sivonen et al., 1992; Namikoshi et al., 1998). Toxic *Microcystis* species are widely distributed, mainly in the fresh water world, and form blooms frequently. The general structure of microcystins is composed of (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), γ-linked D-glutamic acid (D-Glu), *N*-methyldehydroalanine (Mdha), and D-alanine (D-Ala). Four special amino acids are linked in sequential order followed by two variable L-amino acids with D-*erythro*-β-methylaspartic acid (D-MeAsp) inserted between them, forming a cyclic structure (Figure 1). There have been more than 60 different components isolated and determined. These compounds differ primarily in their L-amino acids at the number 2 and number 4 position of the peptide ring skeleton, and in the presence or absence of the methyl group on D-MeAsp and/or Mdha of the 3rd and 7th amino acid, respectively (Namikoshi et al., 1992). L-amino acids in microcystins at the 2nd and 4th position are known to be arginine, leucine, tyrosine, phenylalanine, tryptophan and others. Among the identified microcystins, more than 70% of the structures contained L-Arg at the 4th position. Among these 7 cyclic-linked amino acids in microcystins, Adda has been thought of



	microcystin	X	Z	R
1	MCYST-LR	Leu	Arg	CH_3
2	MCYST-FR	Phe	Arg	CH_3
3	MCYST-WR	Trp	Arg	CH_3
4	[D-Asp^3] MCYST-FR	Phe	Arg	H
5	[D-Asp^3] MCYST-WR	Trp	Arg	H
6	MCYST-RA	Arg	Ala	CH_3
7	MCYST-RR	Arg	Arg	CH_3

Figure 1. Structures of microcystins identified in this research.

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as a key structural component for their biological activities (Dahlem, 1989; Harada et al., 1990a, b; Carmichael, 1992; Saito et al., 1994). Inhibitory activity on the protein phosphatase 1 and 2A of microcystins was generally recognized as the mechanism by which microcystins exerted their hepatotoxicity (Nishiwaki-Matsushima et al., 1992). As a result of these enzyme inhibitions in liver, hepatocyte shrinks and liver tissue dissociates, followed by a deadly internal hemorrhage (Runnegar and Falconer, 1986). Bury et al. (1996) and Zambrano and Canelo (1996) reported the fish mass mortality in the *Microcystis* blooming was due to the toxic effect of microcystins on the disruption of ion homeostasis of the internal medium in fish by blockage of gill function.

In this paper, we report the isolation and identification of seven different microcystins from cultured *M. aeruginosa* strain *M.*TN-2 isolated from an eel pond in southern Taiwan.

Materials and Methods

*Culture of Microcystis aeruginosa M.*TN-2

Microcystis aeruginosa strain *M.*TN-2 was isolated from an eel pond at Duujia in Tainan County in Taiwan, July 1993. This strain was identified according to description of the species in the *Plankton Algae of Reservoirs in Taiwan* (Moriwaka and Chyi, 1996) and in Mizuno (1980). Its toxicity was determined by mouse assay (Lee et al., 1998). The cyanobacteria culture was maintained in modified Fitzgerald media (Hughes et al., 1958) and kept at 23±1°C with an illumination of 26.4 $\mu Ein \bullet m^{-2}s^{-1}$, 12 h by fluorescent light bulbs. Cells of *M.*TN-2 were harvested in their late exponential phase and concentrated by continuous centrifugation, followed by lyophilization before extraction and isolation of microcystins, and high performance liquid chromatography (HPLC) analysis.

Microcystin Extraction and Purification

Dried cells (50 g) were extracted three times with 400 ml methanol for 30 min with sufficient mixing by a magnetic stirrer. The methanol extract was adjusted to 85% of the aqueous solution for hexane partition. The lower layer of the polar solution was then vacuum-evaporated to dryness (3.0 g). For each batch of chromatographic separations, 200 mg dried extract in 10 ml of methanol was applied.

