Seco[D-Asp^3]microcystin-RR and [D-Asp^3,D-Glu(OMe)^6]microcystin-RR, Two New Microcystins from a Toxic Water Bloom of the Cyanobacterium *Planktothrix rubescens*

Olga Grach-Pogrebinsky,[†] Bojan Sedmak,[‡] and Shmuel Carmeli*[,†]

School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv Tel Aviv 69978, Israel, and National Institute of Biology, Vecna pot 111, POB 141, 1001 Ljubljana, Slovenia

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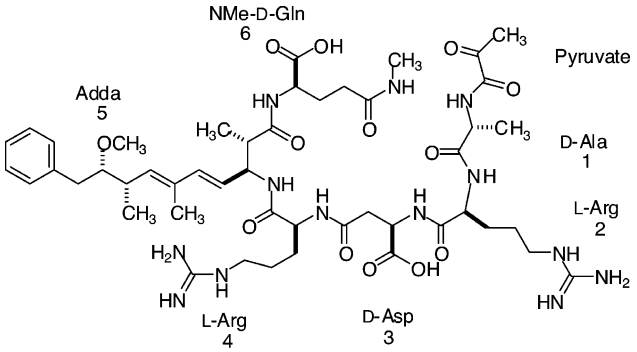
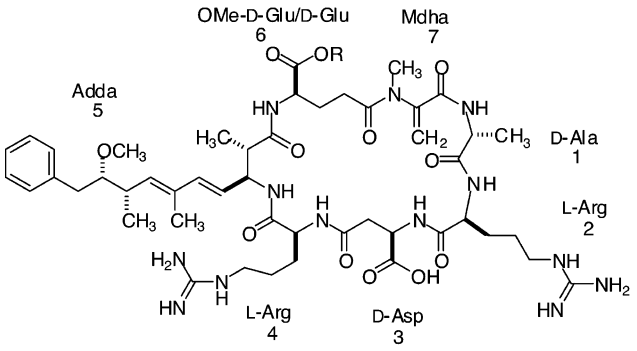
Two novel microcystins, *seco*[D-Asp^3]microcystin-RR (**1**) and [D-Asp^3,D-Glu(OMe)^6]-microcystin-RR (**2**), along with the known [D-Asp^3]microcystin-RR (**3**), were isolated from a *Planktothrix rubescens* toxic bloom collected in Lake Bled, Slovenia. The structures were deduced using one- and two-dimensional NMR techniques, ESIMS/CID/MS analysis, and Marfey's method for determining the amino acids' absolute stereochemistry. Compounds **1** and **3** exhibit weak PP1 inhibitory activity. The NMR data of compound **3** are reported here for the first time.

The hepatotoxic microcystins are the most frequently monitored toxins in cyanobacteria water blooms. These cyclic heptapeptides contain a unique hydrophobic amino acid, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), a D-alanine at a position designated 1, two other D-amino acids at positions 3 and 6, 3-methyl aspartic acid or aspartic acid (Masp/Asp) and glutamic acid (Glu), respectively, that are linked by an iso-linkage, and two variable L-amino acids at positions 2 and 4. Microcystins[1] are produced by several genera of cyanobacteria, including *Microcystis*, *Anabeana*, *Nostoc*, and *Planktothrix* (*Oscillatoria*).[2] The major toxin found in various *Planktothrix* species is [D-Asp^3]microcystin-RR, where the variable amino acids are Arg^2 and Arg^4. [D-Asp^3]microcystin-RR is usually accompanied by closely related derivatives,[3−5] although the presence of microcystin-YR (Tyr^2, Masp^3, and Arg^4) and microcystin-LR (Leu^2, Masp^3, and Arg^4) and related compounds were also reported.[3,6,7] The microcystins are stable in the mammalian gastrointestinal system and are absorbed in the gastrointestinal tract, transported to the liver, and concentrated there.[8] Within the liver cells, the microcystins inhibit specific protein phosphatases (PPs), PP1 and PP2A, which are key components for the control of the cell structure and function. Recently, it was found that the inhibitory activity against protein phosphatases is not always related to the apparent LD_{50} level, and the appearance of toxicity by microcystins depends on the balance between their accumulation and metabolism in the liver.[9] All the toxic microcystins reported thus far are cyclic. The cyclic form was found to be crucial for the toxicity, since the linear peptides isolated from *Nodularia spumigena* and *Microcystis* spp. having the same amino acid sequence as their cyclic analogues do not show apparent toxicity to mice.[10] These linear peptides are presumed to be biogenetic precursors of microcystins. We report here the isolation and structure elucidation of a linear *seco*[D-Asp^3]microcystin-RR (**1**), where the open chain structure is probably obtained through hydrolytic cleavage between the amine and the α-carbon of Dha, to yield a pyruvic acid residue and an ϵ-*N*-methyl glutamine. In addition we isolated two other cyclic microcystins, the novel [D-Asp^3,D-Glu(OMe)^6]microcystin-RR (**2**) and the known [D-Asp^3]microcystin-RR (**3**).[11]



seco[D-Asp^3]microcystin-RR (**1**)



