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Supplementary Material Available: ^1H and ^{13}C NMR spectra for compounds 1 and 2 and crystal data for compound 2 (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

Identification of 12 Hepatotoxins from a Homer Lake Bloom of the Cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: Nine New Microcystins

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Eleven minor components were isolated, together with microcystin-LR (LR, 1, Scheme I) as the principal toxin (ca. 90% of the toxic components), from *Microcystis* cyanobacteria (blue-green algae) collected from Homer Lake (Illinois) in the summer of 1988. The components were characterized by amino acid analysis and HRFABMS, FABMS/MS, ^1H NMR, and UV spectroscopic methods as microcystins-RR (2) and -YR (3) (Scheme I) and nine new microcystins. The structures of seven new microcystins were assigned as [DMAdda^5]microcystin-LR (4), [Dha^7]microcystin-LR (5), microcystin-FR (6), microcystin-AR (7), microcystin-M(O)R (8), [Mser^7]microcystin-LR (9), and microcystin-WR (12). Compound 4 is the first microcystin containing 9-*O*-demethyl-Adda, while phenylalanine, *N*-methylserine, and tryptophan are also new variations in amino acid components of microcystins. Compound 11 was deduced to be a (C_3H_7O) monoester of the α-carboxyl on the Glu unit of LR (1). New microcystin 11 caused no apparent toxic effects in mice dosed ip at 1 mg/kg, while the others had LD_{50}'s of 90–800 μg/kg.

The microcystins[1] are well-known cyclic heptapeptide heptatoxins obtained from cyanobacteria (blue-green algae), which grow worldwide in fresh and brackish waters and cause animal and human water-based toxicosis.[2,3] Nine chemically defined microcystins (1–3 and 13–18, **Scheme** I) have been isolated from the genera *Microcystis*,[4-12] *Anabaena*,[7,8] and *Oscillatoria*.[3,8,13] *Microcystis* is the most common producer of these hepatotoxins, and microcystin-LR (LR, 1, Scheme I) occurs most often.[2,3] The structures of the microcystins differ primarily in the variations in the two L-amino acids at positions 2 and 4 and secondarily in the absence of the methyl groups on D-*erythro*-β-methylaspartic acid (D-MeAsp) and/or *N*-methyldehydroalanine (Mdha) (Scheme I).[2] Nodularin[14] (19, Scheme I), isolated from *Nodularia spumigena*, is thus far the only related cyclic pentapeptide, and it possesses similar hepatotoxicity.[15] Hepatotoxic *Aphanizomenon* and *Gomphosphaeria* species have also been reported.[16] The recently reported[17] inhibition of protein phosphatases 1 and 2A by these toxins makes them important biological tools.

The most unusual feature of nodularin and microcystins is the C_{20} amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda),[14] which plays an important role in their toxicity. Hydrogenation or ozonolysis of the diene system in the Adda unit gives an inactive product,[18] and the stereoisomer at the Δ^6

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Table I. Amino Acid Analysis and HRFABMS Data for 1-12

	amino acids[b]	HRFABMS[a] observed (M + H)	composition	Δ[c]
1	D-Ala, L-Leu, D-MeAsp,[d] L-Arg, D-Glu, $MeNH_2$[e]	995.5571	$C_{49}H_{75}N_{10}O_{12}$	−0.5
2	D-Ala, L-Arg, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1038.5722	$C_{49}H_{76}N_{13}O_{12}$	+1.4
3	D-Ala, L-Tyr, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1045.5345	$C_{52}H_{73}N_{10}O_{13}$	+1.4
4	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	981.5423	$C_{48}H_{73}N_{10}O_{12}$	−1.4
5	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu	981.5405	$C_{48}H_{73}N_{10}O_{12}$	+0.4
6	D-Ala, L-Phe, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1029.5399	$C_{52}H_{73}N_{10}O_{12}$	+1.0
7	D-Ala, L-Ala, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	953.5102	$C_{46}H_{69}N_{10}O_{12}$	−0.6
8	D-Ala, L-Met, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1029.5069	$C_{48}H_{73}N_{10}O_{13}S$	+1.0
9	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu, ($MeNH_2$)	1013.5661	$C_{49}H_{77}N_{10}O_{13}$	+1.1
10	D-Ala, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1049.5656	$C_{52}H_{77}N_{10}O_{13}$	+1.6
11	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1053.5975	$C_{52}H_{81}N_{10}O_{13}$	+1.0
12	D-Ala, D-MeAsp, L-Arg, D-Glu, $MeNH_2$	1068.5494	$C_{54}H_{74}N_{11}O_{12}$	+2.4

[a] ZAB-SE or 70-SE4F. [b] Waters Pico-Tag HPLC system and GC on a chiral capillary column. [c] Difference (mmu) from the calculated value for each composition. [d] D-*erythro*-β-Methylaspartic acid. [e] Methylamine from *N*-methyldehydroalanine.

