Pergamon

Tetrahedron 54 (1998) 463–470

TETRAHEDRON

Two New (*E*)-2-Amino-2-Butenoic Acid (Dhb)-Containing Microcystins Isolated from *Oscillatoria agardhii*

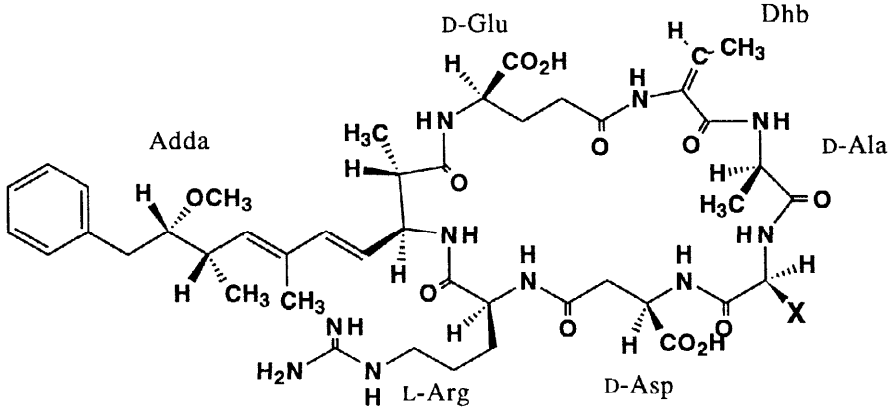
Tomoharu Sano* and Kunimitsu Kaya

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

Received 19 September 1997; accepted 27 October 1997

Abstract: Two new 2-amino-2-butenoic acid (Dhb)-containing microcystins (Dhb-microcystins), [D-Asp^3, (*E*)-Dhb^7]microcystin-LR (**1**) and -HtyR (**2**), were isolated from *Oscillatoria agardhii*. The structures were elucidated by the extensive NMR analyses and confirmed by mass spectral and amino acid analyses. The absolute configuration was determined by chemical degradation and chiral GC analyses. The configuration of the Dhb unit of **1** and **2** was determined as *E* by ROESY experiments.

The freshwater cyanobacterium *Oscillatoria agardhii* forms waterblooms in eutrophicated freshwater lakes and drinking water reservoirs[1]. Some strains of *O. agardhii* produce cyclic heptapeptide toxins, named microcystins[2]. The general structure of microcystins is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L-amino acids, D-MeAsp is D-*erythro*-ß-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and 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. The two acidic amino acids, D-MeAsp and D-Glu, are connected by the isolinkages. In our previous studies of cyanobacterial toxic compounds[3], we isolated the 2-amino-2-butenoic acid (Dhb, dehydrobutyrine)-containing microcystin (Dhb-microcystin), [D-Asp^3, Dhb^7]microcystin-RR (Dhb-microcystin-RR, **3**) (Fig. 1), from *O. agardhii* (CCAP 1459/22 = NIES 610 = CYA 18). The molecular formula of **3** was found to be identical with that of [D-Asp^3] and [Dha^7]microcystin-RR[4,5]. Furthermore, the amino acid composition of **3** agreed well with that of [D-Asp^3]microcystin-RR. However, the ^{1}H-NMR spectrum of **3** was clearly different from those of [D-Asp^3] and [Dha^7]microcystin-RR, strongly suggesting that NMR measurement is essential for precise structure elucidation of microcystins[3-7]. During further investigation of toxic compounds in *O. agardhii*, we were able to isolate two new Dhb-microcystins (**1** and **2**). We now describe the isolation and structure elucidation of **1** and **2**.

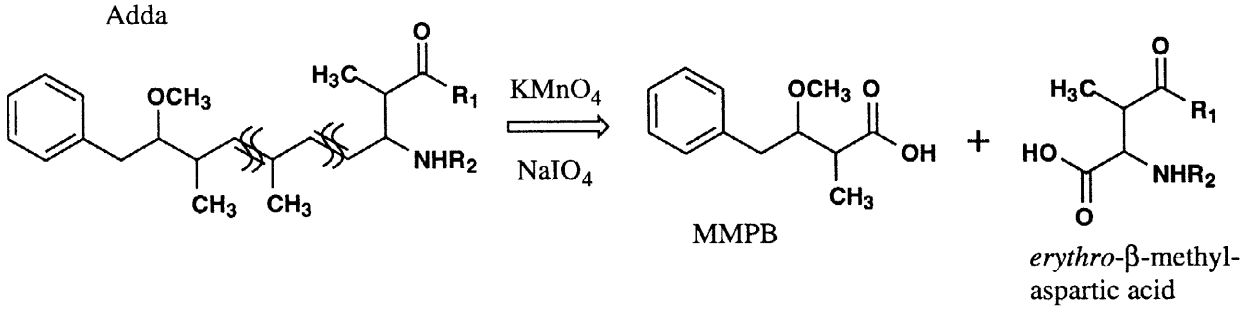


1: X= $CH_2CH_2CH(CH_3)_2$; [D-Asp^3, (*E*)-Dhb^7]microcystin-LR
2: X= CH_2CH_2-*p*-OH-Ph ; [D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR
3: X= $CH_2CH_2CH_2NHC{=}NH(NH_2)$; [D-Asp^3, (*E*)-Dhb^7]microcystin-RR

