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Structures of Three New Homotyrosine-Containing Microcystins and a New Homophenylalanine Variant from *Anabaena* sp. Strain 66

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A hepatotoxic strain of cyanobacterium *Anabaena* sp. 66 was isolated from a hepatotoxic water bloom sample in Lake Kiikkara, Finland. Four cyclic heptapeptide hepatotoxins were isolated and purified by HPLC from cultured cells of this organism. The structures of three new homotyrosine (Hty) containing toxins, [Dha^7]microcystin-HtyR (Dha = dehydroalanine) (**1**), [D-Asp^3,Dha^7]microcystin-HtyR (**2**), and [L-Ser^7]microcystin-HtyR (**3**), were assigned, based upon amino acid analyses using both a Waters Pico Tag HPLC system and chiral capillary GC, ^{1}H NMR, fast atom bombardment mass spectrometry (FABMS), and collisionally induced tandem FABMS. A new homophenylalanine (Hph) variant of **1**, [Dha^7]microcystin-HphR (**4**), was also obtained as a minor component. Compound **3** is most likely a biosynthetic precursor of **1**. The four new toxins did not have an *N*-methyl group at the dehydroamino acid or its precursor unit.

Introduction

Hepatotoxic coccoid *Microcystis* and filamentous *Anabaena*, *Nodularia*, *Nostoc*, and *Oscillatoria* have been isolated from fresh and brackish water cyanobacterial (blue-green algal) blooms (*1–14*). *Nodularia spumigena* produces the cyclic pentapeptide nodularin (*13–15*), and cyclic heptapeptides termed microcystins (*4*) have been detected from the other genera. *Microcystis* is the most common producer of these toxins, and microcystin-LR is the commonly found microcystin (*2*, *3*).

Microcystins have the general structure *cyclo*(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L-amino acids, D-MeAsp[1] is D-*erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine (*4*). Demethyl variants have been reported at the D-MeAsp (i.e., D-Asp) and/or Mdha (i.e., dehydroalanine = Dha) residues (*2*, *6–12*). The two acidic amino acids D-MeAsp and D-Glu are connected by an iso linkage.

These toxins have (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) as the most unusual structural feature (*13*, *16*). Adda plays an important role in their hepatotoxicity since hydrogenation or ozonolysis of the diene system in this unit gives an inactive product[2] and stereoisomers at the Δ^6 double bond in the Adda unit are also inactive (*17*, *18*). The toxins are becoming important biochemical tools (*19*) because of the potent inhibition of protein phosphatases 1 and 2A by these cyclic peptides (*20–24*).

We are interested in the structure–activity relationships of these compounds obtained from both natural and synthetic sources and have reported on the synthesis of Adda (*25*), which showed no toxicity. We have also described the structures of microcystins isolated from *Nostoc* sp. strain 152 (*9*, *26*, *27*) and a water bloom of *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii* collected from Homer Lake (Illinois) (*12*), possessing a modified Adda unit, which retains hepatotoxicity. A nontoxic compound with a monoester at the D-Glu unit [D-Glu(OC_3H_7O)] has been isolated from the Homer Lake cells (*12*) and a new variant (D-Ser) at the D-Ala unit was obtained from *Nostoc* sp. 152 (*27*).

Our efforts toward finding new toxic cyanobacteria have resulted in the isolation of several hepatotoxic *Anabaena* spp. (*5*, *28*). *Anabaena* sp. strain 66 was found to produce four new microcystins 1–4 (Chart I). We applied the fast atom bombardment mass spectrometric (FABMS) method (*12*, *26*), including collisionally induced tandem FABMS (FABMS/MS, *B*/*E* scan), to the structure assignment of these new microcystins as reported in this paper.

Experimental Procedures

Organism and Culturing. *Anabaena* sp. strain 66 was isolated from a hepatotoxic bloom sample taken on July 22, 1986, at Lake Kiikkara, Finland. The strain was isolated and mass-

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[1] Abbreviations: Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Dha, dehydroalanine; DMAP, 4-(dimethylamino)pyridine; FABMS, fast atom bombardment mass spectrometry; FABMS/MS, collisionally induced tandem FABMS; GC, gas chromatography; Hph, L-homophenylalanine; HPLC, high-performance liquid chromatography; HR, high resolution; Hty, L-homotyrosine; Mdha, *N*-methyldehydroalanine; MeAsp, D-*erythro*-β-methylaspartic acid; MS, mass spectra; NMR, nuclear magnetic resonance; TFAA, trifluoroacetic anhydride.