The first separation was carried out by a Sephadex LH-20 column (3 × 55 cm, Pharmacia Biotech., Sweden) of gel filtration chromatography and eluted by MeOH with a flow rate of 13 ml/min. Fractions collected were checked for their compositions by thin layer chromatography (TLC) using Silica gel 60 F_{254} (Merck, Germany) plate (200 μm thickness) and ethyl acetate: isopropanol: H_2O (8:5:3, v/v/v) for development. Contents of the toxic fractions were compared with an authentic sample of MCYST-LR (Sigma, USA). Vanillin-sulfuric acid charring and dark-box observation of UV absorption were applied in the detection of microcystins. Subsequently, toxin fractions from above separation were combined and chromatographed on a 3 cm i.d. flash column, packed with 45 g silica gel for flash column (Baker, USA) and eluted with the same solution as the developer used in TLC, flow rate 36 ml/min. Toxic fractions were pooled into two major partitions, P-I (70 mg) and P-II (10 mg), according to their composition as determined by TLC analysis. Both partitions were subjected for further separation by reversed phase HPLC. P-I was first separated by a semi-preparative C18 column (10 × 250 mm, Vydac, USA) using 0.01 M ammonium acetate: acetonitrile (76:24, v/v) or 0.01 M ammonium acetate: methanol (50:50, v/v) as eluent to give semi-purified fractions. All these fractions were further purified using 0.05% aqueous trifluoroacetic acid: acetonitrile (62:38, v/v) as eluent on the same column to yield pure components: ***1*** (0.5 mg), ***2*** (2.5 mg), ***3*** (2.2 mg), ***4*** (0.3 mg), ***5*** (0.3 mg), and ***6*** (0.8 mg). From P-II, compound *7* (2.0 mg) was purified by elution of 0.01 M ammonium acetate: acetonitrile (78:22, v/v) on an Alltima C18 column (4.6 × 250 mm, Alltech, USA). All purified compounds were vacuum-dried and stored at -20°C before structural analysis.

Structural Elucidation

Structures of the compounds were elucidated by comparing their DQF-COSY nuclear magnetic resonance (NMR) spectra with that of MCYST-LR. The identification of MCYST-LR ***1***, isolated in this study was also confirmed by comparing its chromatographic characteristics with those of authentic MCYST-LR. NMR spectra of compound ***1***, ***6***, and *7* were measured on an FT-NMR spectrometer (AM-400, Bruker, Germany) using methanol-d_4 as solvent and internal standard (δ3.3), while the spectra of compounds ***2***, ***3***, ***4***, and ***5*** were acquired on a Bruker DMX-500 SB FT-NMR spectrometer. Molecular weight of each toxin was determined by G2025A matrix-assisted desorption and ionization-time of flight mass spectrometry (MALDI-TOF/MS, Hewlett-Packard, USA) using α-cyano-4-hydroxycinnamic acid (α-CHC) as the matrix. UV spectra of compounds ***1*** to *7* were measured on a U-2000 Spectrophotometer (Hitachi, Japan).

Amino Acid Analysis

Each toxin component was hydrolyzed by means of gas-phase hydrolysis in 500 μl of 7 M HCl with 10% trifluoroacetic acid containing 0.1% phenol at 158°C for 30 min (Chang and Liu, 1988). The released amino acids were derivatized with 4-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl) to form colored DABS-amino acids, prior to HPLC analysis. HPLC separation of amino acids was performed on an Alltima C18 column (4.6 × 250 mm, Alltech, USA), eluted initially with a gradient of 15 to 40% of acetonitrile in 35 mM sodium acetate solution containing 4% *N', N*-dimethylformamide for 20 min, then eluted with a gradient of 40% to 70% for 12 min, and maintained at 70% for 2 min at a 1 ml/min flow rate. The DABS-derivatives of amino acids were detected at 436 nm for absorption.

Chiral Analysis of the Constituent Amino Acids

Configuration analysis of the amino acids of the acid hydrolysate was performed using a PFP-IPA amino acid derivatization kit (Alltech, USA), and the derivatives were analyzed using a gas chromatography/mass detector (GC/MS) system (5890 series II, Hewlett-Packard, USA). The amino acids derivatives were injected into a Heliflex Chirasil-Val glass capillary column (0.25 mm × 25 m, Alltech, USA) and eluted with helium (0.6 ml/min) for 4 min at 90°C, followed by a linear gradient of 90°C to 210°C for 30 min.