[D-Asp^3,D-Glu(OCH$_3$)^6]microcystin-RR (**2**): R=CH$_3$
[D-Asp^3]microcystin-RR (**3**): R=H

Results and Discussion

As part of our ongoing research on a toxic strain of the cyanobacterium *Planktothrix rubescens*,[12] a sample of the cyanobacterium was freeze-dried and extracted with 70% MeOH in H_2O. The extract was flash chromatographed on an ODS column with gradient elution (water to MeOH in

* To whom correspondence should be addressed. Tel: ++972-3-6408550. E-mail: Carmeli@post.tau.ac.il.
† Tel Aviv University.
‡ National Institute of Biology.

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Table 1. NMR Data of *Seco*[D-Asp^3]microcystin RR (**1**) in DMSO-d_6 [a]

position		δ_C/δ_N, mult.[b]	δ_H, mult., J (Hz)	LR H−C/H−N correlations[c]	NOE correlations[d]
pyruvic acid	1	160.6 s		Ala-2,NH, Pyr.ac.-3	
	2	196.6 s		Pyr.ac.-3	
	3	24.9 q	2.29 s		Ala-2
Ala	1	171.6 s		Ala-3, Arg^2-NH	
	2	48.6 d	4.29 m	Ala-NH	Pyr.ac.-3
	3	18.4 q	1.23 d 7.2	Ala-NH	Arg^2-NH
	NH	114.8 d	8.33 d 7.2	Ala-3	
Arg^2	1	170.8 s		Asp-NH	
	2	52.0 d	4.26 m	Arg^2-NH	Arg^2-3,NH
	3	29.4 t	1.7 m	Arg^2-4,5,NH	Arg^2-2
			1.4 m	Arg^2-4,5,NH	Arg^2-2
	4	25.0 t	1.35 m	Arg^2-3	
	5	41.0 t	3.02 br	Arg^2-3,6	Arg^2-NH
	NH	84.0 d	7.73 m		Arg^2-5
	6	157.0 s		Arg^2-5	
	NH	74.0 d	6.90−7.40 br		
	NH_2	74.0 t	6.90−7.40 br		
	NH	116.1 d	8.20 m		Arg^2-2, Ala-3
Asp	1	172.7 s		Asp-3	
	2	49.2 d	4.50 q 6.8	Asp-3	Asp-NH
	3	37.6 t	2.68 dd 6.8 13.6	Asp-2	
			2.37 dd 6.8 13.6	Asp-2,4	
	4	169.7 s		Arg^4-NH, Asp-2,3	
	NH	117.8 d	8.19 m		Asp-2
Arg^4	1	170.9 s		Adda-NH	
	2	52.6 d	4.15 m		Arg^4-NH, Adda-NH
	3	25.0 t	1.4 m		Arg^4-NH
	4	25.0 t	1.35 m		
	5	41.0 t	3.07 br		Adda-13,13′
	NH	84.0 d	7.68 m		Arg^4-3
	6	157.0 s		Arg^4-5	
	NH	74.0 d	6.90−7.40 br		
	NH_2	74.0 t	6.90−7.40 br		
	NH	121.3 d	8.23 m		Arg^4-2
Adda	1	174.2 s		Adda-15, NMeGlu-NH	
	2	42.8 d	2.57 m	Adda-15	Adda-5
	3	53.2 d	4.33 m	Adda-15	Adda-7,NH
	4	126.2 d	5.39 dd 6.0 15.6		
	5	135.1 d	6.0 d 15.6		Adda-2,15,16
	6	132.1 s		Adda-16	
	7	135.5 d	5.32 d 9.6	Adda-9,17	Adda-3,9,10,15,16,17
	8	35.5 d	2.47 br	Adda-9,10,1617	Adda-12,12′
	9	85.8 d	3.18 q 5.6	Adda-7,8,17,18	Adda-7,12,12′
	10	37.1 t	2.60 m	Adda-8,12,12′	Adda-7
	11	139.4 s		Adda-9,10	
	12,12′	129.4 d (×2)	7.15 d (×2) 8.0	Adda-10	Adda-8,9
	13,13′	128.2 d (×2)	7.22 t (×2) 8.0		Arg4−5
	14	126.0 d	7.13 t 8.0		Adda-17
	15	15.1 q	0.92 d 6.4	Adda-2,3	Adda-5,7,NH
	16	12.5 q	1.46 s	Adda-5,7	Adda-5,7
	17	15.9 q	0.89 d 6.4	Adda-8,9	Adda-7,14
	18(OMe)	57.5 q	3.12 s	Adda-9	
	NH	117.4 d	7.91 d 8.4		Arg^4-2, Adda-3,15
NMeGln	1	173.6 s		NMeGln-3	
	2	51.9 d	4.07 q 7.0		NMeGln-3,4,NH
	3	27.5 t	1.83 m	NMeGln-NH	NMeGln-2,4
			1.71 m	NMeGln-NH	NMeGln-2,4
	4	31.9 t	2.03 m	NMeGln-NMe	NMeGln-NH
	5	172.1 s		NMeGln-NH,NHMe	
	5-NH	103.8 d	7.69 m	NMeGln-4	NMeGln-4,NHMe
	NH*Me*	25.7 q	2.48 d 3.6		NMeGln-NH
	2-NH	123.7 d	8.18 m		NMeGln-2