[2] Dahlem, A. M., Beasley, V. R., Harada, K.-I., Matsuura, K., Suzuki, M., Harvis, C. A., Rinehart, K. L., and Carmichael, W. W. The structure/toxicity relationships of dehydro amino acids in microcystin-LR and nodularin, two monocyclic peptide hepatotoxins from cyanobacteria. Unpublished work.

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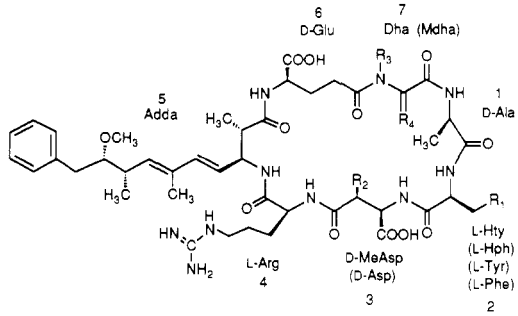
Chart I. Structures of Four New Toxins (1–4) Isolated from *Anabaena* sp. Strain 66 and Compounds 5–7



	R_1	R_2	R_3	R_4
[Dha^7]microcystin-HtyR (**1**):	$CH_2C_6H_4OH$ (*p*)	CH_3	H	CH_2
[D-Asp^3,Dha^7]microcystin-HtyR (**2**):	$CH_2C_6H_4OH$ (*p*)	H	H	CH_2
[L-Ser^7]microcystin-HtyR (**3**):	$CH_2C_6H_4OH$ (*p*)	CH_3	H	H, CH_2OH (*S*)
[Dha^7]microcystin-HphR (**4**):	$CH_2C_6H_5$	CH_3	H	CH_2
microcystin-YR (**5**):	C_6H_4OH (*p*)	CH_3	CH_3	CH2
[D-Asp^3]microcystin-HtyR (**6**):	$CH_2C_6H_4OH$ (*p*)	H	CH_3	CH_2
microcystin-FR (**7**):	C_6H_5	CH_3	CH_3	CH_2

cultivated in the inorganic nutrient medium Z8 minus nitrogen as detailed earlier (*5*, *9*). Cells were lyophilized prior to toxin extraction.

Isolation of Toxins. Toxins were extracted from 1.5 g of dried cells with *n*-BuOH/MeOH/H_2O (1:4:15) (v/v) twice (2 h and overnight) with stirring and centrifuged (8000 rpm, Sorvall GSA rotor). The supernatants were combined and air-dried to half of the original volume and applied to a preconditioned C18 silica gel column (Bond Elut, Analytichem Int., Harbor City, CA). The toxic fraction was eluted with 80% methanol and evaporated to dryness. The sample in water solution was then purified by HPLC using a Beckman Model 421 solvent delivery system and a Beckman Model 165 UV detector (238 nm). A semipreparative C18 silica gel column (19 × 300 mm, μBondapak, Waters Associates, Milford, MA) was used with a mobile phase of CH_3CN/10 mM ammonium acetate (26:74) and a flow rate of 4 mL/min. The toxic fractions were further purified with the same HPLC and column with a methanol gradient from 0% to 50% in 25 min with 10 mM phosphate buffer (pH 6.8). The third purification step was accomplished by using a Beckman Model 406 HPLC with a Model 167 UV detector and an analytical C18 silica gel column (4.6 × 250 mm, Alltech Associates, Deerfield, IL) with CH_3CN/20 mM ammonium acetate (pH 5, 25:75) and flow rate of 1 mL/min. Purity of the compounds was monitored by a Waters 600E HPLC system with a 990 photodiode array detector after each purification step. The column used was an ISRP column (4.6 × 250 mm, Regis Pinkerton, Regis Chemical Co., Morton Grove, IL) with mobile phase CH_3CN/0.1 M phosphate buffer (pH 6.8, 15:85) and a flow rate of 1 mL/min. The pure compounds were desalted and stored at −20 °C.

Amino Acid Analysis. Isolated compounds were hydrolyzed with 6 N HCl at 110 °C for 21 h, and the amino acids obtained, after precolumn derivatization with phenyl isothiocyanate, were analyzed with a Waters Pico Tag HPLC system. The derivatives were separated on a C18 silica gel column (3.9 × 150 mm) using Pico Tag eluents A and B over 13 min and were detected with UV absorption at 254 nm.