Results

*Structure Elucidation of Compound **1**~**7***

The structures of compounds ***1*** to ***7*** isolated from cultured *M*.TN-2 strain are shown in Figure 1. Compounds ***1***, ***6***, and ***7*** were identified as MCYST-LR, -RA, and -RR, respectively according to their DQF-COSY NMR and FAB/MS data assignments, which were repforted previously (Lee et al., 1998) and will not be described in detail here. However, before the compounds ***2*** to ***5*** could be elucidated, proton chemical shifts of MCYST-LR had to be fully assigned according to the homonuclear and heteronuclear correlation of proton and carbon-13 nuclei on this compound through extensive TOCSY, HMQC, and HMBC experiments of modern NMR spectroscopy. Structure of the newly isolated microcystins were then deduced from such an established database of NMR signals, including chemical shifts and splitting patterns of the authentics. In addition, the composition and stereochemistry of the resolved amino acids in these microcystins, based on high resolution liquid chromatographic and gas chromtographic separation of the acid hydrolysate of purified toxins, were further evidence in support of their structure determination.

Compounds ***2*** to ***5*** were collected from several batches of separation until they were sufficient in quantity for NMR experiments. NMR data of each compound from DQF-COSY experiments confirmed the presence of amino acid residues revealed in their amino acid analysis. It was noticed that the resonance levels of each H-2 in the MeAsp, Adda, and Glu moieties of compounds ***2***~***5*** were slightly shifted to the levels of the corresponding protons in MCYST-LR measured in an earlier ^{1}H NMR experiment (with the residual ammonium effect). A similar result was obtained in MCYST-LR when the compound was free from the contamination of ammonium acetate (Table 1).

Amino acid analysis of compound ***2*** revealed a composition of MeAsp, Glu, Ala, Arg, and Phe (Table 2), of which MeAsp, Glu, and Ala were determined as the D-amino acids according to the chiral separation of compound ***2*** acid hydrolysate by GC/MS. Adda and Mdha, the existence of which cannot be resolved from the amino acid analysis, were confirmed by comparing the NMR data with the previous peak assignment of authentic MCYST-LR. We concluded that compound ***2*** is MCYST-FR. The molecule ion peak of compound ***2*** was recorded at 1029 by MALDI/TOF mass spectrometer, which matched that of MCYST-FR as reported by Namikoshi et al. (1992).

Amino acid analysis of compound ***3*** gave only four amino acids, a phenylalanine less than compound ***2***, even with a molecular weight of 1068, higher than MCYST-FR from the MALDI-TOF/MS analysis (Table 2). ^{1}H NMR data also showed the presence of Adda and Mdha in compound ***3***, just as in compound ***2***. In addition, ^{1}H NMR peaks of δ4.45 (H-2), δ3.29 (H_2-3), δ7.30 (H-5), δ7.63 (H-7), δ6.99 (H-8), δ7.06 (H-9), and δ7.31 (H-10) of the compound suggested the presence of tryptophan molecule, which was supported by the absorption maximum (λ_{max}) at 223 nm and the molecular weight of the compound. Moollan et al.

Table 1. Chemical shift differences (Δδ > 0.1) of some specific protons in D-MeAsp[3], Adda[5], and D-Glu[6] moieties of MCYST-LR caused by the existence of residual ammonium acetate in methanol-d_4 while taking a NMR measurement at 500 Hz.

	Proton location		Solvent MeOH-d_4[a]
Δδ	[D-MeAsp[3]]	-H-2	-0.26
	[Adda[5]]	-H-2	+0.35
	[D-Glu[6]]	-H-2	-0.25
	[D-Glu[6]]	-H-4a	-0.12

[a]With residual CH_3COONH_4.

Table 2. Stereochemistry of the amino acids in different microcystins (denoted as compounds ***1***~***7*** accordingly) listed with the protonated molecular ions and λmax from MALDI-TOF mass and UV spectroscopic measurements.