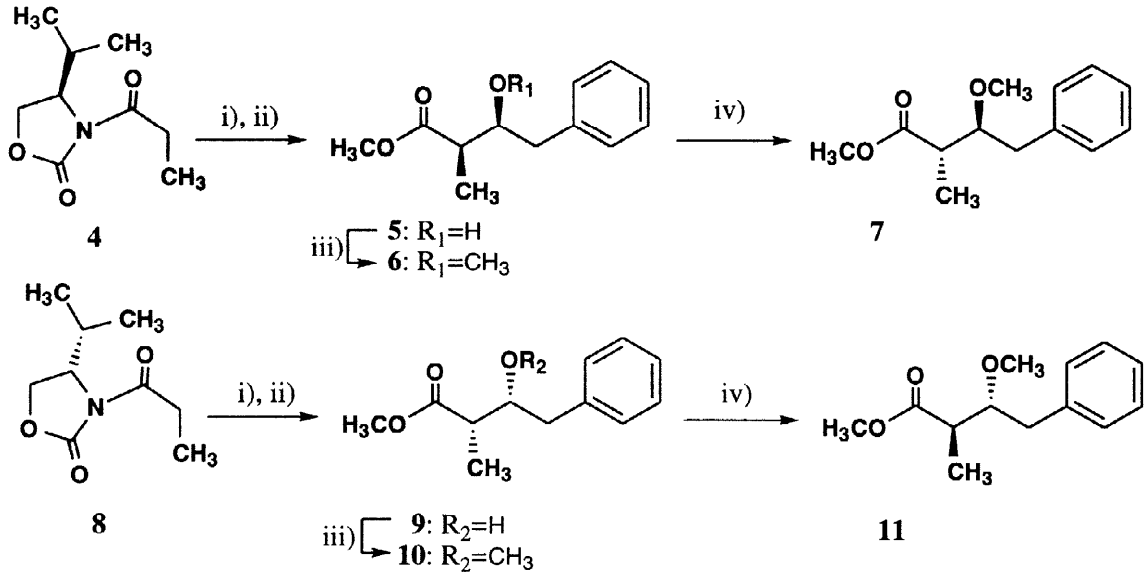
Fig. 1. Structures of (*E*)-Dhb-containing microcystins isolated from *O. agardhii*.

0040-4020/98/$19.00
PII: S0040-4020(97)10291-5

O. agardhii (CCAP 1459/14) was kindly provided by Dr. John G. Day of CCAP, Scotland. The strain was cultured in CT medium[3]. The freeze dried cells were extracted first with 5% acetic acid aqueous solution, then with MeOH. The extracted solution was evaporated *in vacuo*. The extract was fractionated with Sep-Pak ODS cartridges using 20% and 80% MeOH. The fraction eluted with 80% MeOH was analyzed by reverse-phase HPLC using as the eluent 60% MeOH in 50 mM phosphate buffer (pH 3.0) and a photodiode array detector. Two main peaks whose UV spectra resembling those of microcystins were observed. These two peaks were purified by preparative reverse-phase HPLC. Further purification with HPTLC (developed with $CHCl_3$:MeOH:H_2O (6:4:1)) yielded **1** (9.4 mg) and **2** (12.1 mg) as colorless amorphous solids. In the positive FABMS spectrum, the $[M + H]^+$ ion of **1** was observed at *m/z* 981. From the high resolution FABMS spectrum, the molecular formula of **1** was established as $C_{48}H_{73}O_{12}N_{10}$ (calculated for $C_{48}H_{74}O_{12}N_{10}$: 981.5410, Δ +3.4 mmu). The molecular formula of **1** is identical with those of [D-Asp[3]] and [Dha[7]]microcystin-LR[8,9]. Amino acids detected after acid hydrolysis of **1** were D-Ala, L-Leu, D-Asp, L-Arg, D-Glu. This amino acid composition is the same as that of [D-Asp[3]]microcystin-LR. However, the ^{1}H-NMR spectrum of **1** is again clearly different from those of [D-Asp[3]] and [Dha[7]]microcystin-LR[3]. The spectrum of **1** shows a quartet, at 5.69 ppm due to the olefin proton of the Dhb unit (Table 1). In contrast, the ^{1}H-NMR spectra of [D-Asp[3]] and [Dha[7]]microcystin-LR show two singlets at 5-6 ppm, due to the exomethylene group of the dehydroalanine (Dha) unit. The structure of the Dhb unit was confirmed by the analysis of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectra of **1**. In the ROESY spectrum of **1** in DMSO-d_6, a cross peak between the methyl group of the Dhb unit and the amide proton of the Ala unit was observed, suggesting that the geometrical structure of the Dhb unit in **1** is *E*, and, therefore, a geometrical isomer of the Dhb unit in nodularins[10] (cyclic pentapeptide toxins isolated from cyanobacterium *Nodularia* sp.). In the ^{1}H-NMR spectra of nodularins, the olefin proton signal of the (*Z*)-Dhb unit appears at lower field (6.90-6.94 ppm) than that of the (*E*)-Dhb unit in **1**. The geometrical structure of the Dhb unit in **1** was confirmed by 1D NOE experiment. Extensive NMR analysis of 2D-NMR spectra of **1** revealed the presence of the Adda unit. The coupling constants and chemical shifts of the Adda unit suggest that the relative configuration of the Adda unit of **1** is the same as that of microcystins[11]. The absolute configuration of the Adda unit of **1** was determined by oxidation of **1** following chiral GC. Compound **1** was oxidized by treatment with $KMnO_4$ and $NaIO_4$ in the manner previously described[12]. After oxidation, *erythro*-β-methylaspartic acid and 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB) were derived from the Adda unit of **1** (Scheme 1).



