Seven More Microcystins from Homer Lake Cells: Application of the General Method for Structure Assignment of Peptides Containing α,β-Dehydroamino Acid Unit(s)

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Received October 13, 1994 (Revised Manuscript Received April 7, 1995®)

Larger scale isolation of microcystins, cyclic heptapeptide hepatotoxins, from a water bloom of *Microcystis* spp. collected from Homer Lake (Illinois) gave the previously reported **1**–**5**, additional quantities of [L-MeSer^7]microcystin-LR (**6**), and microcystin-(H_4)YR [**8**, (H_4)Y = 1′,2′,3′,4′-tetrahydrotyrosine], which were previously isolated in insufficient amounts to complete the structure assignment, and seven more microcystins, **9**–**15**. A general method for assigning the structures of cyclic peptides containing α,β-unsaturated amino acid unit(s) developed with nodularin, a cyclic pentapeptide hepatotoxin, was applied to confirm the previously assigned structures of **1**–**5** and to assign the structures of [D-Asp^3]microcystin-LR (**9**) and the new microcystin-HilR (**10**, Hil = homoisoleucine). The method consists of linearization of a cyclic molecule by a one-pot reaction sequence (ozonolysis followed by $NaBH_4$ reduction) and tandem FABMS (FABMS/CID/MS) analysis of the product (linear peptide). A new microcystin, **11**, was assigned the structure [L-MeLan^7]-microcystin-LR (MeLan = *N*-methyllanthionine) and synthesized from **1** and L-Cys. Four linear peptides **12**–**15**, which are reasonable biogenetic precursors of the cyclic compounds, were also assigned structures based on their FABMS/CID/MS data.

Potent cyclic heptapeptide hepatotoxins termed microcystins[1a] are produced by some genera of freshwater cyanobacteria (blue-green algae) such as *Anabaena*, *Microcystis*, *Nostoc*, and *Oscillatoria*. More than forty microcystins have been reported thus far.[1b,2,3] Related cyclic pentapeptide hepatotoxins, nodularins, have been isolated from a brackish water cyanobacterium, *Nodularia spumigena*.[1b,4,5] These toxins show strong inhibitory activity against protein phosphatases 1 and 2A and have been reported to be tumor promoters.[6]

Recently, a cyclic pentapeptide, motuporin (= [L-Val^2]-nodularin), was isolated from a sponge, *Theonella swinhoei*, collected from Papua New Guinea as a potent inhibitor of protein phosphatase 1.[7] A new type of hepatotoxin has recently been reported from the cyanobacterium *Cylindrospermopsis raciborskii*.[8]

The most unusual structural feature of microcystins and nodularins is Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid, which plays an important role in their toxicity.[2,5,9]

The general structure of the 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*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine. Demethyl variants have been reported at the D-MeAsp (i.e. D-Asp) and/or Mdha (i.e., dehydroalanine = Dha)

® Abstract published in *Advance ACS Abstracts*, June 1, 1995.

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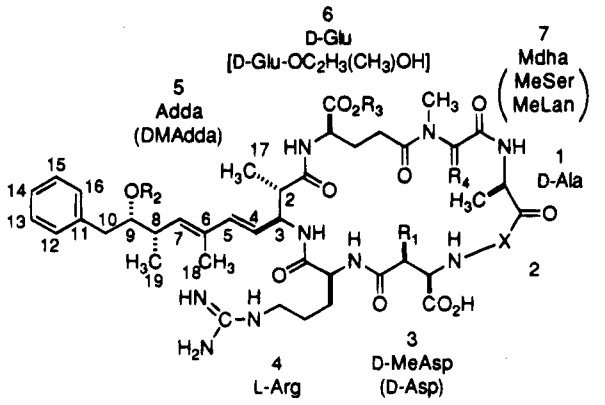
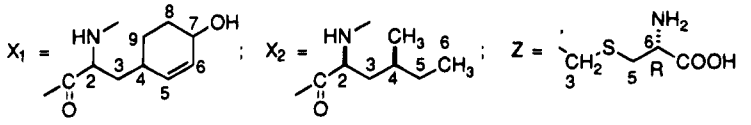
Scheme 1