	Microcystins	Amino acid analysis[a]					M+H$^+$ (*m/z*)	λmax (nm)
1	MCYST-LR	D-MeAsp	D-Glu	D-Ala	Arg	L-Leu	995	238
2	MCYST-FR	D-MeAsp	D-Glu	D-Ala	Arg	L-Phe	1029	238
3	MCYST-WR	D-MeAsp	D-Glu	D-Ala	Arg		1068	223
4	[D-Asp[3]] MCYST-FR	D-Asp	D-Glu	D-Ala	Arg	L-Phe	1015	238
5	[D-Asp[3]] MCYST-WR	D-Asp	D-Glu	D-Ala	Arg		1054	223
6	MCYST-RA	D-MeAsp	D-Glu	D-Ala	Arg	L-Ala	953	238
7	MCYST-RR	D-MeAsp	D-Glu	D-Ala	Arg	Arg	1038	238

[a]Amino acid composition and stereochemistry were determined by the high performance liquid and gas chromatographic separation on the acid hydrolysate of microcystins. Stereochemistry of arginine moiety in microcystins could not be assigned by the method in this research.

(1996) have reported the tryptophan chromophore in microcystin causes a shift from 238 nm, a typical MCYST λ_{max}, to 222 nm. Compound ***3*** was thus assigned as MCYST-WR.

Compound ***4*** had a molecular weight of 1015, 14 daltons (CH_2) less than that of MCYST-FR. Amino acid analysis of compound ***4*** showed the same composition as MCYST-FR from both LC and GC studies except its D-MeAsp was replaced by D-Asp (Table 2). Due to the small quantity of sample available and the embedded signals, peaks of H-2 and H-3 of D-Asp could not be clearly identified in its ^{1}H NMR spectrum. However, all other proton signals from Ala, Phe, Arg, Adda, Glu, and Mdha amino acid moieties of compound ***4*** were able to be assigned completely and be in consistency with those of MCYST-FR. In addition, missing of the significant H_3-5 signal in the ^{1}H NMR spectrum and the crossed signal of H-3 and H_3-5 coupling peaks of MeAsp in the DQF-COSY NMR spectrum revealed compound ***4*** [D-Asp^3] MCYST-FR, a desmethyl variant of compound ***2***.

Compound ***5*** showed a UV maximal absorption at 223 nm, the same as MCYST-WR, suggesting a similar presence of tryptophan in the amino acid composition as MCYST-WR of compound ***5***. Molecular weight (1054, 12 daltons less than 1068 of MCYST-WR) from MALDI-TOF/MS and the ^{1}H NMR signals of tryptophan moiety similar to those of MCYST-WR showed in the experiments of compound ***5***, confirming it as a desmethyl variant of MCYST-WR, [D-Asp^3] MCYST-WR.

Discussion

Structures of compounds ***1***~***7*** were mainly determined by their ^{1}H NMR and DQF-COSY NMR data. The elucidated structures were further confirmed by their MALDI-TOF/MS and amino acid analysis. Two-dimensional DQF-COSY NMR spectra gave strong evidence in determining the correlated protons within the molecule of these cyclic heptapeptides. Thus the proton signals within and the nature of each amino acid moiety of the toxin were assigned. The amino acid sequence of the cyclic peptides was speculated to be identical in all the toxins due to the similar proton signals among the respective amino acids in different toxins. Identification of the isolated microcystins was confirmed by comparing the chemical shifts and splitting patterns of peaks with those of the published ones (Namikoshi et al., 1990; Harada et al., 1991b; Namikoshi et al., 1992).

Table 1 showed the difference in chemical shifts of each H-2 proton in the MeAsp, Adda and Glu moieties of MCYST-LR, with and without the residual ammonium effect during its NMR measurement. This is an experiment designed to explain the shifted energy levels of the respective protons in compounds ***2***~***5***, while they were measured for the chemical shifts right after the isolated using ammonium acetate as eluent (Lee et al., 1998). The difference in chemical shifts of certain protons was due to the deprotonation reaction of the residual ammonium ions on the free carboxylic acid of MeAsp and Glu. It was reported that deprotonation of carboxylic acid in Glu and MeAsp moieties occurred when ND_3/D_2O solution was added to the deuteriated solvent in the NMR measurment, and led to the signal shifts of certain protons near the affected functional group (Rudolph-Böhner et al., 1993). In this study we observed an up-field shift of the H-2 proton signals in Glu and MeAsp and a down-field shift of the H-2 proton signal in Adda of the microcystins deprotonated. We speculate that a conformational change may have happened to the molecule due to the coincident shift of the H-2 of Adda while deprotonations were occurring on the free acid groups. The spatial distance of the H-2 of Adda to the nearby carboxylic acid group seems to rule out bond transmission. What kind of conformational change induced the signal shift of the H-2 in Adda due to deprotonation of the carboxylic acid groups needs further study.