Scheme 1. Oxidation of the Adda unit.



Scheme 2. Diasteroselective synthesis of MMPB.
i) Bu_2BOTf, iPr_2NEt, $PhCH_2CHO$ in CH_2Cl_2 at -78 °C; ii) NaOMe in MeOH at 0 °C;
iii) MeI, NaOH in DMSO-THF at 0 °C; iv) NaOH in MeOH at rt.

The absolute configuration of MMPB was confirmed using chiral GC by comparing the retention time of four diastereoisomers of MMPB. These four diastereoisomers of MMPB were diastereoselectively synthesized *via* an Evans aldol condensation[13] (Scheme 2). The absolute structure of MMPB derived from the Adda unit of **1** was established as (2*R*,3*S*)-MMPB by chiral capillary GC analysis. The configuration of *erythro*-β-methylaspartic acid was confirmed as D by chiral GC analysis of its *N*-trifluoroacetyl-*O*-isopropyl ester derivative. These results suggest that the absolute configuration of the Adda unit in **1** is (2*S*,3*S*,8*S*,9*S*), i.e. the same as that in microcystins. The sequence of **1** was mostly deduced by HMBC correlations from α-H to C=O. The connection between the Dhb and Glu units was confirmed by a decoupled-HMBC experiment, described in our previous paper[3]. From these data, the structure of **1** was established as [D-Asp[3], (*E*)-Dhb[7]]microcystin-LR ((*E*)-Dhb-microcystin-LR) (Fig. 1).

The pseudomolecular ion ($[M + H]^+$) of **2** in the FABMS spectrum was observed at *m/z* 1045. From the high resolution FABMS spectrum, the molecular formula of **2** was deduced as $C_{52}H_{72}N_{10}O_{13}$ (calculated for $C_{52}H_{73}N_{10}O_{13}$: 1045.5359, Δ ±0.0 mmu), identical with those of [D-Asp[3]] and [Dha[7]]microcystin-HtyR[14,15]. The amino acids detected after acid hydrolysis of **2** were D-Ala, L-homotyrosine (Hty), D-Asp, L-Arg, D-Glu. The amino acid composition of **2** is the same as that of [D-Asp[3]]microcystin-HtyR. In the ^{1}H-NMR spectrum of **2**, a quartet was observed at 5.73 ppm. This quartet is similar to that observed with **1** and **3**. Extensive NMR spectra and chiral GC analysis suggested that the structure of **2** was [D-Asp[3], (*E*)-Dhb[7]]microcystin-HtyR ((*E*)-Dhb-microcystin-HtyR) (Fig. 1).

The absolute structure of **3** was also confirmed as [D-Asp[3], (*E*)-Dhb[7]]microcystin-RR ((*E*)-Dhb-microcystin-RR) (Fig. 1) in the same manner as **1**. Dhb-microcystins were also isolated from CCAP strains of *O. agardhii* (CCAP 1459/11A, 1459/11B, and 1459/16).