Table II. FABMS/MS Data for 1-12

composition	fragment ion, m/z 1	2	3	4	5	6	7	8	9	10	11	12
M + H	995	1038	1045	981	981	1029	953	1029	1013	1049	1053	1068
M − 135	859[a]	902	909	–	845	893	817	893	877	913	917	932
$C_{11}H_{14}O$-Glu-Mdha[b]	375	375[c]	375	375	361[d]	375	375	375[c]	393[e]	375	433[f]	375
CO-Glu-Mdha − H	239	239	239	239	225[d]	239	239	239	257[c,e] (239)	239	297[f]	239
Glu-Mdha + H	213[a]	213	213	213	199[d]	213	213	213	231[e] (213)	213	271[f]	213
Mdha-Ala + H	155[a]	155	155	155	141[d]	155	155	155	173[e] (155)	155	155	155
$PhCH_2CH(OMe)$	135[a]	135	135	–	135	135	135	135	135	135	135	135
other fragments				860[h]				1013[i] 965[j]	995[i]		995[k]	

[a] Ion formula was confirmed by HRFABMS. [b] $CH_3CH{=}CHC(CH_3){=}CHCH{=}CHCH(CH_3)$CO-Glu-Mdha − H. [c] Weak. [d] Contains Dha instead of Mdha. [e] Contains Mser instead of Mdha. The peak in parentheses, probably obtained via dehydration, was also detected at nearly the same intensity, but the intensities of both peaks were weaker than that of the (dehydrated) peak of other microcystins. [f] 58 mu shifted to higher mass. [g] Ion formula was confirmed by HRFABMS of the dihydro derivative of 1.[20] [h] (M + H − 121). [i] (M + H − O). [j] (M + H − H_2O). [k] (M + H − 58). [l] [M − $S(O)CH_3$].

double bond has been reported to show no toxicity.[10,11]

We recently described new microcystins **20–22** (Scheme I), isolated from *Nostoc* sp. strain 152,[19] which have an acetoxyl group instead of a methoxyl group at the C-9 position in Adda.[20] These compounds have toxicity similar to that of the corresponding methoxyl compounds and were the first to show a modification in the Adda unit which allows retention of hepatotoxicity. We are interested in the structure–activity relationships of nodularin and microcystins, both from synthetic and natural sources, and have synthesized Adda itself,[21] which showed no toxicity. We present here the isolation and characterization of twelve microcystins, including nine with new structures, of which seven have been assigned, from a *Microcystis* bloom at Homer Lake (Illinois, summer 1988). Of par-

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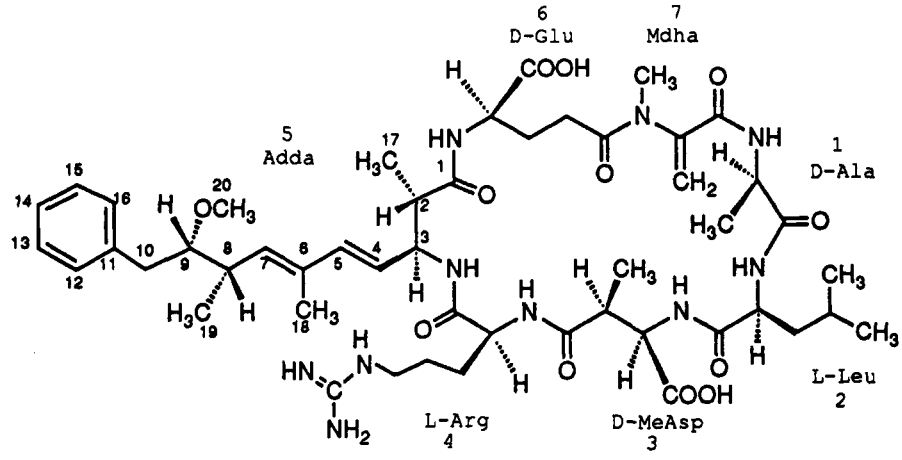
Scheme I



microcystin-LR (1)

		1	2	3	4	5	6	7
microcystin-RR	(2):	cyclo(-D-Ala-	-L-Arg-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
microcystin-YR	(3):	cyclo(-D-Ala-	-L-Tyr-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
[DMAdda^5]microcystin-LR	(4):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Arg-	DMAdda-	-D-Glu-	-Mdha-)
[Dha^7]microcystin-LR	(5):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Dha-)
microcystin-FR	(6):	cyclo(-D-Ala-	-L-Phe-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
microcystin-AR	(7):	cyclo(-D-Ala-	-L-Ala-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
microcystin-M(O)R	(8):	cyclo(-D-Ala-	-L-Met(O)-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
[Mser^7]microcystin-LR	(9):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mser-)
microcystin-WR	(12):	cyclo(-D-Ala-	-Trp-	-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
microcystin-LA	(13):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Ala-	-Adda-	-D-Glu-	-Mdha-)
microcystin-YA	(14):	cyclo(-D-Ala-	-L-Tyr-	-D-MeAsp-	-L-Ala-	-Adda-	-D-Glu-	-Mdha-)
microcystin-YM	(15):	cyclo(-D-Ala-	-L-Tyr-	-D-MeAsp-	-L-Met-	-Adda-	-D-Glu-	-Mdha-)
[D-Asp^3]microcystin-LR	(16):	cyclo(-D-Ala-	-L-Leu-	-D-Asp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
[D-Asp^3]microcystin-RR	(17):	cyclo(-D-Ala-	-L-Arg-	-D-Asp-	-L-Arg-	-Adda-	-D-Glu-	-Mdha-)
[D-Asp^3,Dha^7]microcystin-RR	(18):	cyclo(-D-Ala-	-L-Arg-	-D-Asp-	-L-Arg-	-Adda-	-D-Glu-	-Dha-)
Nodularin	(19):			cyclo(-D-MeAsp-	-L-Arg-	-Adda-	-D-Glu-	-Mdhb-)
[ADMAdda^5]microcystin-LR	(20):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Arg-	ADMAdda-	-D-Glu-	-Mdha-)
[ADMAdda^5]microcystin-LHar	(21):	cyclo(-D-Ala-	-L-Leu-	-D-MeAsp-	-L-Har-	ADMAdda-	-D-Glu-	-Mdha-)
[D-Asp^3,ADMAdda^5]microcystin-LR	(22):	cyclo(-D-Ala-	-L-Leu-	-D-Asp-	-L-Arg-	ADMAdda-	-D-Glu-	-Mdha-)