		X	R_1	R_2	R_3	R_4
microcystin-LR	(1):	L-Leu	CH_3	CH_3	H	CH_2
[DMAdda5]microcystin-LR	(2):	L-Leu	CH_3	H	H	CH_2
microcystin-RR	(3):	L-Arg	CH_3	CH_3	H	CH_2
microcystin-AR	(4):	L-Arg	CH_3	CH_3	H	CH_2
microcystin-FR	(5):	L-Phe	CH_3	CH_3	H	CH_2
[L-MeSer^7]microcystin-LR	(6):	L-Leu	CH_3	CH_3	H	H, CH_2OH (*S*)
[D-Glu-$OC_2H_3(CH_3)OH^6$]microcystin-LR	(7):	L-Leu	CH_3	CH_3	$C_2H_3(CH_3)OH$	CH_2
microcystin-H_4YR	(8):	X_1	CH_3	CH_3	H	CH_2
[D-Asp^3]microcystin-LR	(9):	L-Leu	H	CH_3	H	CH_2
microcystin-HilR	(10):	X_2	CH_3	CH_3	H	CH_2
[L-MeLan^7]microcystin-LR	(11):	L-Leu	CH_3	CH_3	H	H, Z (*R,R*)



DMAdda, *O*-demethyl-Adda; MeSer, *N*-methylserine; $OC_2H_3(CH_3)OH$, mixture of four isomers (stereo and regio) of 1,2-propanediol mono-ester; (H_4)Y, 1',2',3',4'-tetrahydrotyrosine; Hil, L-homoisoleucine; MeLan, *N*-methyllanthionine.

residues. The two acidic amino acids, D-MeAsp and D-Glu, are isolinked. Microcystin-LR (**1**, Scheme 1) is found most often among the microcystins.

Studies on these hepatotoxins have revealed some structure–activity relationships, other than variable L-amino acids and demethyl variants. Stereoisomers at the Δ^6 double bond (6*Z*-isomer) in the Adda unit of microcystins and nodularin are nontoxic,[5,9] while acetoxyl variants at the C-9 position, replacing the methoxyl group in Adda (ADMAdda) of microcystins isolated from *Nostoc*[3a,10] and hydroxyl variants at the same position of nodularin[5] and **1**,[2] isolated from a water bloom of *N. spumigena* collected from New Zealand and of *Microcystis* spp. obtained from Homer Lake (Illinois), respectively, retain the activity. Dihydro derivatives at the *N*-methyldehydroamino acid units of **1** and nodularin (two stereoisomers each) obtained by $NaBH_4$ reduction showed toxicity comparable to the parent compounds, which revealed that saturation and stereoisomerism at this unit (amino acid 7 in microcystins and 5 in nodularins) do not affect the activity.[11] Linear peptides isolated from Homer Lake cells and cultured *N. spumigena* L-575, possible biogenetic precursors of microcystins and nodularin, have no toxicity to mice,[5,12] which clearly shows that the cyclic structure of these compounds is essential for the activity.

Recent progress in research on these cyclic peptides includes biosynthesis of **1**[13] and nodularin[12] and computer molecular modeling of both toxins.[14]

We have reported the use of FABMS including tandem FABMS (FABMS/CID/MS) for the structure assignment of microcystins.[2,3,10,11] FABMS/CID/MS of the intact microcystins, illustrated for **1** in Figure 1, showed sequential information on the upper four amino acids (5-6-7-1), but sequences of the rest of three amino acids (2-3-4) were presumed by the comparison of ^{1}H NMR spectra with those of known microcystins, since intense fragment ion peaks due to this part were not detected in their FABMS/CID/MS spectra. A general method for assigning the structures of cyclic peptides possessing α,β-unsaturated amino acid unit(s) developed with nodularin[5] was, therefore, applied to microcysins previously isolated from Homer Lake cells.[2] The method consists of (1) determination of a molecular weight and assignment of a molecular formula by FABMS and high-resolution (HR) FABMS; (2) identification of amino acid components by ^{1}H NMR spectroscopy and FABMS/CID/MS of the parent compound (assigning Adda and Mdha), and GC analysis of a derivatized acid hydrolysate on a chiral capillary column (assigning the other amino acids and stereochemistry); (3) ozonolysis followed by $NaBH_4$ reduction and HCl acidic workup to form a linear peptide; and (4) sequence analysis by FABMS/CID/MS of the linear peptide. We report here the isolation of seven more