The configuration of the Dhb unit of Dhb-microcystins isolated from the CCAP strains of *O. agardhii* is *E*, while that of nodularins from *Nodularia spumigena* has been determined as *Z*[10]. The Dhb unit in Dhb-

Table 1. ^{1}H and ^{13}C NMR Data for [D-Asp^{3}, (*E*)-Dhb^{7}]microcystin-LR (**1**) in CD_3OD at 500 MHz.

position		^{1}H	*J* (Hz)	^{13}C	position		^{1}H	*J* (Hz)	^{13}C
Dhb	1			166.5	Adda	1			176.4
	2			132.0		2	3.00	(m)	45.0
	3	5.69	(q, 7.3)	123.8		3	4.52	(t, 9.5)	56.5
	4	1.86	(d, 7.3)	13.5		4	5.37	(dd, 9.5, 15.6)	127.1
Ala	1			175.3		5	6.18	(d, 15.6)	138.2
	2	4.61	(q, 7.0)	49.5		6			134.1
	3	1.31	(d, 7.0)	17.5		7	5.37	(d, 9.8)	136.7
Leu	1			174.7		8	2.58	(m)	37.7
	2	4.28	(dd, 3.9, 11.6)	55.1		9	3.24	(m)	88.4
	3	1.99	(m)	40.6		10	2.82	(m)	39.0
		1.59	(m)				2.67	(dd, 7.2, 13.9)	
	4	1.78	(m)	26.0		11	1.08	(d, 7.0)	16.1
	5	0.93	(d, 6.7)	23.8		12	1.62	(s)	13.0
	5'	0.89	(d, 6.4)	21.3		13	0.99	(d, 6.7)	16.6
Asp	1			176.7		14	3.23	(s)	58.7
	2	4.56	(t, 4.8)	53.3		15			140.6
	3	2.79	(m)	39.8		16, 20	7.18	(d, 6.7)	130.5
		2.33	(m)			17, 19	7.24	(dd, 6.7, 7.6)	129.2
	4			175.0		18	7.16	(t, 7.6)	127.0
Arg	1			172.4	Glu	1			179.3
	2	4.48	(dd, 4.3, 9.0)	52.7		2	4.27	(dd, 5.8, 9.2)	56.0
	3	2.07	(m)	28.7		3	2.03	(m)	29.9
		1.52	(m)				1.92	(m)	
	4	1.60	(m)	26.0		4	2.47	(m)	34.4
		1.49	(m)				2.23	(m)	
	5	3.15	(m)	41.4		5			175.3
	6			158.7					

Table 2. ^{1}H and ^{13}C NMR Data for [D-Asp^{3}, (*E*)-Dhb^{7}]microcystin-HtyR (**2**) in CD_3OD at 500 MHz.

position		^{1}H	*J* (Hz)	^{13}C	position		^{1}H	*J* (Hz)	^{13}C
Dhb	1			166.7	Adda	1			176.4
	2			132.0		2	2.98	(m)	45.0
	3	5.73	(q, 7.3)	123.8		3	4.52	(dd, 8.6, 9.2)	56.4
	4	1.87	(d, 7.3)	13.5		4	5.51	(dd, 8.6, 15.6)	127.0
Ala	1			175.5		5	6.17	(d, 15.6)	138.2
	2	4.61	(q, 7.0)	49.7		6			134.0
	3	1.34	(d, 7.0)	17.4		7	5.37	(d, 8.8)	136.8
Hty	1			174.4		8	2.57	(m)	37.7
	2	4.14	(dd, 3.7, 11.3)	56.0		9	3.24	(m)	88.4
	3	2.24	(m)	34.1		10	2.80	(dd, 4.9, 14.0)	39.0
		2.10	(m)				2.65	(dd, 7.3, 14.0)	
	4	2.73	(m)	32.6		11	1.06	(d, 7.0)	16.2
		2.52	(m)			12	1.60	(s)	13.0
	5			133.2		13	0.99	(d, 7.0)	16.6
	6, 10	7.01	(d, 8.5)	130.7		14	3.22	(s)	58.7
	7, 9	6.66	(d, 8.5)	116.2		15			140.6
	8			156.6		16, 20	7.16	(d, 7.0)	130.5
Asp	1			176.8		17, 19	7.23	(t, 7.0)	129.2
	2	4.57	(t, 4.6)	53.2		18	7.15	(t, 7.0)	127.0
	3	2.77	(m)	39.8	Glu	1			179.2
		2.34	(m)			2	4.24	(dd, 5.8, 9.2)	56.1
	4			175.1		3	2.03	(m)	29.9
Arg	1			172.4			1.95	(m)	
	2	4.44	(dd, 4.0, 8.5)	52.9		4	2.47	(m)	34.2
	3	2.03	(m)	28.8			2.26	(m)	
		1.53	(m)			5			175.3
	4	1.58	(m)	26.3					
		1.54	(m)						
	5	3.12	(m)	41.7					
	6			158.6					

microcystins will be biosynthesized *via* a dehydration of Thr or *allo*Thr. It is thought the biosynthesis enzymes of the Dhb unit of the *Oscillatoria* strains are different from those of the *Nodularia*.

(*E*)-Dhb-microcystin-LR, -HtyR, and -RR were toxic. Their LD_{50} values (mice, i.p.) were 70, 70, and 250 μg/kg, respectively. These values are similar to those of the corresponding normal microcystins which contain the Dha unit instead of the Dhb unit. These results suggest that toxicity is affected little by the methyl group of the Dhb unit of the microcystins.