Gas Chromatography (GC). Capillary GC analyses were carried out on a Varian 3700 gas chromatograph using a Chirasil Val III column (*29*) (0.32 mm × 25 m, Alltech Associates, Deerfield, IL) and helium as a carrier gas (flow rate: 37 mL/min; split ratio: 20:1). The program rate for the analysis of amino acid derivatives, except Arg, was 90 °C (2 min) to 190 °C (20 min) at 8 °C/min. The homotyrosine (Hty) and Tyr derivatives were also analyzed by isothermal chromatography at 170 and 150 °C, respectively. The Arg derivative was detected at 200 °C. The other conditions were as follows: injector temperature 210 °C; detector temperature 220 °C; makeup gas He (20 mL/min).

Each amino acid (100 μg) was treated with 300 μL of 4 N HCl/MeOH at 110 °C for 15 min in a screw-capped vial. The reaction mixture was evaporated in a stream of N_2, CH_2Cl_2 (200 μL) and trifluoroacetic anhydride (TFAA, 200 μL) were added, and the mixture was heated at 110 °C for 5 min and then evaporated by N_2. The residue was dissolved in CH_2Cl_2 for GC analysis.

Arg was converted to a dimethylpyrimidine derivative prior to the above treatment. The amino acid (100 μg) was heated at 110 °C for 4 h with a mixture of H_2O (25 μL), EtOH (50 μL), triethylamine (Et_3N, 25 μL), and acetylacetone (50 μL) (*30*). The reaction mixture was evaporated to dryness by N_2.

Acid Hydrolysis of Toxin and Derivatization of the Hydrolysate. Compounds **1** (100 μg), **2** (60 μg), and **3** (60 μg) were hydrolyzed with 6 N HCl (200 μL) at 110 °C for 21–24 h. The reaction mixture was cooled to room temperature and divided into two portions. Each portion was evaporated to dryness at 110 °C by N_2. One portion was treated with 4 N HCl/MeOH (200 μL) followed by CH_2Cl_2 and TFAA (each 100 μL) as above. Another portion was treated with acetylacetone (30 μL), H_2O (15 μL), EtOH (30 μL), and Et_3N (15 μL) followed by esterification and acylation as above. The residue was dissolved in CH_2Cl_2 for GC.

Racemization of Tyr and Hty (*26*). L-Tyr (180 μg) and 4-(dimethylamino)pyridine (DMAP, 60 μg) in methanol (200 μL) and water (200 μL) were heated in a sealed vial at 110 °C for 27 h. The mixture was evaporated to dryness by N_2 and then derivatized as above. The GC chromatogram showed about a 20:1 L:D ratio.

The dried acid hydrolysate of **1** (100 μg) was dissolved in methanol and water (each 200 μL), DMAP (120 μg) was added, and the mixture was heated at 110 °C for 29 h, evaporated to dryness, and then derivatized as above.

Mass Spectrometry and ^{1}H NMR Spectroscopy. Mass spectra (MS) were run on either a VG ZAB-SE (FABMS) or a VG 70-SE4F (HRFABMS) mass spectrometer operating in the FAB mode, using xenon atoms (accelerated to 8 keV) and a matrix of dithiothreitol/diethioerythritol ("magic bullet") (*31*). Collisionally induced tandem mass spectra in the FAB mode were obtained on a four-sector tandem mass spectrometer (70-SE4F) using helium as the collision gas: resolution of the first and second mass spectrometers, both 1000; accelerating potential 8 kV; collision potential reduced to 4 kV; 90% attenuation. Approximately 5–10 μg of each sample was applied as a methanol solution. High-resolution (HR) FABMS (resolution 10 000) and FABMS/MS data are summarized in Tables I and II, respectively. ^{1}H NMR spectra were recorded on a GE GN-500 FT NMR spectrometer using CD_3OD as solvent and an internal standard (δ 3.30 ppm).

Toxicity Testing. Toxicity of the cells and fractions after the first purification step and the purified compounds were tested by mouse bioassay. Cells, fractions from HPLC, and purified compounds were injected intraperitoneally in water solution to mice (20–25 g, female NMRI mice at the University of Helsinki; 20–25 g, male ICR Swiss mice at Wright State University). Mice were observed for 4 h, and signs of poisoning typical for the hepatotoxic microcystins were observed and recorded.