MeAsp = *erythro*-β-Methylaspartic acid. Mdha = *N*-Methyldehydroalanine. DMAdda = *O*-DemethylAdda. Dha = Dehydroalanine. Mser = *N*-Methylserine. Mdhb = *N*-Methyldehydroaminobutyric acid. ADMAdda = *O*-Acetyl-*O*-demethylAdda. Har = Homoarginine.

ticular interest are the first chemically characterized microcystins containing 9-*O*-demethyl-Adda (DMAdda) (4), as well as the first containing phenylalanine (6), *N*-methylserine (9), or tryptophan (12).

Isolation, Molecular Formulas, and Amino Acid Analyses. A toxic bloom containing *Microcystis aeruginosa* (dominant), *M. viridis*, and *M. wesenbergii* was lyophilized and extracted with MeOH, and the component microcystins were separated as described in the Experimental Section and analyzed by HRFABMS. The elemental compositions (molecular formulas) of the compounds isolated were assigned by combining HRFABMS with amino acid analyses (Table I). The stereochemistry of amino acids listed in Table I was determined by GC analysis on a chiral column (see the Experimental Section).

Tandem Mass Spectrometric Analysis. The (M + H) ions of 1–12 were subjected to collisionally induced tandem FABMS (FABMS/MS, B/E scan) to give daughter ion peaks. Significant fragment ions observed, including a number with Mdha (*m/z* 375, 239, 213, and 155 in the FABMS/MS spectrum of 1),[20] revealed the structural modifications summarized in Table II.

Compounds 1–3. Compound 1, the principal component, was identified as microcystin-LR (LR), compound 2 as microcystin-RR (RR), and compound 3 as microcystin-YR (YR) on the basis of HRFABMS and amino acid analyses being identical with those of the known compounds. In each case, ^{1}H NMR (see the supplementary material) and FABMS/MS (Table II) supported the assignment.

Compound 4. Amino acid analyses for 4 were the same as those for LR (1) (Table I), while the molecular weight (formula) of 4 is 14 mu (CH_2) less than that of LR, suggesting a demethyl variant of LR. Since 4 gave D-MeAsp by amino acid analysis, the compound does not correspond to the previously reported [D-Asp^3]microcystin-LR (16).[3,8,10,13]

FABMS/MS of 4 showed neither a peak at *m/z* 135 nor one at M − 135, as would be characteristic of the Adda-containing microcystins,[20] but a peak detected at *m/z* 860 (M − 121) suggested a demethyl derivative of Adda. The ^{1}H NMR spectrum of 4 (supplementary material) lacked an *O*-methyl signal but contained signals ascribable to DMAdda (Table III), a new variation on the Adda unit, confirming the FABMS/MS results. The ^{1}H NMR data for the DMAdda unit in 4 (Table III) also allow assignment

Table III. ^{1}H NMR Data for Adda and Dehydroalanine in 1-12[a]