The structure elucidation of microcystins has been mainly performed by the relative t_R in HPLC, amino acid analyses and FABMS analyses of pseudomolecular ions. The LC/MS method was believed to be potentially one of the most useful methods for microcystin analyses[16]. However, the molecular formulae and amino acid composition of Dhb-microcystins are the same as those of the corresponding [D-Asp^3]microcystins. Our findings strongly suggest that 1D ^{1}H-NMR analysis is necessary for the distinction between Dhb-microcystins and the corresponding [D-Asp^3]microcystins.

ACKNOWLEDGMENT

The authors are grateful to Drs. H. Ito, J. Hayashi, A. Sakata, and S. Serizawa for measurements of GC/MS and FABMS spectra. The authors thank Dr. John G. Day for providing CCAP strains of *Oscillatoria agardhii*. We also thank Dr. Mark Bradley for gifts of D- and L-Hty.

EXPERIMENTAL SECTION

General Procedures. NMR spectra were recorded on a Jeol JNM A-500 spectrometer (500 MHz). ^{1}H and ^{13}C chemical shifts are referenced to tetramethylsilane. Homonuclear ^{1}H connectivities were determined with the COSY and HOHAHA experiments and heteronuclear ^{1}H-^{13}C connectivities were determined by HSQC and HMBC experiments. Low and high resolution FABMS were performed with a Jeol JMS-700 spectrometer. Specific rotations were obtained on a Atago POLAX-D polarimeter.

Chemicals. D- and L-Hty were gifts of Dr. Mark Bradley at University of Southampton, UK. DL-*threo*-β-Methylaspartic acid was purchased from SIGMA Chemical Co. (St. Louis, USA). DL-Methylaspartic acid was purchased from ICN Biomedicals Inc. (Aurora, USA). (4*R*)- and (4*S*)-4-isopropyl-2-oxazolidone, dibutylboron triflate, and n-butyllithium were obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). All other solvents and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Culture Conditions. *O. agardhii* (CCAP 1459/14) was kindly provided by Dr. John G. Day of CCAP, Scotland. The strain was cultured in 10 L culture bottles with CT medium of the following composition: 15 mg $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg KNO_3, 5 mg β-Na_2 glycerophosphate, 4 mg $MgSO_4 \cdot 7H_2O$, 0.01 μg vitamin B_{12}, 0.01 μg biotin, 1 μg thiamin HCl, 0.06 mg $FeCl_3 \cdot 6H_2O$, 0.01 mg $MnCl_2 \cdot 4H_2O$, 7 μg $ZnSO_4 \cdot 7H_2O$, 1.2 μg $CoCl_2 \cdot 6H_2O$, 0.75 μg $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg $Na_2EDTA \cdot 2H_2O$, 40 mg TAPS(*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid), and 100 mL distilled water, pH 8.2. The cells were grown isothermally at 20 °C (light intensity, below 250 μmol photon m^{-2} s^{-1}; aeration rate, 1.5 L min^{-1}). After 2 weeks, the alga was harvested by centrifugation and freeze-dried. Yields of lyophilized alga averaged 0.09 g/L.

Extraction and Isolation. The freeze-dried cells (7.7 g) was extracted first with 5% aqueous acetic acid solution, then with methanol. The supernatant were combined and evaporated *in vacuo*. *The* extract was

suspended with aqueous 5% acetic acid solution. The suspension was filtered and the filtrate was passed through Sep-Pak ODS cartridges. The cartridges were washed with 20% MeOH and eluted with 80% MeOH. The fraction eluted with 80% MeOH was separated by reverse-phase HPLC (Mightysil RP-18, 20 × 250 mm, Kanto Chemical, Japan, flow rate, 9.0 mL/min) with methanol (65%) containing 0.05 M phosphate (pH 3.0). Further purification with HPTLC (Silica gel 60 F_{254}, 0.25 mm, 10 x 25 cm, Merck, developed with $CHCl_3$:MeOH:H_2O (6:4:1)) yielded [D-Asp^3, (*E*)-Dhb^7]microcystin-LR ((*E*)-Dhb-microcystin-LR, **1**, 9.4 mg) and [D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR ((*E*)-Dhb-microcystin-HtyR, **2**, 12.1 mg) as colorless amorphous solids.

Toxicity test. Toxicity was tested on male BALB/C mice(7 weeks old, 23-29 g, obtained from JCL, Japan) by i.p. injection using four doses and six animals per dose. The calculation of LD_{50} value was performed with Weil's method[17].

Hydrolysis and Amino acid analysis. Dhb-microcystins in 6N HCl were heated at 110 °C for 20 h. The amino acid hydrolysate was heated with 6N HCl (0.2 mL) and iPrOH (0.2 mL) at 110 °C for 1h. The mixture was evaporated to dryness under a gentle stream of nitrogen (N_2). The residue was treated with trifluoroacetic anhydride (100 μL) and CH_2Cl_2 (100 μL) at 100 °C for 5 min and again evaporated by N_2. The mixture in CH_2Cl_2 was analyzed by GC/MS using a Chirasil-L-Val capillary column (0.25 mm × 25 m) and the following conditions: column temperature 40 to 200 °C at 8 °C/min.