Both MCYST-FR and its desmethyl variant [D-Asp^3] MCYST-FR were found in the same culture in this study, as were MCYST-WR and its desmethyl variant [D-Asp^3] MCYST-FR. Although MCYST-WR has been described by Namikoshi et al. (1992), and MCYST-FR by Luukkainen et al. (1994), the [D-Asp^3] MCYST-FR and [D-Asp^3] MCYST-WR found in this study are new, emerging compounds to this vast group of heptapeptides. Ratio of the content of microcystin to its desmethylaspartic acid variant seems to be regulated by the same factors in living cells since they are identical in both MCYST-FR and MCYST-WR pairs. However, this hypothesis needs further confirmation. The question has been raised as to whether the desmethylaspatic acid variant of MCYST-LR or MCYST-RR is produced in the same culture. A careful review of the toxin profile confirmed that desmethylaspartic acid derivatives of MCYST-LR and MCYST-RR were not produced by the cell culture. It is not necessary for the peptide synthetase responsible for the formation of [D-Asp^3] MCYST-FR and [D-Asp^3] MCYST-WR to have the same function on MCYST-LR and MCYST-RR in the same organism, although [D-Asp^3] MCYST-LR and [D-Asp^3] MCYST-RR are quite common in certain strains of *M. aeruginosa* (Rinehart et al., 1994). From the list summarized by Rinehart et al. (1994), we can also find that most of the reported desmethyl replacement in MeAsp and Mdha residue of microcystins are found in cyanobacteria from Finland and Norway, while a small portion of the desmethyl microcystins were in *Microcystis* from Japan. Our report enriched the toxin list studied in Asia.

It was reported that MCYST-LR is the most common component among the known microcystins in the studied cyanobacterial strains all over the world (Carmichael et al., 1988; Watanabe et al., 1991; Namikoshi et al., 1992). We also found that MCYST-RR was also a major component, in addition to MCYST-LR, in most of the *Microcystis* strains isolated in Taiwan (Lee et al., 1999). However, in *M*.TN-2 strain MCYST-LR was found to be a minor component. This was the reason we selected this strain in our search for new toxin molecules.

Recent studies showed a non-ribosomal thio-template mechanism, namely the peptide synthetase, a multifunctional enzyme, was responsible for the production of different microcystins (Arment and Carmichael, 1996), and its genes were identified (Dittmann et al., 1997; Nishizawa et al., 1999). With the help of various *Microcystis* strains that produce diferent microcystins, the biosynthetic regulation of the synthesis of various toxin derivatives will be clarified.

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銅綠微囊藻株 *M*.TN-2 所含七種微囊藻毒的分離與化學結構鑑定

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1993 年夏季單離自臺灣南部鰻魚池銅綠微囊藻華（*Microcystis aeruginosa*）之 *M*.TN-2 藻株，經大量培養後，藻體內所含之微囊藻毒（microcystin, MCYST）經一系列的 LH-20 管柱膠濾層析、矽膠吸附性速分管柱層析及逆相高效液相層析等的分離、純化，共分離出七種不同的微囊藻毒，其結構續經紫外光譜、介質輔助激光去吸附及離子化飛行時間質譜（MALDI-TO/FMS）、^{1}H 和 DQF-COSY 核磁共振波譜的解析，佐以氨基酸組成的高效液相層析與手性（chirality）分離的氣相層析，始確認其結構分別為 MCYST-LR、-RR、-RA、-FR、-WR 及［D-Asp^3］MCYST-FR、-WR，其中［D-Asp^3］MCYST-FR 及 -WR 為過去文獻中所未曾發表的新化合物。

關鍵詞：銅綠微囊藻；微囊藻毒；肝臟毒素；藍綠細菌。