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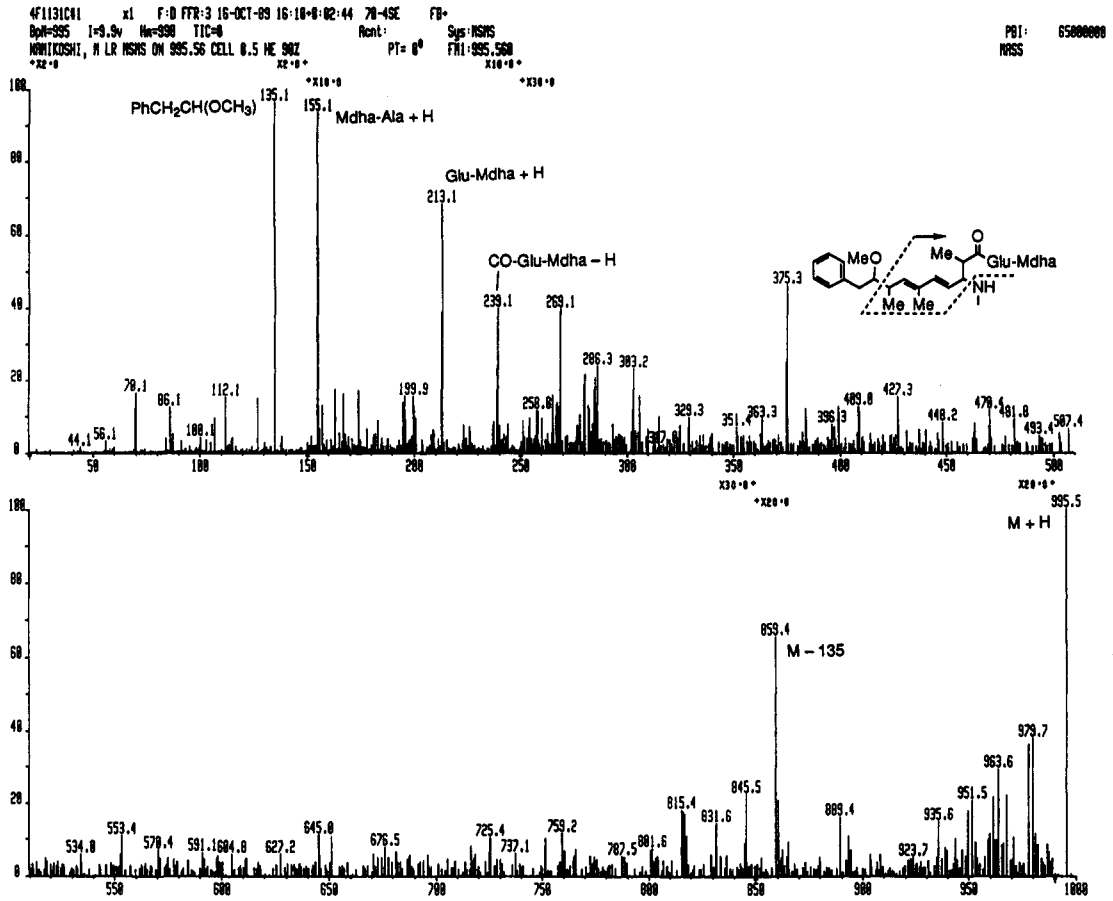
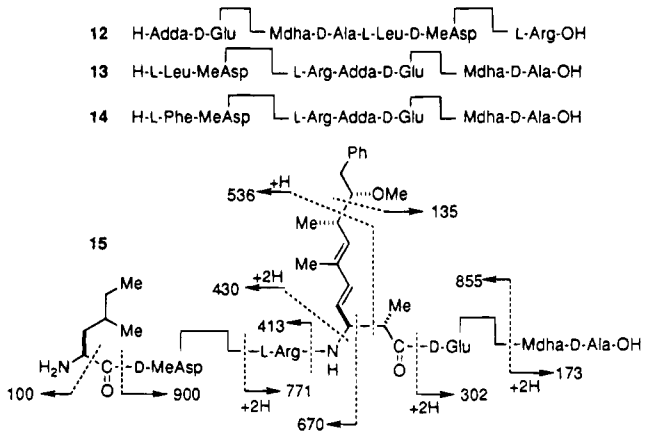


Figure 1. FABMS/CID/MS spectrum of **1**.

Scheme 2



microcystins (**9-15**) from Homer Lake cells results from application of the general method to microcystins **1-5** (Scheme 1) isolated previously,[2] and the structures of **6–8**, which were not assigned in the previous report because of inadequate amounts isolated. Compound **9** and the new microcystin **10** (Scheme 1) were assigned structures utilizing the general method. The structures of two more new microcystins, **11** (Scheme 1) and **15** (Scheme 2), are also suggested in this report.