[D-Asp^3, (*E*)-Dhb^7]microcystin-LR ((*E*)-Dhb-microcystin-LR) (1). Retention time, 14.0 min; $[\alpha]^{25}_D$ -133° (c 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 239 nm (4.5); FABMS, *m/z* 981 $[M + H]^+$; HRFABMS, *m/z* 981.5444 ($[M + H]^+$, calculated for $C_{48}H_{74}O_{12}N_{10}$, 981.5410).

[D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR ((*E*)-Dhb-microcystin-HtyR) (2). Retention time, 11.7 min; $[\alpha]^{25}_D$ -100° (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 239 nm (4.5); FABMS, *m/z* 1045 ($[M + H]^+$); HRFABMS, *m/z* 1045.5359 ($[M + H]^+$, calculated for $C_{52}H_{74}O_{13}N_{10}$, 1045.5359).

Methyl (2*R*,3*S*)-3-hydroxy-2-methyl-4-phenyl-butanoate (5). Compound **5** was synthesized *via* an Evans aldol condensation following NaOMe treatment at 0 °C. $[\alpha]^{30}_D$ -52° (c 0.6, MeOH); UV (MeOH) λ_{max} 259 nm (ε, 230); ^{1}H-NMR ($CDCl_3$) δ 1.21 (d, 3H, *J* = 7.0 Hz, H-5), 2.41 (br, 1H, 3-OH), 2.54 (m, 1H, H-2), 2.73 (dd, *J* = 5.5, 13.7 Hz, 1H, H-4a), 2.77 (dd, *J* = 7.9, 13.7 Hz, 1H, H-4b), 3.68 (s, 3H, Me ester), 4.14 (m, 1H, H-3), and 7.20-7.29 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 10.9 (C-5), 40.4 (C-4), 43.6 (C-2), 51.8 (C-Me ester), 72.7 (C-3), 126.6 (C-*p*-Ph), 128.6 (C-*m*-Ph), 129.3 (C-*o*-Ph), 138.1 (C-Ph), and 176.3 (C-1); EIMS, *m/z* 57, 91, 117, and 190 ($[M - H_2O]^{+\bullet}$); CIMS, *m/z* 209 ($[M + H]^+$) and 191 ($[M - H_2O + H]^+$); HRCIMS, *m/z* 209.1187 ($[M + H]^+$, calculated for $C_{12}H_{17}O_3$, 209.1178).

Methyl (2*R*,3*S*)-3-methoxy-2-methyl-4-phenyl-butanoate (6). Compound **5** (16 mg, 77 μmole) was dissolved in 1 mL of 0.1% H_2O-containing DMSO:THF (2:1) solution, and iodomethane (0.2 ml) was added to the solution. NaOH powder (0.1 g) was added to the mixture in ice-water bath, and the reaction mixture was stirred in ice-water bath for 1.5 hr. To the reaction mixture, 3 mL of CH_2Cl_2 and then 0.5 M phosphate buffer (pH 3.0) were added. The organic layer was washed with sat. NaCl three times. After evaporation, the organic layer was applied to four HPTLC plates(Silica gel 60 F_{254}, 0.25 mm, 10 x 25 cm, Merck). The plates were developed with n-hexane:EtOAc (5:1). Compound **6** (12 mg, 54 μmole, yield 71%) was obtained as a colorless oil. $[\alpha]^{30}_D$ -68° (c 0.8, $CHCl_3$); UV ($CHCl_3$) λ_{max} 260 nm (ε, 260); ^{1}H-NMR ($CDCl_3$) δ 1.21 (d, 3H, *J* = 7.0 Hz, H-5), 2.51 (m, 1H, H-2), 2.72 (dd, 1H, *J* = 6.0, 14.0 Hz, H-4a), 2.85 (dd, 1H, *J* = 7.0, 14.0 Hz, H-4b), 3.24 (s, 3H, 3-O-Me), 3.65 (s, 3H, Me ester), 3.75 (m, 1H, H-3), and 7.18-7.29 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 11.4 (C-5), 38.3 (C-4), 42.9 (C-2), 51.6 (C-Me ester), 58.4 (C-3-O-Me), 83.2 (C-3), 126.3 (C-*p*-Ph), 128.4 (C-*m*-Ph), 129.3 (C-*o*-Ph), 138.5 (C-Ph), and 175.3 (C-1);

EIMS, *m/z* 75, 91, 131, and 190 ([M - MeOH]$^{+\bullet}$); CIMS, *m/z* 223 ([M + H]$^+$) and 191 ([M - MeOH + H]$^+$); HRCIMS, *m/z* 223.1349 ([M + H]$^+$, calculated for $C_{13}H_{19}O_3$, 223.1334).