proton (multiplicity)	δ (J, Hz)											
	1	2	3	4[b]	5[c]	6	7	8	9	10	11	12
[Adda]												
H-2 (m)	3.05 (10.5, 7.0)	3.15 (10.5, 7.0)	3.14 (10.5, 7.0)	3.11 (10.5, 7.0)	3.06 (10.5, 6.5)	3.15 (10.5, 7.0)	3.12 (10.5, 6.5)			3.15 (10.5, 7.0)	3.21 (10.5, 7.0)	3.15 (10.5, 6.5)
H-3 (dd)	4.56 (10.5, 9.0)	4.54 (10.5, 9.0)	4.58 (10.5, 9.0)	4.56 (10.5, 9.0)	4.52 (10.5, 9.0)	4.58 (10.5, 8.5)	4.54 (10.5, 9.0)			4.56 (10.5, 9.0)	4.53 (10.5, 9.0)	4.60 (10.5, 9.0)
H-4 (dd)	5.48 (15.5, 9.0)	5.56 (15.5, 9.0)	5.51 (15.5, 9.0)	5.52 (15.5, 9.0)	5.51 (15.5, 9.0)	5.51 (15.5, 8.5)	5.56 (15.5, 9.0)	5.51	5.61	5.54 (15.5, 9.0)	5.51 (15.5, 9.0)	5.49 (15.5, 9.0)
H-5 (d)	6.24 (15.5)	6.22 (15.5)	6.24 (15.5)	6.24 (15.5)	6.23 (15.5)	6.24 (15.5)	6.23 (15.5)	6.23 (15.5)	6.21 (15.5)	6.23 (15.5)	6.23 (15.5)	6.25 (15.5)
H-7 (d)	5.42 (9.5)	5.39 (10.0)	5.41 (10.0)	5.43 (10.0)	5.40 (9.5)	5.41 (10.0)	5.42 (10.0)	5.39 (10.0)	5.39 (9.5)	5.40 (10.0)	5.42 (10.0)	5.42 (10.0)
H-8 (m)	2.58 (9.5, 7.0, 7.0)	2.57 (10.0, 6.5, 6.5)	2.58 (10.0, 7.0, 6.5)	2.52 (10.0, 7.0, 6.5)	2.57 (9.5, 6.5, 6.5)	2.58 (10.0, 7.0, 6.5)	2.58 (10.0, 6.5, 6.5)	2.58	2.57	2.58 (10.0, 7.0, 7.0)	2.59 (10.0, 7.0, 6.5)	2.59 (10.0, 6.5, 6.0)
H-9 (m)	3.27 (7.5, 7.0, 3.5)	3.26 (7.5, 6.5, 5.0)	3.26 (7.5, 6.5, 4.5)	3.58 (8.0, 6.5, 4.0)	3.25 (7.0, 6.5, 4.5)	3.26 (7.5, 6.5, 4.5)	3.26 (7.5, 6.5, 4.5)			3.26 (7.5, 7.0, 4.5)	3.26 (7.5, 6.5, 4.5)	3.26 (7.5, 6.0, 4.5)
H-10 (dd)	2.68 (14.0, 7.5)	2.68 (14.0, 7.5)	2.68 (14.0, 7.5)	2.58 (14.0, 8.0)	2.68 (14.0, 7.0)	2.68 (14.0, 7.5)	2.67 (14.0, 7.5)	2.68 (14.0, 7.5)	2.66 (14.0, 7.5)	2.68 (14.0, 7.5)	2.68 (14.0, 7.5)	2.68 (14.0, 7.5)
(dd)	2.81 (14.0, 3.5)	2.82 (14.0, 5.0)	2.82 (14.0, 4.5)	2.81 (14.0, 4.0)	2.81 (14.0, 4.5)	2.82 (14.0, 4.5)	2.82 (14.0, 4.5)	2.81 (14.0, 5.0)	2.81 (14.0, 4.5)	2.82 (14.0, 4.5)	2.82 (14.0, 4.5)	2.82 (14.0, 4.5)
H-12, 16 (d)	7.18	7.17	7.18	7.18	7.18	7.18	7.18	7.18	7.18	7.18	7.18	7.18
H-13, 15 (t)	7.24	7.23	7.24	7.24	7.24	7.24	7.24	7.24	7.24	7.24	7.24	7.24
H-14 (t)	7.15	7.15	7.15	7.15	7.15	7.15	7.16	7.16	7.16	7.16	7.16	7.16
H_3-17 (d)	1.03 (7.0)	1.03 (7.0)	1.04 (7.0)	1.02 (7.0)	1.05 (6.5)	1.07 (7.0)	1.03 (6.5)	1.03 (7.0)	1.02 (6.5)	1.03 (7.0)	1.03 (7.0)	1.05 (6.5)
H_3-18 (s)	1.61	1.61	1.62	1.69	1.62	1.62	1.62	1.62	1.61	1.62	1.61	1.62
H_3-19 (d)	1.00 (7.00	0.99 (6.5)	1.00 (7.0)	1.02 (7.0)	0.99 (6.5)	1.00 (7.0)	1.00 (6.5)	1.00 (7.0)	1.00 (7.0)	1.00 (7.0)	1.00 (7.0)	1.00 (6.5)
H_3-20 (s)	3.24	3.24	3.24	–	3.24	3.24	3.24	3.24	3.23	3.24	3.24	3.24
[Mdha][d]												
H-3 (s)	5.43	5.37	5.46	5.40	5.26	5.46	5.38	5.41	–	5.41	5.42	5.46
(s)	5.89	5.80	5.89	5.84	5.49	5.89	5.80	5.84	–	5.85	5.89	5.89
N-CH_3 (s)	3.33	3.36	3.33	3.33	–	3.34	3.37	3.37	–	3.35	3.35	3.33

[a] 500 MHz, CD_3OD (δ 3.30). [b] *O*-DemethylAdda instead of Adda. [c] Dehydroalanine instead of Mdha. [d] *N*-Methyldehydroalanine.

of the stereochemistry of DMAdda.[14,20,22]

The FABMS/MS fragment ion peaks for **4** at m/z 239, 213, and 155 (Table II) suggested the presence of Mdha as the seventh amino acid;[20] this was also indicated by the peak for $MeNH_2$, which was generated by decomposition of Mdha during acid hydrolysis prior to the amino acid analysis (Table I) and confirmed by the ^1H NMR spectrum, showing three singlets due to the Mdha unit (Table III).