[a] Carried out on a Bruker Avance 400 spectrometer. [b] Multiplicity and assignment from HMQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s. The HMBC correlations are reported as correlations of the protons printed in the column with the carbons or nitrogen in the rows. [d] By ROESY experiment.

10% steps). Fractions 6 and 7 were further separated on a reversed-phase HPLC column. A number of subsequent separations yielded the new *seco*[D-Asp^3]microcystin-RR (**1**) (23.6 mg) and [D-Asp^3, D-Glu(OMe)^6]microcystin-RR (**2**) (2.1 mg), along with the known [D-Asp^3]microcystin-RR (**3**) (22.5 mg).

Seco[D-Asp^3]microcystin-RR, **1**, was isolated from the HPLC as an amorphous white solid. The MALDI TOF MS of **1** showed a protonated molecular ion peak at *m*/*z* 1042 amu. High-resolution MALDI TOF MS measurement of the protonated molecular ion cluster established for **1** a molecular formula of $C_{48}H_{75}N_{13}O_{13}$, requiring 18 degrees of unsaturation. A cursory examination of the ^{1}H NMR of **1** in DMSO revealed signals of a monosubstituted aromatic ring at 7.10−7.25 ppm (5H), a trisubstituted diene system at 5.3−6.0 ppm (3H), and a methoxyl at 3.12 ppm, a clue