Methyl (2*S*,3*S*)-3-methoxy-2-methyl-4-phenyl-butanoate (7). Compound **6** (5 mg) was dissolved in 0.2 mL of MeOH. To the solution, 10 μL of 28% NaOMe in MeOH was added at rt. After 2 hr stirring, 5 mL of 0.1 M phosphate buffer (pH 3.0) was added, then the reaction mixture was extracted with n-hexane. The extract was applied to HPLC (Lichrosorb Si-60, 20 x 250 mm, Merck, n-hexane:$CHCl_3$ (20:3), 9 mL/min.). Compound **7** (1 mg, yield 20%) was obtained as a colorless oil. $[\alpha]^{30}_D$ +26° (c 0.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 1.16 (d, 3H, *J* = 7.0 Hz, H-5), 2.69 (dd, 1H, *J* = 7.0, 14.0 Hz, H-4b), 2.70 (m, 1H, H-2), 2.85 (dd, 1H, *J* = 4.0, 14.0 Hz, H-4a), 3.20 (s, 3H, 3-O-Me), 3.62 (m, 1H, H-3), 3.65 (s, 3H, Me ester), and 7.18-7.28 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 12.5 (C-5), 37.1 (C-4), 43.1 (C-2), 51.6 (C-Me ester), 58.2 (C-3-O-Me), 83.8 (C-3), 126.2 (C-*p*-Ph), 128.3 (C-*m*-Ph), 129.5 (C-*o*-Ph), 138.5 (C-Ph), and 175.1 (C-1); EIMS, *m/z* 75, 91, 131, and 190 ([M - MeOH]$^{+\bullet}$); CIMS, *m/z* 223 ([M + H]$^+$) and 191 ([M - MeOH + H]$^+$); HRCIMS, *m/z* 223.1348 ([M + H]$^+$, calculated for $C_{13}H_{19}O_3$, 223.1334).

Synthesis of the antipodes of compounds 5, 6, and 7. The antipodes (compounds **9**, **10**, and **11**) were synthesized in the same manner as those of compounds **5**, **6**, and **7** except the chirality of oxazolidone.

Methyl (2*S*,3*R*)-3-hydroxy-2-methyl-4-phenyl-butanoate (9). Colorless oil. $[\alpha]^{30}_D$ +53° (c 1.5, MeOH); UV (MeOH) λ_{max} 259 nm (ε, 230); ^{1}H-NMR ($CDCl_3$) δ 1.26 (d, 3H, *J* = 7.0 Hz, H-5), 2.41 (d, 1H, *J* = 3.4 Hz, 3-OH), 2.54 (m, 1H, H-2), 2.73 (dd, *J* = 5.5, 13.7 Hz, 1H, H-4a), 2.78 (dd, *J* = 8.0, 13.7 Hz, 1H, H-4b), 3.68 (s, 3H, Me ester), 4.15 (m, 1H, H-3), and 7.20-7.31 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 10.9 (C-5), 40.4 (C-4), 43.6 (C-2), 51.8 (C-Me ester), 72.7 (C-3), 126.6 (C-*p*-Ph), 128.6 (C-*m*-Ph), 129.3 (C-*o*-Ph), 138.1 (C-Ph), and 176.3 (C-1); EIMS, *m/z* 57, 91, 117, and 190 ([M - H_2O]$^{+\bullet}$); CIMS, *m/z* 209 ([M + H]$^+$) and 191 ([M - H_2O + H]$^+$); HRCIMS, *m/z* 209.1196 ([M + H]$^+$, calculated for $C_{12}H_{17}O_3$, 209.1178).

Methyl (2*S*,3*R*)-3-methoxy-2-methyl-4-phenyl-butanoate (10). Colorless oil. $[\alpha]^{30}_D$ +71° (c 1.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} 260 nm (ε, 260); ^{1}H-NMR ($CDCl_3$) δ 1.21 (d, 3H, *J* = 7.3 Hz, H-5), 2.51 (m, 1H, H-2), 2.72 (dd, 1H, *J* = 6.0, 14.0 Hz, H-4a), 2.85 (dd, 1H, *J* = 7.0, 14.0 Hz, H-4b), 3.24 (s, 3H, 3-O-Me), 3.65 (s, 3H, Me ester), 3.75 (m, 1H, H-3), and 7.18-7.29 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 11.3 (C-5), 38.3 (C-4), 42.9 (C-2), 51.6 (C-Me ester), 58.4 (C-3-O-Me), 83.2 (C-3), 126.3 (C-*p*-Ph), 128.4 (C-*m*-Ph), 129.3 (C-*o*-Ph), 138.5 (C-Ph), and 175.3 (C-1); EIMS, *m/z* 75, 91, 131, and 190 ([M - MeOH]$^{+\bullet}$); CIMS, *m/z* 223 ([M + H]$^+$) and 191 ([M - MeOH + H]$^+$); HRCIMS, *m/z* 223.1314 ([M + H]$^+$, calculated for $C_{13}H_{19}O_3$, 223.1334).