Taken together, the FABMS/MS and ^1H NMR spectra of **4** allow assignment of the sequence Adda-Glu-Mdha-Ala and agree with assignment of **4** as *O*-demethylmicrocystin-LR, i.e., [DMAdda^5]microcystin-LR.[23]

Compound 5. HRFABMS suggested that **5** is also a demethyl variant of LR (**1**), and amino acid analyses for **5** lack $MeNH_2$. FABMS/MS of **5** showed peaks at m/z 225, 199, and 141 (Table II), each 14 mu (CH_2) less than corresponding peaks of LR (**1**), RR (**2**), YR (**3**), and **4**. This locates the missing CH_2 in the Mdha unit. The ^1H NMR spectrum of **5** (supplementary material) revealed two one-proton singlets at δ 5.26 and 5.49 (lower field than Mdha), but no *N*-methyl signal (Table III), in agreement with the lack of a $MeNH_2$ peak in the amino acid analysis (Table I). Therefore, **5** has dehydroalanine (Dha) instead of Mdha.

The presence of Adda as the seventh amino acid unit was suggested by fragment ion peaks at m/z 135 and 845 (M − 135) and confirmed by ^1H NMR data (Table III).[22]

The sequence Adda-Glu-Dha-Ala was shown by the FABMS/MS fragment ion peaks for **5** at m/z 361, 225, 199, and 141. The ^1H NMR spectrum of **5** was similar to that of LR (**1**), which argued that **5** is *N*-demethylmicrocystin-LR, that is, [Dha^7]microcystin-LR. After we had submitted the present manuscript, Harada et al. reported **5** from *M. aeruginosa*, K-139.[24]

Compound 6. Amino acid analysis of **6** gave L-Phe and L-Arg as the variable L-amino acid components (Table I).[2] The FABMS/MS fragment ion peaks for **6** at m/z 135 and 893 (M − 135) suggested the presence of Adda, which was confirmed by ^1H NMR data (Table III).[22] The peak for $MeNH_2$ in the amino acid analysis (Table I) and the fragment ion peaks at m/z 239, 213, and 155 (Table II) suggested Mdha as the seventh amino acid unit, and ^1H NMR data confirmed the assignment (Table III).

FABMS/MS and ^1H NMR (supplementary material) spectra suggested that **6** is microcystin-FR,[23] which appeared in a report[25] on liquid chromatographic determination of cyanoginosins (microcystins)[1] by Gathercole and Thiel, but was undefined by physicochemical or biological properties.

Compound 7. Five amino acids (D-Ala, L-Ala, D-MeAsp, L-Arg, and D-Glu) and $MeNH_2$ were found in **7** (Table I). In addition, Adda and Mdha units were assigned as components of **7** by FABMS/MS (Table II) and ^1H NMR (Table III) data.[22] FABMS/MS and the ^1H NMR spectrum (supplementary material) of **7** established the sequence Adda-Glu-Mdha-Ala and suggested that **7** is an L-Ala variant of L-Leu in **1**, that is, microcystin-AR.[23]

Compound 8. Amino acid analysis, FABMS/MS, and ^1H NMR data for **8** revealed the presence of D-Ala, L-Met, D-MeAsp, L-Arg, D-Glu, and Mdha. Amounts of compound **8** were inadequate for a good ^1H NMR spectrum, and a few positions of the Adda unit could not be assigned, but the signals listed in Table III confirmed the presence of Adda.[22] The sum of unit weights of these seven amino acid residues was 16 mu (O) less than the molecular weight (formula) of **8**. FABMS/MS fragment ion peaks at m/z 1013 and 965 suggested that the difference is ascribable to the presence of the L-Met unit as a sulfoxide. A three-proton singlet at δ 2.61 in the ^1H NMR spectrum of **8** confirmed this assignment.

FABMS/MS (Table II) revealed the sequence Adda-Glu-Mdha-Ala, so that, by analogy with other components, compound **8** could be deduced as microcystin-M(O)R.[23]

Compound 9. The amino acid profile of **9** (Table I) was like that of LR (**1**), but the $MeNH_2$ peak was smaller than in the hydrolyzate of **1**. FABMS/MS showed strong peaks at m/z 877 (M − 135) and 135, indicative of the presence of Adda, which was confirmed by ^1H NMR (Table III) even though a few signals for the Adda unit could not be assigned from the poor ^1H NMR spectrum.[22] Several weak fragment ion peaks were observed at m/z 257, 239, 231, 213, 173, and 155 together with the other strong peak at m/z 995 (M + H − H_2O) (Table II). The peaks at m/z 239, 213, and 155 corresponded to but were less intense than the same fragment ions for other microcystins. The peaks at m/z 257, 231, and 173 were each 18 mu (H_2O) higher than the above peaks, and the relative intensities of each pair (m_n, $m_n - H_2O$) were approximately the same. These data suggested that the increase in mass (18 mu, H_2O) is ascribable to the N-methylated amino acid unit and that the lower peaks are generated through dehydration from the corresponding fragment ions, or through dehydration of the parent ion followed by fragmentation. The relatively weak $MeNH_2$ peak in the amino acid analysis agreed with this presumption. The ^1H NMR spectrum of **9** revealed a singlet for an *N*-methyl at δ 3.34, but not singlets in the olefinic region. Although other signals due to the N-methylated amino acid unit were not detected because of the inadequate amount of **9** and the amino acid was not detected in the hydrolyzate, FABMS/MS (peaks at m/z 257, 231, and 173) showed the presence of the N-methylated hydroxyamino acid, presumably *N*-methylserine (Mser), whose stereochemistry cannot be assigned. It should be noted that serine is the biosynthetic precursor of dehydroalanine.[26]