Isolation of Microcystins from Homer Lake Cells. Further separation of the fractions remaining from the previous study gave two more new microcystins, **10** and **13**, in addition to 12 compounds reported previously.[2] Since the structure assignment of several compounds had not been completed in the previous paper because of inadequate amounts isolated, larger scale isolation was carried out. Separation of a minor components fraction was repeated several times from lyophilized cells (total 950 g). The fraction (total 682 mg) was subjected to repeated HPLC on an ODS column and silica gel TLC to afford **9**, two new microcystins **10** and **11** (Scheme 1) and four linear peptides **12–15** (Scheme 2) together with previously reported compounds **1–6** and **8** (Scheme 1). However, **7**, a monoester with the alkoxyl group OC_3H_6-OH on the Glu unit of **1**, was not detected in any batches of cell materials used in the present study. Compounds **6** (1.4 mg) and **8** (2.7 mg) were purified to assign their structures, which had not been completed previously since the amounts isolated were insufficient.[2]

The structures of **1–5** assigned peviously[2] were confirmed utilizing the general method.[5] The method was also applied to **8**, **9**, and a new microcystin **10**. Linear peptides **12–14** were assigned structures by analysis of their FABMS/CID/MS data as reported previously.[12] The alcohol unit of the Glu monoester unit in **7** was also determined. The structure of **11** was assigned by semisynthesis from **1** and L-Cys.

Application of the General Method. The FABMS spectra of microcystins **1-5** obtained with a matrix of dithiothreitol/dithioerythritol ("magic bullet", $M_r = 154$)[15] showed strong molecular ion peaks at $(M + H)^-$ and matrix adducts [$(M + 154 + H)^-$ and $(M + 154 \times 2 + H)^+$]. The FABMS/CID/MS spectrum on the $(M + H)^+$ ion of **1** (Figure 1) revealed sequential information on the four amino acids (Adda-Glu-Mdha-Ala) by the product ion peaks listed in Table 1. No intense product ion peaks containing the other three amino acid units were observed in the FABMS/CID/MS spectrum of **1**, in contrast

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Table 1. FABMS/CID/MS Data for 1, 9, and 10

composition	product ion, *m/z* 1	9	10
M + H	995	981	1009
M − 135	859	845	873
$C_{11}H_{15}O$-Glu-Mdha[a]	375	375	375
CO-Glu-Mdha − H	239	239	239
Glu-Mdha + H	213	213	213
Mdha-Ala + H	155	155	155
$PhCH_2CH(OMe)$	135	135	135

[a] $C_{11}H_{15}O$ = Adda − 135 − NH (see Figure 1).

to nodularin.[5] The elemental compositions of the product ion peaks at *m/z* 135 [$PhCH_2CH(OCH_3)$] and 859 (M − 135), which are characteristic peaks for Adda-containing compounds,[2,10] were confirmed by HRFABMS. The presence of the Mdha unit in the product ion peaks at *m/z* 155, 213, 239, and 375 was assigned by FABMS/CID/MS data for dihydro derivatives of **1** at this unit obtained by $NaBH_4$ reduction, which showed each of the corresponding peaks at 2 Da higher mass.[11] The product ion peaks at *m/z* 157 and 215 were detected in the low-resolution FABMS spectra of dihydro derivatives of **1**, and the isotopic compositions were confirmed by HRFABMS.[10,11]

The sequence of **1** was confirmed by the general method.[5] Compound **1** afforded aromatic alcohol **16** and linear peptide **17** [theoretical $(M + H)^+$ for $C_{33}H_{59}N_{10}O_{13}$ = 803.4263, found 803.4276 (HRFABMS)] upon ozonolysis followed by $NaBH_4$ reduction and HCl acidic workup (Scheme 3). Acid hydrolysis of **17** gave amino lactone **18** and small amounts of its C-2 epimer (ca. 9:1 ratio), together with the five amino acids, similar to the results from linear peptides obtained from nodularins.[5] In the previous papers, we have assigned the stereochemistry of aromatic alcohol **16** and aminolactone **18** obtained from **1** (Scheme 3) by comparison to the four synthetic stereoisomers.[4a,5] Ozonolysis of microcystins (the Mdha unit) was faster and completed within a few minutes in contrast to that of nodularins [the α-(methylamino)-dehydrobutyric acid unit], which required longer (>10 min).[5] Linear peptides obtained by $NaBH_4$ reduction and HCl workup from the ozonides of microcystins were adsorbed on an ODS cartridge with H_2O and retrieved by MeOH elution, although linear peptides obtained from nodularins were partially eluted with H_2O containing inorganic salts.[5] The minimum amounts of microcystins required to give adequate results were, therefore, smaller than those of nodularins.