Methyl (2*R*,3*R*)-3-methoxy-2-methyl-4-phenyl-butanoate (11). Colorless oil. $[\alpha]^{30}_D$ -22° (c 0.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 1.16 (d, 3H, *J* = 7.0 Hz, H-5), 2.69 (dd, 1H, *J* = 7.0, 14.0 Hz, H-4b), 2.70 (m, 1H, H-2), 2.85 (dd, 1H, *J* = 4.0, 14.0 Hz, H-4a), 3.20 (s, 3H, 3-O-Me), 3.62 (m, 1H, H-3), 3.65 (s, 3H, Me ester), and 7.18-7.28 (m, 5H, H-Ph); ^{13}C-NMR ($CDCl_3$) δ 12.5 (C-5), 37.1 (C-4), 43.1 (C-2), 51.6 (C-Me ester), 58.2 (C-3-O-Me), 83.8 (C-3), 126.2 (C-*p*-Ph), 128.3 (C-*m*-Ph), 129.5 (C-*o*-Ph), 138.5 (C-Ph), and 175.1 (C-1); EIMS, *m/z* 75, 91, 131, and 190 ([M - MeOH]$^{+\bullet}$); CIMS, *m/z* 223 ([M + H]$^+$) and 191 ([M - MeOH + H]$^+$); HRCIMS, *m/z* 223.1314 ([M + H]$^+$, calculated for $C_{13}H_{19}O_3$, 223.1334).

Chiral GC analysis of MMPB methyl esters. Methyl esters of MMPB were dissolved in CH_2Cl_2, and injected in a chiral capillary GC column (Chiraldex B-PM, 0.25 mm x 30 m, ASTEC Inc., USA). The column temperature was held at 135 °C.

REFERENCES

1. Sivonen, K.; Niemelä, S. I.; Niemi, R. M.; Lepistö, L.; Luoma, T. H.; Räsänen, L. A. *Hydrobiologia* **1990**, 190, 267-275.
2. Luukkainen, R.; Sivonen, K.; Namikoshi, M.; Färdig, M.; Rinehart, K. L.; S. I. Niemelä, S. I. *Appl. Environ. Miclobiol.* **1993**, 59, 2204-2209.
3. Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, 36, 8603-8606.
4. Meriluoto, J. A. O.; Sandström, A.; Eriksson, J. E.; Remaud, G.; Craig, A. G.; Chattopadhyaya, J. *Toxicon* **1989**, 27, 1021-1034.
5. Kiviranta, J.; Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rinehart, K. L. *Toxicon* **1992**, 30, 1093-1098.
6. Kusumi, T.; Ooi, T.; Watanabe, M. M.; Takahashi, H.; Kakisawa, H. *Tetahedron Lett.* **1987**, 28, 4695-4698.
7. Beattie, K. A.; Kaya, K.; Sano, T.; Codd, G. A. *Phytochemistry* **1997**, in press.
8. Harada, K.-I.; Matsuura, K.; Suzuki, M.; Watanabe, M. F.; Oishi, S.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W. *Toxicon* **1990**, 28, 55-64.
9. Harada, K.-I.; Ogawa, K.; Matsuura, K.; Nagai, H.; Murata, H.; Suzuki, M.; Itezono, Y.; Nakayama, N.; Shirai, M.; Nakano, M. *Toxicon* **1991**, 29, 479-489.
10. Namikoshi, M.; Choi, B. W.; Sakai, R.; Sun, F.; Rinehart, K. L.; Carmichael, W. W.; Evans, W. R.; Cruz, P.; Munro, M. H. G.; Blunt, J. W. *J. Org. Chem.* **1994**, 59, 2349-2357.
11. Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Stotts, R. R.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W.; Evans, W. R. *J. Org. Chem.* **1992**, 57, 866-872.
12. Sano, T.; Nohara, K.; Shiraishi, F.; Kaya, K. *Intern. J. Environ. Anal. Chem.* **1992**, 49, 163-170.
13. Beatty, M. F.; Jennings-White, C.; Avery, M. A. *J. Chem. Soc. Perkin Trans. I* **1992**, 1637-1641.
14. Harada, K.-I.; Ogawa, K.; Kimura, Y.; Murata, H.; Suzuki, M.; Thorn, P. M.; Evans, W. R.; Carmichael, W. W. *Chem. Res. Toxicol.* **1991**, 4, 535-540.
15. Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. *Chem. Res. Toxicol.* **1992**, 5, 661-666.
16. Kondo, F.; Ikai, Y.; Oka, H.; Ishikawa, N.; Watanabe, M. F.; Watanabe, M.; Harada, K.-I.; Suzuki, M. *Toxicon* **1992**, 30, 227-237.
17. Weil, C. S. *Biometrics* **1952**, 8, 249-263.