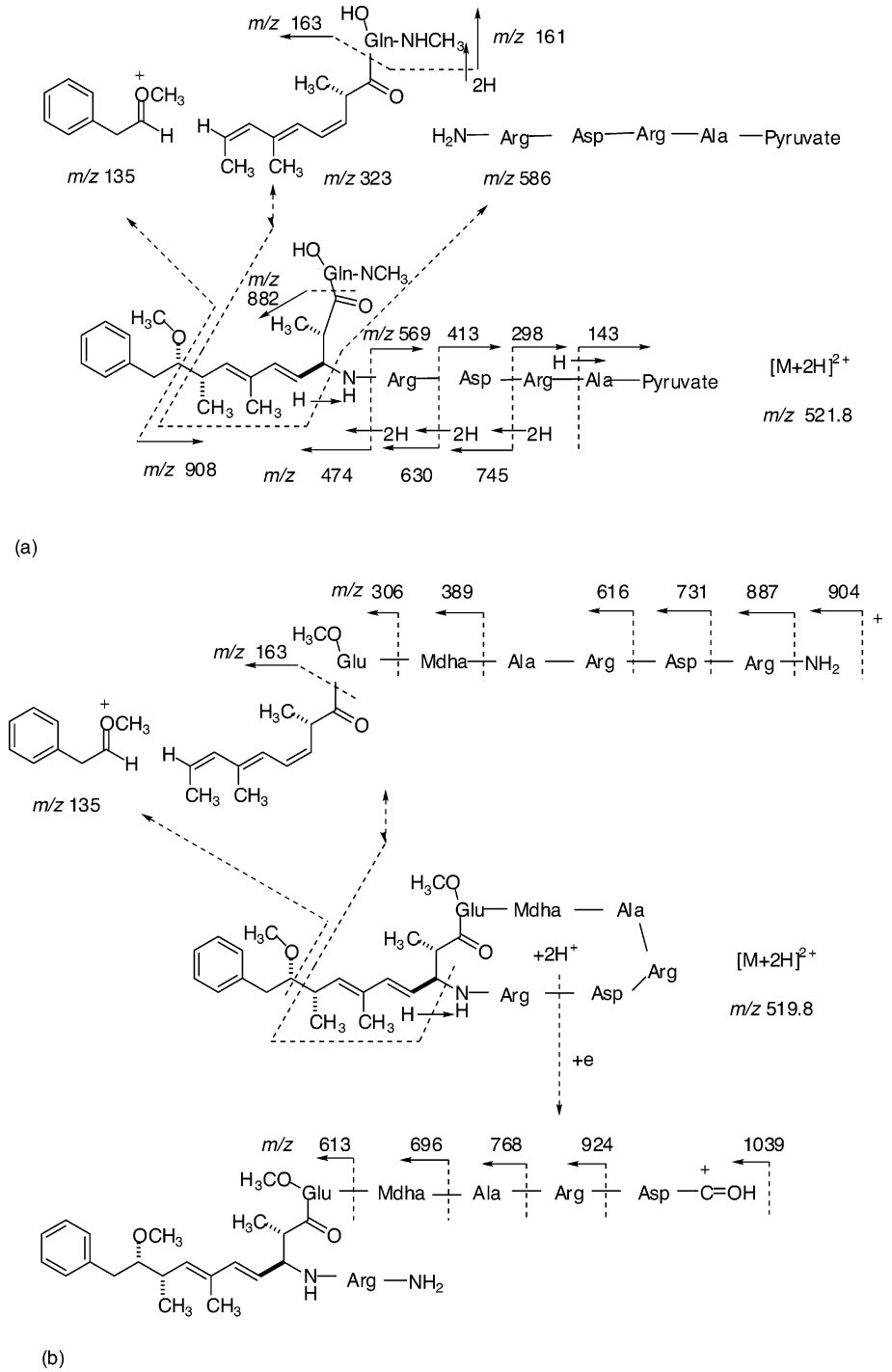

Figure 1. Characteristic fragmentation patterns observed in ESIMS/CID/MS of $[M + 2H]^{2+}$ of compounds **1** (a) and **2** (b).

for the presence of an Adda (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid) residue in **1**. Comparison of the ^{1}H NMR data of **1** with the published data for [D-Leu^1]microcystin-LR[13] proposed that **1** indeed belongs to the microcystins. A literature search revealed two possible known structures, [D-Asp^3,Mser^7]microcystin-RR[14] and [L-Ser^7]microcystin-RR[7] with a molecular mass of 1041 amu and a molecular formula of $C_{48}H_{75}N_{13}O_{13}$. However, a detailed 1D and 2D NMR study revoked the possibility that **1** is one of these microcystins, since it does not contain a serine group at position 7. The ^{13}C NMR spectrum revealed a signal with an unusual chemical shift for a microcystin (δ_C 196.6 ppm, s), which indicated the presence of a conjugated ketone. This signal was assigned as C-2 of a pyruvate residue. Analysis of the 1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **1** (see Table 1) established six amino acid units and an α-keto acid unit that built *seco*[D-Asp^3]-microcystin-RR (**1**), namely, alanine, arginine^2, aspartic acid, arginine^4, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), ϵ-*N*-methyl glutamine, and pyruvic acid. The amino acid sequence of **1** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (pyruvate−Ala, Ala−Arg^2, Arg^2−Asp, Asp C-4−Arg^4, Arg^4−Adda, Adda−NMeGln), where the aspartic acid is bound with an iso-linkage (C-4) to the neighboring arginine^4 (NH). The amino acid sequence could also be assembled from the ROESY data (see Table 1). These seven acid units account for all 18 degrees of unsaturation of **1**, suggesting that it

Table 2. Assignment of the Peaks in the ESIMS/CID/Mass Spectra of $[M + 2H]^{2+}$ Ions of [D-Asp[3],D-Glu(OMe)[6]]Microcystin RR (**2**) and [D-Asp[3]]Microcystin RR (**3**)[a]

ion composition	ion type	**2**	**3**
$[M+2H]^{2+}$		519.7732	512.7680
$[M+2H-CO]^{2+}$		505.8365	498.8111
$[M+2H-135]^{2+}$		452.7564	445.7492
$[M+2H-NH_3]^{2+}$		511.3384	504.2959
$[M+2H-2NH_3]^{2+}$		503.8268	496.7982
[Arg−Adda−X*−Mdha−Ala−Arg+2H]$^{2+}$		462.2689	455.2717
[Arg−Adda−X−Mdha−Ala−Arg−NH_2+2H]$^{2+}$		471.3024	464.2834
[CO−Arg−Adda−X−Mdha−Ala−Arg−NH_2+2H]$^{2+}$		485.3199	477.2898
[Mdha−Ala−Arg−Asp−Arg+2H]$^{2+}$		291.1737	291.1745
[Arg−Asp−Arg−Adda−X−Mdha+2H]$^{2+}$		484.3036	477.2898
$[M+2H-135]^{+}$	B	904.5014	890.5101
$[M+2H-135-NH_3]^{+}$	B	887.4778	873.4764
$[M+2H-135-NH_3-Arg]^{+}$	B	731.4107	717.4053
$[M+2H-135-NH_3-Arg-Asp]^{+}$	B	616.3636	602.3469
$[M+2H-135-NH_3-Arg-Asp-Arg-Ala-Mdha]^{+}$	B	306.1769	292.1673
$[X+H]^{+}$	B	144.0722	130.0518
$[X-Mdha+H]^{+}$	B	227.1057	213.0956
$[Mdha-Ala+H]^{+}$	B	155.0875	155.0874
$[Mdha-Ala-Arg+H]^{+}$	B	311.1894	311.1901
$[Mdha-Ala-Arg-Asp+H]^{+}$	B	426.2185	426.2149
$[Mdha-Ala-Arg-Asp-Arg+H]^{+}$	B	582.3253	582.3210
$[Mdha-Ala-Arg-Asp-Arg-NH_3+H]^{+}$		565.2985	565.2940
$[Arg-Asp+H]^{+}$	B	272.1405	272.1382
$[Arg-Adda-X+H]^{+}$	B	613.3902	599.3658
$[Arg-Adda-X-Mdha+H]^{+}$	B	696.4231	682.4086
$[Arg-Adda-X-Mdha-Ala+2H]^{2+}$		768.4605	754.4660
$[Arg-Adda-X-Mdha-Ala-Arg+2H]^{2+}$		924.5123	910.5224
$[Arg-(Adda-135+H)-X-Mdha-Ala-NH_2+2H]^{+}$	C″	634.3742	620.3539
$[Mdha-Ala-Arg-Asp-Arg-NH_2+2H]^{+}$	C″	599.3559	599.3658
$[Asp-Arg-NH_2+2H]^{+}$	C″	289.1667	289.1669
$[Ala-Arg-CO+H]^{+}$		200.1244	200.1266