Results and Discussion

Hepatotoxic *Anabaena* sp. Strain 66. A hepatotoxic strain isolated from a hepatotoxic waterbloom at Lake Kiikkara, Finland, was tentatively identified as *Anabaena lemmermannii* (P. Richter). Typical hepatotoxic symptoms were observed when the cultured cells were intraperitoneally administered in mice; death occurred within 1–2 h after injection, and a dark, blood-engorged liver was found in autopsy. There are only two reports concerning the characterization of hepatotoxins from two *Anabaena*

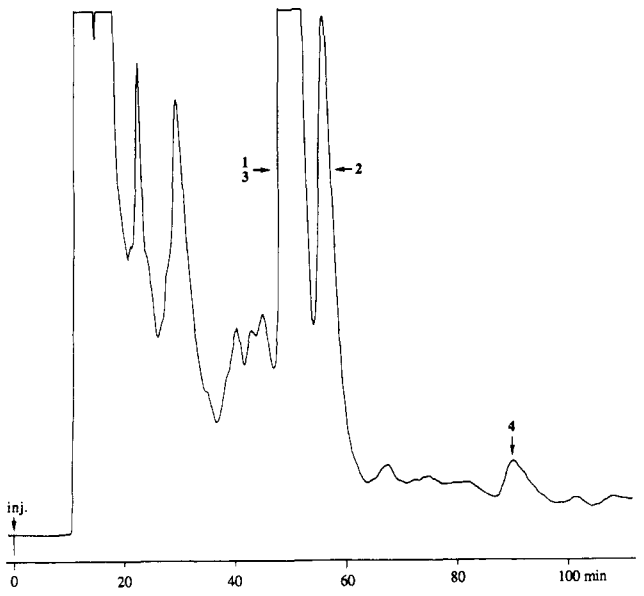


Figure 1. High-performance liquid chromatogram of *Anabaena* sp. 66 showing retention times for compounds 1 and 3 of ~50 min, for compound 2 of ~56 min, and for compound 4 of ~90 min. Peaks eluted within 40 min after injection were pigments and showed no toxicity in the mouse bioassay. Column, C18 silica gel (19 × 300 mm); mobile phase, CH_3CN/10 mM ammonium acetate (26:74); flow rate, 4 mL/min.

strains, which originated from lakes in Canada (*6*, *11*). The survey made in Finland showed, however, that *Anabaena* species like *Anabaena flos-aquae*, *Anabaena circinalis*, and *Anabaena lemmermannii* are important hepatotoxin producers, at least in that particular area (*5*). Toxins from seven strains were isolated and analyzed (*28*). Four microcystins produced by *Anabaena* sp. strain 66 described in this paper have not been previously reported.

Isolation of Toxins. Lyophilized cells (1.5 g) were extracted with the mixture of *n*-BuOH/MeOH/H_2O (1:4:15), and the four compounds **1** (0.4 mg), **2** (0.1 mg), **3** (0.15 mg), and **4** (0.08 mg) were separated by HPLC (Figure 1). Retention times of purified compounds using the ISRP column were (**1**) 6.8, (**2**) 6.3, (**3**) 5.9, and (**4**) 7.9 min. Of the four microcystins isolated from *Anabaena* 66, sufficient amounts of toxins **1**–**3** were purified for preliminary determination of the effect of intraperitoneal bioassay on mice (20–25 g, male ICR Swiss). Symptoms consistent with hepatotoxin poisoning caused by the other microcystins were observed; i.e., death occurred within 1–3 h following injection with internal hemorrhage of the livers, resulting in liver weights of 9–12% of body weights (controls 5–7%). Further determinations of an accurate LD_{50} were not possible because the compounds had been consumed by the structural analysis. Lethal concentrations of microcystins reported in literature vary according to the toxin between 40 and 1000 μg/kg (ip, in mice) (*2*, *3*).

Molecular Weights and Molecular Formulas. The molecular weights of **1**–**4** were determined from the protonated molecular ions, $(M + H)^+$, obtained by FABMS (*31*), and the molecular formulas of **1** ($C_{52}H_{72}N_{10}O_{13}$), **2** ($C_{51}H_{70}N_{10}O_{13}$), **3** ($C_{52}H_{74}N_{10}O_{14}$), and **4** ($C_{52}H_{72}N_{10}O_{12}$) were deduced from HRFABMS data on the $(M + H)^+$ ions as shown in Table I.

Structure of 1. The molecular weight and molecular formula of **1** were identical to those of microcystin-YR (YR, **5**) (*12*, *32*). The amino acid analysis of **1** showed the presence of Ala, MeAsp, Arg, Glu, and an unknown amino acid, but Tyr and methylamine peaks were not detected in the amino acid analysis. Methylamine is generated from the Mdha unit as a degradation product in the acid hydrolysis (*12*).

Table I. High-Resolution FABMS Data for Compounds 1–4

compd	*m/z* (M + H)	composition	Δ[a]
1	1045.5347	$C_{52}H_{73}N_{10}O_{13}$	+1.2
2	1031.5212	$C_{51}H_{71}N_{10}O_{13}$	−1.0
3	1063.5447	$C_{52}H_{75}N_{10}O_{14}$	+1.7
4	1029.5417	$C_{52}H_{73}N_{10}O_{12}$	−0.8

[a] Difference (mmu) from the calculated value for each composition.