FABMS/MS (Table II) showed the sequence Adda-Glu-Mser-Ala, indicating that **9** contains an Mser substituent in place of the Mdha unit of LR (**1**), i.e., **9** is [Mser^7]microcystin-LR.[23]

Compound 10. Amino acid analysis data (Table I) identified only four amino acids in **10**. FABMS/MS

(22) The chemical shifts of all DMAdda protons in **4** [except for those near the 9-*O*-methyl group (H-8, H-10, and especially H-9)] and of all the Adda protons in **5**–**12** are very close to those of Adda in LR (**1**), RR (**2**), and YR (**3**). Moreover, the coupling constants for all DMAdda and Adda protons are also very close to those for Adda in **1**, **2**, and **3**, confirming the relative stereochemistry in DMAdda and Adda in **4**–**12**. Since the absolute stereochemistry of all the other amino acids identified in **4**–**12** is the same as that in **1**–**3**, a complete inversion of all stereocenters in DMAdda or Adda in **4**–**12** would stretch the imagination and would in any event alter the chemical shifts and coupling constants of the ring protons H-2 and H-3 in DMAdda or Adda.

(23) The other three amino acids (L-Leu, D-MeAsp, and L-Arg) are not actually sequenced by the present data and are assigned by similarity of ^1H NMR and by analogy to all the previous microcystins. Strictly speaking, only five microcystins have been definitively sequenced by extensive NMR experiments (**2**, **13**)[4c,6] and by FABMS/MS of a ring-opened (linear) peptide (**1**, **16**, **18**).[8,14] We are currently developing a general method for the complete sequential analysis of microcystins by FABMS/MS on a linear peptide obtained by a one-pot reaction (ozonolysis followed by reduction) of microcystins. An application to the new microcystins reported in this paper will be made as more materials become available. The method and results of its application will be reported in the future.

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(Table II) and ^{1}H NMR (Table III) data confirmed the presence of Adda[22] and Mdha and the sequence Adda-Glu-Mdha-Ala. The ^{1}H NMR spectrum of **10** (supplementary material) showed two olefinic doublets (J = 9.0 Hz) at δ 5.54 and 5.59 overlapping the H-4 signal of the Adda unit, and these signals are ascribable to an unknown amino acid residue.

These data were very similar to those of an undefined toxin Ma_1 obtained from a Chinese *M. aeruginosa*.[9] A structural analysis will be made as more material becomes available.

Compound 11. The seven amino acid residues of **11** identified by amino acid analyses, FABMS/MS (Table II) and ^{1}H NMR (Table III)[22] data were the same as those of LR (**1**).

The total mass derived from summing the seven amino acid residues, however, was 58 mu (C_3H_6O) less than the molecular weight (formula) of **11** established by FABMS (HRFABMS), which corresponds to the difference between the molecular formulas of LR (**1**) and **11**. The fragment ion peak observed at m/z 995 (Table II) corresponds to the (M + H) peak for LR (**1**), indicating a ready loss of the C_3H_6O unit. Peaks at m/z 433, 297, and 271 for **11** were 58 mu higher than the corresponding peaks for LR, which contain Glu and Mdha, but the fragment ion Mdha-Ala + H was observed at the same position (m/z 155) as that of LR. These data argued that C_3H_6O must be attached to the D-Glu unit.

The ^{1}H NMR spectrum of **11** (supplementary material) revealed two one-proton multiplets around δ 4.0. Therefore, the extra unit is probably attached through an ester bond on the α-carboxyl of the Glu unit, and **11** probably has the structure cyclo(-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu(C_3H_7O)-Mdha-).[23]

Compound 12. Although **12** has a rather high molecular weight, amino acid analyses identified only four amino acids (Table I). FABMS/MS (Table II) and ^{1}H NMR (Table III) revealed the presence of Adda[22] and Mdha.

The UV spectrum of **12** (supplementary material) suggested Trp (stereochemistry not determined) as the seventh amino acid, as confirmed by the ^{1}H NMR spectrum (supplementary material) containing signals due to the indole ring [δ 6.98 (1 H, dd, J = 7.5 and 8.0 Hz, H-5), 7.05 (1 H, dd, J = 7.5 and 8.0 Hz, H-6), 7.28 (1 H, s, H-2), 7.29 (1 H, d, J = 8.0 Hz, H-7), and 7.66 (1 H, d, J = 8.0 Hz, H-4)].[27]

FABMS/MS confirmed the sequence Adda-Glu-Mdha-Ala, and the compound is assigned as microcystin-WR.[23]