[a] X = Glu(OMe) in **2** and Glu in **3**.

has a linear peptide structure. This finding is supported by the multiplicity of the amide *N*-methyl signal of NMeGln, a doublet, which is coupled with an amide proton signal (δ_H 7.69 ppm, m), and the pyruvic acid residue, which substitutes the N-terminus, alanine. $^1H-^{15}N$ HSQC and HMBC experiments allowed the assignment of the ^{15}N signals of the various amino acids of **1** (see Table 1). All the secondary amide nitrogen signals resonated in the expected region (δ_N 110−125 ppm, relative to ammonia). The guanidine residues of the two arginine moieties resonated in the expected region of 70−85 ppm when the same chemical shift was recorded for the two terminal guanidinium nitrogens, possibly indicating a fast resonance of the double bond. Acid hydrolysis of *seco*[D-Asp[3]]microcystin RR (**1**) and derivatization with Marfey's reagent,[15] followed by HPLC analysis, demonstrated the L-stereochemistry of arginine and D-stereochemistry of alanine, aspartic acid, and *N*-methyl-substituted glutamine. The stereochemistry of Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid] was determined to be identical to the same residue in the known [D-Asp[3]]-microcystin-RR (**3**) by comparison of their retention times under the same conditions. The low-energy CID/MS spectrum obtained for **1** from the $[M + 2H]^{2+}$ ion presented a set of strong b- and y-type fragmentations (see Figure 1a). A typical fragmentation of the Adda residue next to the *O*-methyl group yielded the fragments at *m*/*z* 135, 908, 323 and 586 mass units. This MS/MS spectrum is different from the spectra measured for the cyclic microcystines, which typically produce only abundant b-type fragmentations (see below).

[D-Asp[3],D-Glu(OMe)[6]]microcystin RR (**2**) presented ^1H NMR and 2D COSY spectra almost identical to [D-Asp[3]]-microcystin RR (**3**). The only difference was an additional methoxyl group in the spectrum of **2**. Due to the small amount of **2**, we could not obtain good ^{13}C, HMQC, and HMBC NMR spectra and thus could not elucidate the structure of **2**, unequivocally. The MALDI TOF MS measurement furnished a sodiated molecular ion cluster at *m*/*z* 1060 amu, for which a $C_{49}H_{75}N_{13}NaO_{12}$ molecular formula was calculated. This molecular formula is in agreement with the finding from the NMR data, explained above. To solve the structure of **2** unequivocally, we measured an ESIMS/CID/MS spectrum for **2**. The low-energy CID/MS spectrum obtained for **2** from the $[M + 2H]^{2+}$ ion presented a set of strong b-type fragmentations (see Figure 1b and Table 2) that assigned the methyl group to the carboxyl of the glutamic acid at position 6. Two sets of b-type fragmentations derived from the cleavage of the peptide ring between Adda-NH and Adda-C-3 and between Arg[4]-NH and Asp[3]-4-carboxyl (see Figure 2b) were the most abundant mono-charged fragments in the CID spectrum. In addition to these two sets of fragments, the occurrence of two b-type fragments, $[Glu(OMe) + H]^+$ (*m*/*z* 144) and [Glu-

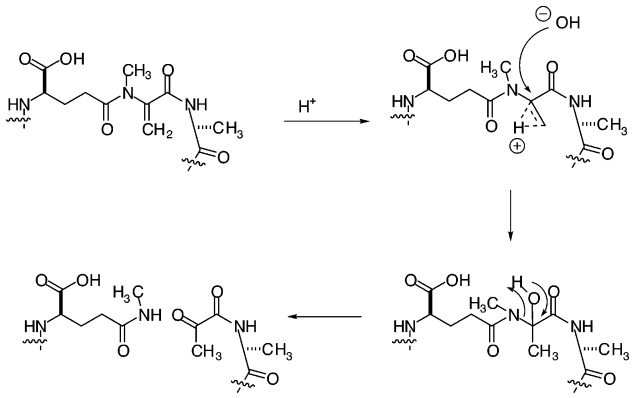

Figure 2. Possible hydrolytic scheme of compound **3**.