The ^1H NMR spectrum of **1** (Figure 2) showed two one-proton singlets ascribable to *exo*-methylene protons at δ 5.34 and 5.53, which were detected at higher field than those of **5** (δ 5.46 and 5.89) (*10*, *12*). An *N*-methyl signal was not detected in the spectrum of **1**, which was observed at δ 3.33 in YR (**5**) (*12*). These data suggested that **1** has a Dha residue instead of Mdha, and the lack of a methylamine peak in the amino acid analysis of **1** supported this assignment.

The presence of the Adda residue in **1** was revealed by the ^1H NMR spectrum of **1** (Figure 2) and confirmed by FABMS/MS (Table II) as described below.

The unknown amino acid found in the amino acid analysis of **1** has a para-substituted phenyl ring as shown by two doublets at (J = 9.0 Hz) δ 6.65 and 7.00 (indicated by a and b, respectively, in the ^1H NMR spectrum of **1**, Figure 2), which strongly suggested that the unknown amino acid is a Tyr analogue.

The total mass of the six amino acid residues is 867 Da. Subtraction of this value from its molecular mass gives 177 Da (Ala, MeAsp, Glu, Arg, Dha, Adda) ($C_{10}H_{11}NO_2$). The residual mass (formula) of the unknown amino acid is 14 Da (CH_2) higher than that of a Tyr residue (163 Da, $C_9H_9NO_2$), suggesting either a methyl or methylene homologue of Tyr. Since there is no methyl signal due to the unknown amino acid residue (besides the signals ascribed to the Ala, Adda, and MeAsp residues) in the ^1H NMR spectrum of **1** (Figure 2), only Hty as shown in Figure 1 is the possible structure for the seventh amino acid. Any of the other possibilities has to show a singlet or a doublet due to a methyl group in the ^1H NMR spectrum.

The Hty residue has been detected in two microcystins recently isolated by Harada et al. (*11*). One of those toxins (**6**, toxin 1 in ref 11)[3] was hydrolyzed and analyzed by GC on a chiral capillary column (Chirasil Val III, 0.32 mm × 25 m), together with the hydrolysate of **1**. The toxins were hydrolyzed with 6 N HCl at 110 °C for 21–24 h, and the hydrolysates were derivatized as their *N*-(trifluoroacetyl) methyl esters (*27*). The GC trace of the hydrolysate of **1** showed D-Ala, D-MeAsp, D-Glu, and a component at longer retention time, which was identified as Hty by coelution with the hydrolysate of **6**. L-Arg was detected as its pyrimidine derivative in the chromatogram operated at 200 °C (*12*, *30*).

The stereochemistry of Hty was determined as L by racemization with DMAP (*26*, *33*). The reaction conditions were first examined using L-Tyr as a standard amino acid, and the reaction mixture showed about a 20:1 L:D ratio after heating at 110 °C for 27 h in a mixture of methanol and water (1:1). The hydrolysate obtained from **1** was treated in a similar manner, which converted L-Hty to a

[3] Toxin **6** was kindly supplied by Dr. Ken-ichi Harada at the Faculty of Pharmacy, Meijo University, Japan.