Discussion

In the summer of 1988, many states in the U.S., including Illinois, suffered from drought. A dense bloom of toxic *Microcystis*, due in part to the warm, dry weather, was found in Homer Lake, located approximately 20 miles east of the University of Illinois.[28] The algal bloom material contained large amounts of pigments and glycoproteins, which interfered with several steps of earlier isolation procedures. Although large amounts of pigments were contained in the extract, MeOH extraction suppressed the extraction of glycoproteins and water-soluble materials, thereby improving solid-phase extraction (ODS silica gel) and isolation procedures. Most pigments were removed by LH-20 column chromatography with MeOH. More recently we found that 80% MeOH–water elution from an ODS column is more effective in removing the pigments, which remain on the column. Use of the LH-20 column after solid-phase extraction with ODS silica gel was very effective in removing not only the pigments but also many impurities. This allowed better separation in the following silica gel column chromatography, and the microcystins were concentrated in a very small volume of eluate. HW-40 column chromatography[11] was more efficient than LH-20 for the separation of individual microcystins.

We have been examining the application of tandem mass spectrometry to the chemistry of peptides and proteins.[14,20,29] As cyclic peptides, the intact microcystins did not give sufficient fragment ion peaks by FABMS/MS to determine a complete sequence. Several fragment ions characteristic of the microcystins (Table II) are, however, very useful in identifying components and partial sequences of any new microcystins.[20] The strong peak at m/z 135, usually the base peak in daughter ion spectra, corresponds to a $PhCH_2CH(OMe)$ ion and is used to verify the presence of ordinary Adda; this is also supported by an ion at (M – 135). Compounds **4** and **5**, for example, have the same molecular weight, but are distinguished from each other and also from [D-Asp^3]microcystin-LR (**16**) by FABMS/MS. This method is quick and easy and uses <5 μg of compound.

Compound **4** is the first microcystin to possess a free hydroxyl group at C-9 in the Adda unit. Mser in **9** and Trp in **12** are new amino acid variations, and a monoester on the Glu unit in **11** is a new modification in microcystins. Compounds **4**, **5**, and **9** could be biogenetic precursors of LR (**1**).

We recently reported three toxins with an acetoxyl group replacing the methoxyl group on the Adda unit.[20] Compound **4** is the second example of a modified Adda unit. These compounds show clearly that neither a methyl nor an acetyl group is necessary for toxicity, and that the hydrophilic OH group on Adda does not change its toxicity; this amends slightly an earlier inference of structural requirements in the Adda unit.[28]

Compound **9** showed that the animals' livers revealed signs of toxication similar to the pathological changes caused by hepatotoxic microcystins.[30] Since dihydro derivatives obtained by borohydride reduction of the Mdha unit of microcystin-LR are toxic,[18,31] the toxicology of **9** should be reexamined in detail when a sufficient amount is obtained. Compound **11** was not toxic at concentrations of 1 mg/kg (ip, mouse), and the liver did not show any pathological change. The free carboxylic acid on the D-Glu unit seems then to be essential for toxicity.

The other microcystins obtained in this study showed hepatotoxicity and their approximate LD_{50}'s (μg/kg, ip, mice) were as follows: 500–800 (**2**), 150–200 (**3**), 90–100 (**4**), 250 (**5**), 250 (**6**), 250 (**7**), 700–800 (**8**), 300–400 (**10**), and 150–200 (**12**). Details of the toxicity data and pathological

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observations will be described elsewhere.[30]

Several more microcystins detected by FABMS of remaining fractions do not correspond to known or presently isolated compounds. These compounds, as well as the complete sequences of the remaining new microcystins in the present paper, will be described in future reports.

Experimental Section

General. Mass spectra were run on either a ZAB-SE or a 70-SE4F spectrometer operating in the FAB mode, using Xe atoms and a matrix of dithiothreitol/dithioerythritol (magic bullet).[32] Collisionally induced tandem mass spectra (MS/MS, B/E scan) in the FAB mode were obtained on a four-sector tandem mass spectrometer (70-SE4F) using Ar or He as a collision gas. ^{1}H NMR spectra were recorded on a GN-500 FT NMR spectrometer using CD_3OD as solvent and an internal standard; assignments (Table III and supplementary material) were based on the analysis of ^{1}H-^{1}H COSY spectra and single-frequency decoupling experiments. UV spectra were measured on a Lambda 3 UV/vis spectrophotometer in MeOH. Specific rotations were obtained on a DIP-370 digital polarimeter.

TLC was performed on precoated silica gel plates (Kieselgel $60F_{254}$), 0.25 mm thickness for analytical and preparative, and 1 mm thickness for preparative separation. Solvents (A) $CHCl_3$–MeOH–H_2O, 13:7:1.5, (B) EtOAc–2-PrOH–H_2O, 4:2:1.5, (C) EtOAc–2-PrOH–H_2O, 4:3:2, and (D) BuOH–AcOH–H_2O, 4:1:1, were used for chromatography. Adsorbed spots or bands were detected under UV light at 254 nm and by spraying phosphomolybdic acid followed by heating for analytical samples. R_f values for isolated compounds were as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
(A)	0.26	0.12	0.20	0.18	0.27	0.29	0.26	0.12	0.14	0.17	0.62	0.26
(B)	0.15	0.05	0.15	0.11	0.21	0.18	0.14	0.05	0.05	0.06	0.35	0.19
(C)	0.40	0.12	0.40	0.35	0.49	0.42	0.36	0.14	0.16	0.29	0.38	0.44
(D)	0.42	0.19	0.47	0.47	0.55	0.50	0.38	0.17	0.28	0.40	0.51	0.51

***Microcystis*.** Field samples collected from Homer Lake (Illinois) on August 13, 1988, consisted primarily of *M. aeruginosa* plus smaller quantities of *M. viridis* and *M. wesenbergii*. The cells were lyophilized and stored in a freezer pending extraction.