(OMe) − Mdha + H]$^+$ (*m*/*z* 227), further supports the suggested structure for **2**. Acid hydrolysis of **2** and derivatization with Marfey's reagent,[15] followed by HPLC analysis, gave a chromatogram identical to that of **1**, thus suggesting an identical stereochemistry for all of the amino acids in **1** and **2**.

The structure of compound **3** was elucidated using similar methods. The molecular formula of **3**, $C_{48}H_{73}N_{13}O_{12}$, was deduced from a high-resolution MALDI TOF MS measurement. Analysis of 1D (see Experimental Section) and 2D NMR data revealed that **3** is the known toxic heptapeptide [D-Asp^3]microcystin RR.[11] ESIMS/CID/MS data (see Table 2) verified the identification of **3**.

The cleavage of the Mdha in **3** to afford **1** may occur spontaneously under acidic conditions (see Figure 2) or by a unique enzymatic cleavage. To preclude the possibility that **3** was converted to **1** or **2** under the acidic conditions used in the isolation procedure of **1**−**3**, a sample of **3** was dissolved in 6:4 0.1% TFA in water/acetonitrile and another in 1:1 MeOH/water, and the solutions were allowed to stand at room temperature for two weeks. Using ^{1}H NMR we observed no detectable amounts of either **1** or **2** in these samples. We thus believe that compounds **1**−**3** are natural products produced by the cyanobacteria.

A preliminary examination of the inhibitory activity of **1** and **3** for the recombinant protein phosphatase 1 established that *seco*[D-Asp^3]microcystin RR (**1**) and [D-Asp^3]-microcystin RR (**3**) are weak inhibitors of PP1 with an IC_{30} of 0.8 and 7.8 μM, respectively. This is the first report of a linear microcystin (**1**) with a biological activity comparable with the cyclic ones. Further studies of the toxicology of these compounds are under investigation.

Experimental Section

General Experimental Procedures. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). UV spectra were recorded on an Agilent 8453 spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^{1}H and 125.76 MHz for ^{13}C and a Bruker Avance 400 spectrometer at 400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C, and 40.55 MHz for ^{15}N. ^{1}H, ^{13}C, DEPT, gCOSY, gTOCSY, gROESY, gHMQC, gHMBC, NHHSQC, and NHHMBC spectra were recorded using standard Bruker pulse sequences. High-resolution MALDI TOF MS spectra were recorded on an Applied Biosystems Voyager System 4312 instrument. MS/MS data were recorded on an Applied Biosystems Inc. QSTAR Pulsar I mass spectrometer. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystems Inc. diode-array detector and Merck-Hitachi HPLC system (model L-4200 UV−vis detector and model L-6200A Intelligent pump).

Electrospray Ionization Mass Spectrometry. Samples were dissolved in 50% (v/v) methanol/0.5% (v/v) formic acid and diluted 1:1 with a calibration mixture [3 μM sex pheromon inhibitor iPD1 (Bachem)/6 μM CsI (Sigma)/50% (v/v) methanol/1% (v/v) formic acid]. ESI mass spectra were obtained on a QSTAR Pulsar mass spectrometer using a nano-spray source (Protana). The sample was introduced directly into the ion source through a QSTAR capillary. Full scan spectra were acquired in the positive ion mode, using a source potential of 900 V, over the mass range of 50−1700 at 1 s. ESIMS/CID mass spectra were measured using nitrogen as a collision gas (collision energy, 20−40 eV) in the pressure range of 0.1−1 μTorr and scanned at a rate of 1 s per decade through the required mass range. A total of 70−100 scans were accumulated and averaged.

Water Bloom Material. A sample of the cyanobacterium *Planktothrix rubescens* was collected from Lake Bled,[16] Slovenia, on September 30, 1999. The bloom sample (ca. 10 L) was collected by skimming the water surface with a 25-meshplankton net. It was kept cool and brought to the laboratory. Larger particles and zooplankton were removed by using different sized sieves. The sample was additionally purified by sedimentation, achieved by placing the cyanobacteria in glass cylinders under natural light. In this way the cell buoyancy was increased and the cyanobacteria floated to the surface while the remaining algae and zooplankton settled down to the bottom. The cyanobacteria were collected, frozen, and lyophilized.