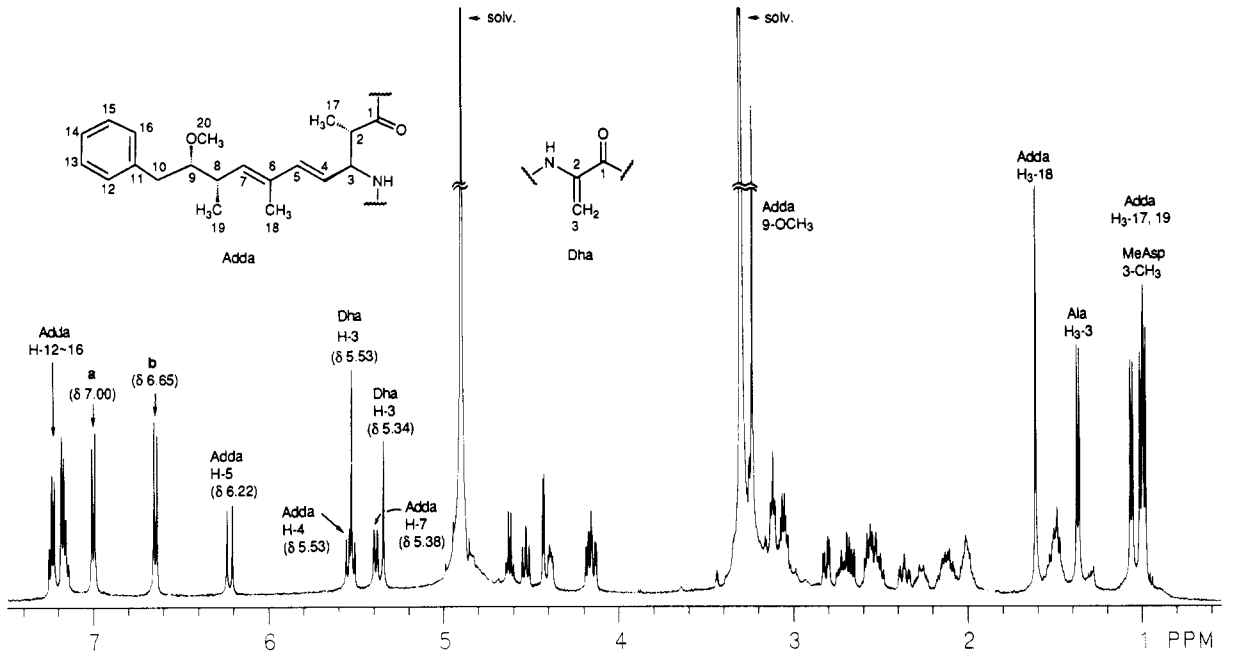


Figure 2. ^{1}H NMR spectrum of 1 obtained on a GE GN-500 FT NMR spectrometer in CD_3OD (δ 3.30 ppm).

Table II. FABMS/MS Data for Compounds 1–3

sequence	fragment ion, m/z 1	2	3
M + H	1045	1031	1063
M − 135	909	895	927
Arg-MeAsp-Hty + H	463	449[a]	463
$C_{11}H_{14}O$-Glu-Dha[b]	wk[c]	361	379[d]
Glu-Dha + H	199	199	217[d]
Dha-Ala + H	141	141	wk[c]
$PhCH_2CH(OCH_3)$	135[e]	135[e]	135[e]

[a] Contains Asp instead of MeAsp (14 Da less than for 1 and 3). [b] $CH_3CH/CHC(CH_3)/CHCH/CHCH(CH_3)CO$-Glu-Mdha − H. [c] Intensity of the corresponding peak was weak. [d] Contains Ser instead of Dha (18 Da higher than for 1 and 2). [e] Base peak of daughter ions.

20:1 mixture after 29 h. The smaller peak eluted faster than the main peak, confirming the L-configuration (*11*). D-Ala and D-Glu showed ratios of 20:1 and 9:1, respectively, and D-MeAsp was detected as a mixture of four stereoisomers in a 9:1:9:1 D-erythro:L-erythro:D-threo:L-threo ratio.

Thus, the seven amino acid components of 1 were determined to be D-Ala, L-Hty, D-MeAsp, L-Arg, Adda, D-Glu, and Dha. The structure of 1 was deduced from the similarity of the ^{1}H NMR spectrum of 1 to that of YR (**5**), except for the signals due to the dehydroamino acid residue, and confirmed by the analysis of FABMS/MS data.

The $(M + H)^+$ ion of 1 obtained by FABMS was subjected to tandem MS to afford product ion peaks. Some useful peaks are summarized in Table II (*12*, *26*, *27*). The fragment ion peaks at m/z 135 and M − 135 confirmed the presence of Adda (*12*). The sequences Glu-Dha-Ala and Arg-MeAsp-Hty were revealed by the fragment ion peaks at m/z 141 and 199 and at m/z 463, respectively. The structure of 1 is, therefore, assigned as shown in Chart I and named [Dha^7]microcystin-HtyR.

Structure of 2. The molecular mass (1030 Da) and molecular formula ($C_{51}H_{70}N_{10}O_{13}$) of **2** were respectively 14 Da and CH_2 less than those of 1 (Table I). The difference was shown in the amino acid analyses, in which **2** gave D-Asp instead of the D-MeAsp detected in 1, together with D-Ala, L-Hty, L-Arg, and D-Glu. A methylamine peak was not detected in the amino acid analysis, suggesting the presence of Dha. The fragment ion peaks at m/z 135 and M − 135 observed by FABMS/MS (Table II, Figure 3) confirmed the Adda residue as the seventh amino acid component.