Isolation of Microcystins. Dried cells (240 g) were extracted with MeOH (4.8 L × 3, 15 h each). Each extract was filtered from cells and evaporated in vacuo. The residue was suspended in H_2O (600 mL) and centrifuged (9000*g*, 1 h). The supernatant was applied to an ODS silica gel column (100–200 mesh, 120 g), and the column was washed with H_2O (2 L) and then eluted with MeOH (1.5 L). The toxin-containing fractions were collected and evaporated to dryness (6.55 g), redissolved in MeOH, and chromatographed on an LH-20 column (25–100 mesh) to give the toxin fraction (4.38 g). Microcystin-LR (1) was monitored by TLC throughout these procedures as a representative of all microcystins. The toxin fraction was chromatographed on silica gel (230–400 mesh, 150 g) with $CHCl_3$–MeOH–H_2O (13:7:1) to afford fractions 1 (620 mg), 2 (1.42 g), and 3. The silica gel column, after elution of LR, was washed with MeOH, and the eluate was combined with fraction 3 (total 1.39 g). Fraction 1 was then subjected to LH-20 and HW-40F (MeOH) column chromatography and repeated preparative TLC separation (solvents A, B, and D) to give 11 [$[\alpha]^{26}_D$ −51.3° (*c* 0.460, MeOH), 2.8 mg] and 12 [$[\alpha]^{26}_D$ −62.4° (*c* 0.173, MeOH), 1.3 mg]. Fraction 2 was chromatographed on LH-20 (MeOH) followed by silica gel (100 g; $CHCl_3$–MeOH–H_2O, 13:7:1.5) and then purified by preparative TLC (solvents A, B, and D), affording 1 [$[\alpha]^{26}_D$ −80.2° (*c* 0.620, MeOH), 218 mg]; 5 [$[\alpha]^{26}_D$ −73.3° (*c* 0.060, MeOH), 0.8 mg]; 6 [$[\alpha]^{26}_D$ −67.0° (*c* 0.026, MeOH), 1.2 mg]; and 7 [$[\alpha]^{26}_D$ −80.0° (*c* 0.040, MeOH), 1.1 mg]. ODS silica gel (30 g) column chromatography (MeOH–0.05 M Na_2SO_4, 6:4) of fraction 3 gave four fractions. Each fraction was then subjected to HW-40F and LH-20 column chromatography (MeOH) followed by preparative TLC separation (solvents A, B, C, and D) to give 1 (35 mg); 2 [$[\alpha]^{26}_D$ −68.2° (*c* 0.086, MeOH), 0.7 mg]; 3 [$[\alpha]^{26}_D$ −71.2° (*c* 0.093, MeOH), 0.8 mg]; 4 [$[\alpha]^{26}_D$ −58.6° (*c* 0.506, MeOH), 8.0 mg]; 8 (0.3 mg); 9 (0.3 mg); and 10 [$[\alpha]^{26}_D$ −46.6° (*c* 0.060, MeOH), 0.9 mg].

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 an ODS 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. Capillary GC analyses were carried out on a Varian 3700 gas chromatograph using a Chirasil Val III column (0.32 mm × 25 m) and He as a carrier gas (flow rate, 37 mL/min; split ratio, 18:1). The program rate for the analysis of amino acid derivatives, except Arg, was 100 °C (2 min) to 200 °C at 8 °C/min. The Arg derivative was detected by isothermal chromatography at 200 °C.

Amino Acid Derivatives for GC Analysis. Each amino acid (100 μg) was treated with 300 μL of 4 N HCl–*i*-PrOH 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 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. Arg (100 μg) was heated at 110 °C for 4 h with a mixture of H_2O (25 μL), EtOH (50 μL), Et_3N (25 μL), and acetylacetone (50 μL).[33] The reaction mixture was evaporated to dryness by N_2.

Hydrolysis of Microcystins and Derivatization of Amino Acids. Each microcystin (100 μg) was hydrolyzed with 6 N HCl (200 μL) at 110 °C for 21 h or at 140 °C for 40 min. The reaction mixture was cooled to rt and divided into two aliquots. Each portion was evaporated to dryness at 110 °C by N_2. One portion was treated with 4 N HCl–*i*-PrOH (200 μL) followed by TFAA (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.

Toxicity Testing. Aqueous solutions (100 μg/mL) were adjusted by dilution with H_2O to lower concentrations. Solutions of 0.2–0.8 mL were injected (ip) into each of three male white mice (22–28 g; Swiss-Webster).

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Supplementary Material Available: ^{1}H and ^{13}C NMR spectra of 1, ^{1}H NMR spectra of 2–7 and 10–12, and UV spectra of 1, 12, and tryptophan (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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