Isolation Procedure. The freeze-dried cells (180 g) were extracted with 7:3 MeOH/H_2O. The crude extract (20 g) was evaporated and separated on an ODS (YMC-GEL, 120A, 4.4 × 6.4 cm) flash column with an increasing amount of MeOH in water. Fractions 6 and 7 separated in a similar manner. Fraction 6 (60:40 MeOH/H_2O) was subjected to reversed-phase HPLC (YMC ODS-A, 5 μm, 250 × 20 mm, DAD at 238 nm, 65:35 water/acetonitrile, flow rate 5.0 mL/min). A semipure fraction with a retention time of 18 min was subsequently separated on another column (YMC C-8, 5 μm, 250 × 20.0 mm, DAD at 238 nm, 60:40 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compounds **1** and **3** were eluted from the column with retention times of 15.0 and 18.7 min, respectively. The overall quantities and yields (from fractions 6 and 7) based on the dry weight of the bacteria of the compounds **1** and **3** are 23.6 mg (0.013%) and 22.5 mg (0.0125%), respectively. In addition to the pure compounds, a semipure sample, with a retention time of 26 min, was obtained. The latter fraction was subsequently separated on a reversed-phase HPLC column (YMC ODS-AQ 5 μm, 250 × 20.0 mm, DAD at 238 nm, 60:40 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **2** (2.1 mg, 0.0012%) was eluted from the column with a retention time of 13.9 min.

***Seco*[D-Asp^3]microcystin-RR (1):** amorphous white solid; $[\alpha]^{28}_D$ −12° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (ϵ) 203 (45200), 239 (22300) nm; for ^{1}H, ^{13}C, and ^{15}N NMR data, see Table 1; HR MALDI TOF MS *m*/*z* 1042.5725 [MH$^+$] (calcd for $C_{48}H_{76}N_{13}O_{13}$, 1042.5680).

[D-Asp^3,D-Glu(OMe)^6]microcystin-RR (2): colorless oil; $[\alpha]^{25}_D$ +17° (*c* 0.05, MeOH); UV (MeOH) λ_{max} (ϵ) 203 (21000), 239 (13600) nm; NMR data δ_H (500 MHz, CD_3OD) Ala: 4.36 (m, 2-H), 1.24 (d, J = 7.0 Hz, 3-H_3); Arg^2: 4.12 (m, 2-H), 1.88 (m, 3-H_2), 1.60 (m, 4-H_2), 3.07 (m, 5-H_2); Asp: 4.72 (m, 2-H), 2.80 (m, 3-H), 2.45 (m, 3-H′); Arg^4: 4.21 (m, 2-H), 1.90 (m, 3-H), 1.46 (m, 3-H′), 1.46 (m, 4-H_2), 3.05 (m, 5-H_2); Adda: 2.60 (m, 2-H), 4.47 (t, J = 9.5 Hz, 3-H), 5.41 (m, 4-H), 6.13 (d, J = 15.5 Hz, 5-H), 5.33 (d, J = 9.5 Hz, 7-H), 2.51 (m, 8-H), 3.19 (m, 9-H), 2.71 (dd, J = 15.0,5.0 Hz, 10-H), 2.58 (m, 10-H′), 7.08 (d × 2, J = 7.7 Hz, 12,12′-H), 7.16 (t × 2, J = 7.7 Hz, 13,13′-H), 7.07 (t, J = 7.7 Hz, 14-H), 0.97 (d, J = 7 Hz, 15-H3), 1.51 (s, 16-H3), 0.91 (d, J = 7.0 Hz, 17-H_3), 3.14 (s, 18-H3); Glu-(OMe): 4.26 (m, 2-H), 1.85 (m, 3-H), 2.04 (m, 3-H′), 2.59 (br, 4-H), 2.49 (m, 4-H′), 3.59 (s, OCH_3); Mdha: 5.75 (s, 3-H), 5.35 (s, 3-H′), 3.24 (s, *N*-CH_3); HR MALDI TOF MS *m*/*z* 1060.5656 [MNa$^+$] (calcd for $C_{49}H_{75}N_{13}NaO_{12}$, 1060.5550).

[D-Asp^3]microcystin-RR (3): colorless oil; $[\alpha]^{28}_D$ −38° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (ϵ) 203 (22500), 239 (16000) nm; NMR data δ_H (500 MHz, CD3OH) Ala: 4.38 (brt, 2-H), 1.26 (d, J = 7.0 Hz, 3-H_3), 7.97 (d, J = 7.0 Hz, NH); Arg^2: 4.16 (q, J = 5.0 Hz, 2-H), 8.17 (m, 2-NH); 1.90 (m, 3-H_2), 1.64 (m, 4-H_2), 3.09 (m, 5-H_2), 7.34 (brt, J = 5.0 Hz, NH), 6.50−7.20 (brm, NH,NH_2); Asp: 4.70 (m, 2-H), 2.80 (brdd, 3-H), 2.45 (brdd, 3-H′), 8.17 (m, 2-NH); Arg^4: 4.21 (brd, 2-H), 1.92 (m, 3-H), 1.50 (m, 3-H′), 1.49 (m, 4-H_2), 3.08 (m, 5-H_2), 8.75 (d, J = 8.0 Hz, 2-NH), 7.39 (brt, J = 5.2 Hz, NH), 6.50−7.20 (brm, NH,NH_2); Adda: 2.64 (m, 2-H), 4.51 (t, J = 10.0 Hz, 3-H), 5.37 (m, 4-H), 6.16 (d, J = 15.0 Hz, 5-H), 5.35 (d, J = 10.3 Hz, 7-H), 2.53 (m, 8-H), 3.17 (m, 9-H), 2.73 (dd, J = 15.0, 5.1 Hz, 10-H), 2.58 (brd, J = 15.0 Hz, 10-H′), 7.10 (d × 2, J = 8.1 Hz, 12,12′-H), 7.16 (t × 2, J = 8.1 Hz, 13,13′-H), 7.09 (t, J = 8.1 Hz, 14-H), 1.01 (d, J = 7.3 Hz, 15-H_3), 1.53 (s, 16-H_3), 0.93 (d, J = 10.1 Hz, 17-H_3), 3.16 (s, 18-H_3), 7,72 (d, J = 9.0 Hz, NH); Glu: 4.27 (brt, 2-H), 1.85 (m, 3-H), 2.04 (m, 3-H′), 2.60 (br, 4-H), 2.5 (m, 4-H′), 7.85 (brd, J = 5.5 Hz, NH); Mdha: 5.75 (s, 3-H), 5.36 (s, 3-H′), 3.26 (s, *N*-CH_3); δ_C (125 MHz,CD_3OH) Ala: 175.4 (s, C-1),