The amounts isolated were not sufficient for NMR experiments, but the sequence could be assigned by FABMS/MS analysis. Fragment ion peaks at m/z 141 and 199 revealed the sequence Glu-Dha-Ala, and a fragment ion peak at m/z 361 was ascribable to the tripeptide consisting of Glu, Dha, and a residual component of Adda generated by the loss of $PhCH_2CH(OCH_3)$ (*12*). The sequence Arg-Asp-Hty was shown by the fragment ion peak at m/z 449, which was observed at 14 Da lower mass than the corresponding peak of 1, showing the replacement of MeAsp in 1 by Asp in **2**. Thus, compound **2** is assigned the structure shown in Chart I, namely, [D-Asp^3,Dha^7]-microcystin-HtyR.

Structure of 3. The amino acid analyses of **3** showed D-Ala, L-Hty, D-MeAsp, L-Arg, D-Glu, and L-Ser. The presence of the Adda residue was revealed by the fragment ion peaks at m/z 135 and M − 135 obtained by FABMS/MS of **3** (Table II). The total mass of the seven amino acid residues explained its molecular mass (1062 Da), which is 18 Da (H_2O) higher than the molecular mass (formula) of 1 (Table I). The difference is ascribable to the replacement of Dha in 1 with L-Ser in **3**.

These data suggest that **3** is an L-Ser variant of 1 at the Dha residue. The fragment ion peaks at m/z 379 ($C_{11}H_{14}O$ − Glu − Ser) and 217 (Glu − Ser + H) in the FABMS/MS spectrum of **3** were each 18 mu higher than the corresponding peaks of 1 and **2**, while the peak at m/z 463 (Arg − MeAsp − Hty + H) in the spectrum of **3** was detected at the same mass as that of 1 (Table II), confirming the presumed structure of **3**, [L-Ser^7]microcystin-HtyR, as shown in Chart I.

Structure of 4. The molecular mass and molecular formula of **4** (1028 Da, $C_{52}H_{72}N_{10}O_{12}$) were the same as those of microcystin-FR (**7**) (*12*); however, **4** showed neither a Phe nor a methylamine peak in its amino acid analysis. The amino acid analysis of **4** showed Ala, Arg, Glu, MeAsp, and an unknown amino acid, which had a longer retention

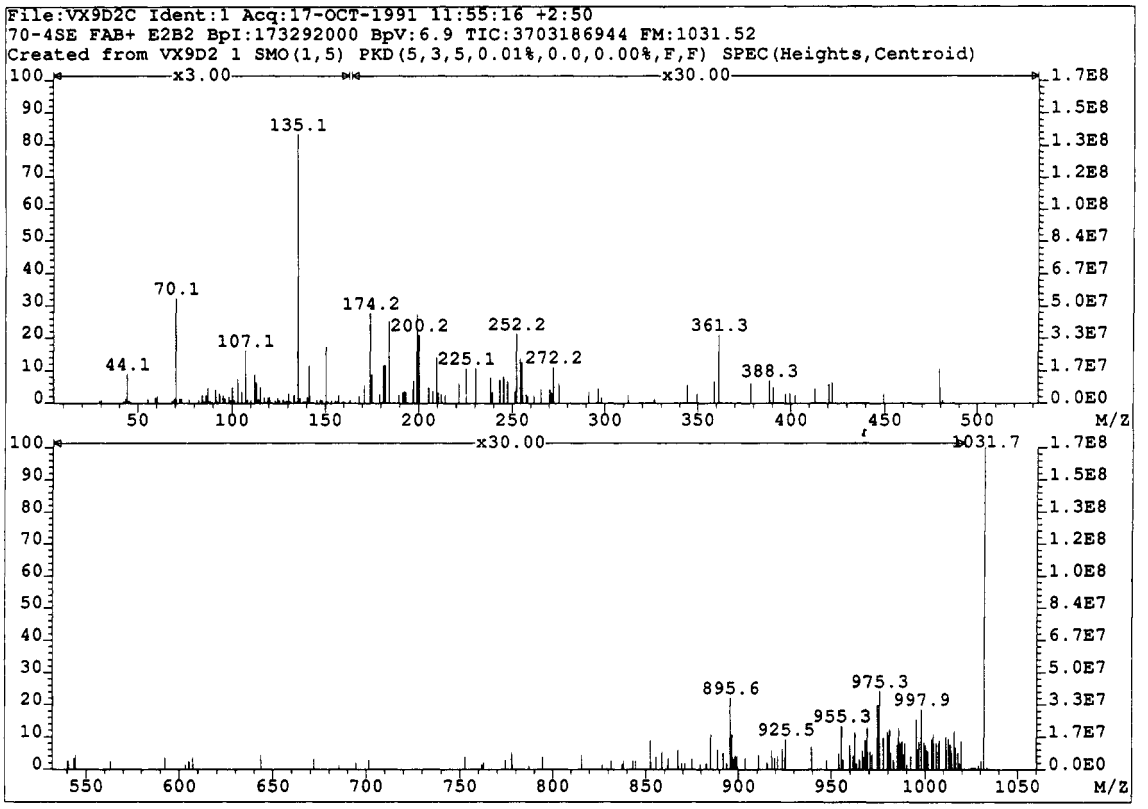


Figure 3. FABMS/MS spectrum of compound **2** obtained on a VG70-SE4F mass spectrometer.