50.5 (d, C-2), 16.9 (q, C-3); Arg[2]: 174.0 (s, C-1), 55.9 (d, C-2), 29.3 (t, C-3), 26.5 (t, C-4), 41.9 (t, C-5), 158.6 (s, C-6); Asp: 174.7 (s, C-1), 50.5 (d, C-2), 37.4 (t, C-3), 176.5 (s, C-4), Arg[4]: 172.2 (s, C-1), 54.0 (d, C-2), 29.1 (t, C-3), 26.6 (t, C-4), 41.9 (t, C-5), 158.6 (s, C-6); Adda: 176.9 (s, C-1), 45.7 (d, C-2), 56.1 (d, C-3), 126.1 (d, C-4), 139.0 (d, C-5), 133.8(s, C-6), 137.4 (d, C-7), 37.6 (d, C-8), 88.3 (d, C-9), 38.9 (t, C-10), 140.5 (s, C-11), 130.5 (d × 2, C-12,12′), 129.2 (d × 2, C-13,13′), 127.1 (d, C-14), 16.4 (q, C-15), 12.9 (q, C-16), 16.5 (q, C-17), 58.7 (q, C-18); Glu: 175.4 (s, C-1), 53.5 (d, C-2), 28.5 (t, C-3), 32.9 (t, C-4), 175.4 (s, C-5); Mdha: 166.7 (s, C-1), 146.4 (s, C-2), 114.3 (t, C-3), 38.5 (q, C-4). HR MALDI TOF MS *m*/*z* 1024.5592 [MH^+] (calcd for $C_{48}H_{74}N_{13}O_{12}$, 1024.5574).

Determination of the Absolute Configuration of the Amino Acids. Compounds **1**, **2**, and **3** (0.5 mg) were dissolved in 6 N HCl (1 mL). The reaction mixture was placed in a sealed glass bomb and kept at 110 °C overnight. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (100 μL) and derivatized with Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA). The *N*-[(-dinitrophenyl)-5-L-alanine amide]-1-amino acid (AA) derivatives, from hydrolysates, were compared with similarly derivatized standard AA by HPLC analysis: Purospher STAR RP-18e 5 μm, 4.6 × 250 mm, flow rate 1.00 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/acetonitrile to 1:1 TEAP/acetonitrile within 60 min. The absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids. Retention times of the derivatized amino acids were as follows: L-Arg, 23.3 min; D-Arg, 25.6 min; L-Asp, 28.9 min; D-Asp, 31.0 min; L-Glu, 29.0 min; D-Glu, 31.7 min; L-Ala, 39.5 min; D-Ala, 44.0 min. HPLC analysis of derivatized hydrolysates of **1**, **2**, and **3** established L-Arg, D-Asp, D-Glu, and D-Ala. For the determination of the stereochemistry of Adda, the hydrolysates of compounds **1** and **2** were compared with that of **3**, and the retention time of FDAA-derivatized Adda under the same Marfey's analysis conditions was identical.

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References and Notes

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(16) Sampling site: Lake Bled: Latitude N (°) 46.362839, Longitude E (°) 14.098068, height a.s.l. (m) 475. The lake is 2120 m long, 1080 m wide, 30 m deep, and ca. 14 000 years old. Presently contains 31 × 10^6 m^3 water with a retention time of 3 years. The average water temperature is 12 °C, in summer reaches 24 °C, and is covered with ice in winter. The productivity of the lake is normally low, and the inflows are rich and permanent. The nutrients in the phase of summer stratification diffuse from the lake bottom and support metalimnetic blooms. Almost every year *Planktothrix rubescens* blooms appear in the lake, which in favorable meteorological and climatic conditions migrate to the surface, frequently covering almost the entire lake surface (Sedmak, B.; Kosi G. *Acta Biol. Slovenica* **2002**, *45*, 17−30).

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