time and was not identical with Hty. The lack of a methylamine peak in the amino acid analysis of **4** suggested the presence of a Dha residue in **4**. The unknown amino acid was, therefore, suggested as a Phe homologue, and the commercially available homophenylalanine (Hph) was identical with this amino acid. The stereochemistries of the amino acid components were determined by the same procedure as the other toxins, **1–3**, using the chiral GC capillary column (Chirasil Val III), and Hph was compared by its retention time on GC with those of commercial DL-Hph, all as their *N*-(trifluoroacetyl) methyl ester derivatives (Figure 4). Although the stereochemistry of Arg could not be assigned because the sample amount was not enough, it should be the L-configuration by analogy to the other components (**1–3**).

Subtraction of the total mass (formula) for the six amino acid residues from the molecular mass (formula) of **4** indicated an Adda residue (313 Da, $C_{20}H_{27}NO_2$). Compound **4** did not give good FABMS/MS data because of the inadequate sample size; but the data argued the presence of the Adda unit by a peak at *m/z* 135. The Adda unit should also be in the molecule on the basis of its hepatotoxicity, similar to that of other toxic microcystins.

The structure of **4** is tentatively assigned as shown in Chart I by analogy to the other toxin components, **1–3**. A rigorous structure assignment of **4** will be made as more material is obtained.

Conclusion. The hepatotoxic filamentous *Anabaena* sp. strain 66 isolated from a hepatotoxic water bloom in Lake Kiikkara, Finland, was found to contain four new microcystins that were assigned using HRFABMS and FABMS/MS. Three toxins, **1–3**, contained L-Hty as a variable L-amino acid. Another toxin, **4**, had a new amino acid variation among microcystins, with L-Hph at the same position as L-Hty. Compound **3** is possibly a biosynthetic precursor of **1**, since dehydroalanine residues are biosynthetically derived from serine (*12*, *34*). The organism produced the demethyl variant at the dehydroamino acid or its derivative residue.

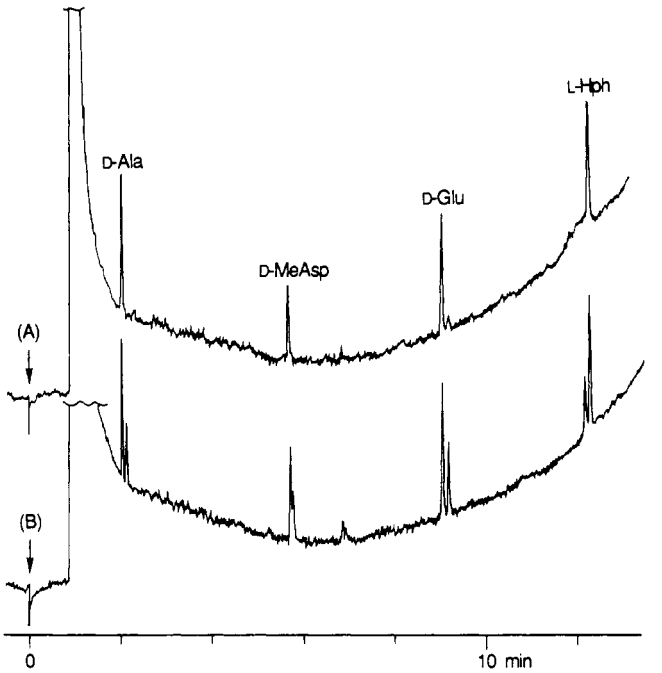


Figure 4. Gas chromatograms of (A) the hydrolysate of toxin **4** and (B) a mixture of the hydrolysate of **4** and authentic DL-amino acids detected as *N*-(trifluoroacetyl) methyl ester derivatives on a Varian 3700 gas chromatograph with a Chirasil Val III column (0.32 mm × 25 m). The D-isomer elutes faster than the L-isomer, which was also confirmed by the coelution of DL mixtures and L-isomers of authentic amino acids.

Several additional hepatotoxic strains of *Anabaena* spp. have been isolated (*28*), and the hepatotoxic components of these organisms are being analyzed, which will be

described in future papers.

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