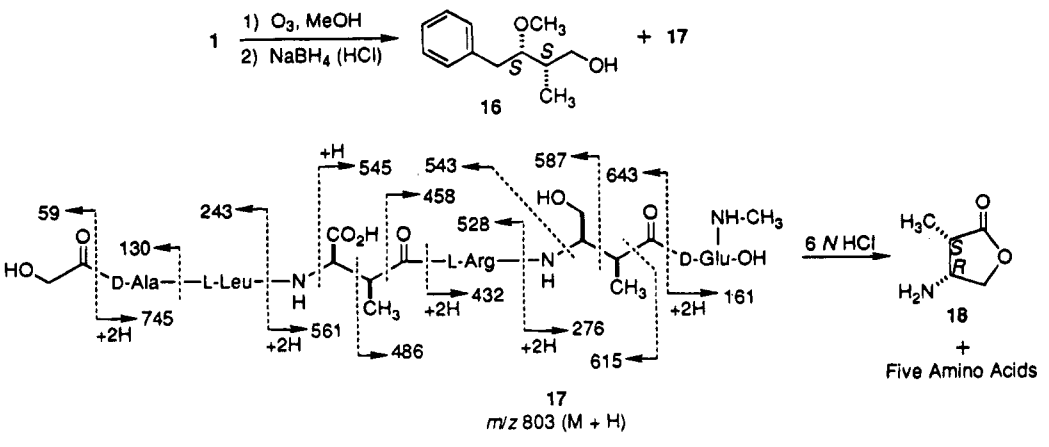
The yields of **17** from **1** were between 65 and 80%. A larger scale reaction (>2 mg) gave a better yield than the reaction with a smaller sample (<1 mg). The main side product was a cyclic compound [*m/z* 801 $(M + H)^+$, FABMS] having a carbinolamine group with an N−C bond formed between the *N*-methyl nitrogen of the N^{δ}-methylglutamine unit and the aldehyde carbon (C-2) from the Mdha unit. This cyclic compound, an intermediate in the reaction sequence, gave **17** upon treatment with HCl followed by a second $NaBH_4$ reduction. The reaction sequence yield is influenced by the acid hydrolysis step: a longer reaction time or larger amount of HCl gave a lower yield and unidentified byproducts. This cyclic peptide was also obtained by the reaction of the ozonide of **1** with $NaBH_4$ followed by AcOH to quench the reaction. Its structure was confirmed by FABMS, FABMS/CID/MS and NMR spectra. Limitations of this reaction sequence involve compounds with acid- and/or ozone-sensitive functional groups. Since compound **8** has a double bond in the H4Tyr unit, the corresponding product **22** was obtained in lower yield than those from the other microcystins. The reduction of the ozonide from **1** with $NaB(CN)H_3$ (pH 3−4 with HCl) gave a poor yield of **17** and a complicated product mixture, similar to the result obtained with nodularin.[5]

The FABMS/CID/MS spectrum of **17** obtained from **1** (Figure 2) showed product ion peaks at *m/z* 545, 486, 458, 543, 587, and 615 generated by fragmentation in the MeAsp and 3-amino-4-hydroxy-2-methylbutanoic acid units, together with peaks obtained by cleavage at each peptide bond (Scheme 3). Compound **2** gave the same linear peptide **17** [found 803.4269 (HRFABMS)] as that isolated from **1**, and acid hydrolysis of **17** afforded amino lactone **18**, assigned by capillary GC as the trifluoroacetyl (TFA) derivative, completing the structure of **2** including the absolute configuration of the *O*-demethyl-Adda (DMAdda) unit. Linear peptides **19**−**21** were obtained from **3**−**5**, respectively, and confirmed the amino acid sequences by their FABMS/CID/MS data as shown in Scheme 4. The same amino lactone **18** was detected on each GC after acid hydrolysis of **19**−**21** followed by TFA-derivatization.

Thus, the structures of **1**−**5** assigned previously[2] were confirmed by the general method for the structure assignment of cyclic peptides possessing α,β-unsaturated amino acid unit(s).

Stereochemistry of *N*-Methylserine (MeSer) in 6. The structure of **6** was assigned previously as [MeSer[7]]-microcystin-LR.[2] Since the amounts obtained were inadequate to give a good ^1H NMR spectrum, the stereochemistry of the MeSer unit in **6** remained unknown. The isolation from 950 g of cell materials afforded 1.4 mg of

Scheme 3



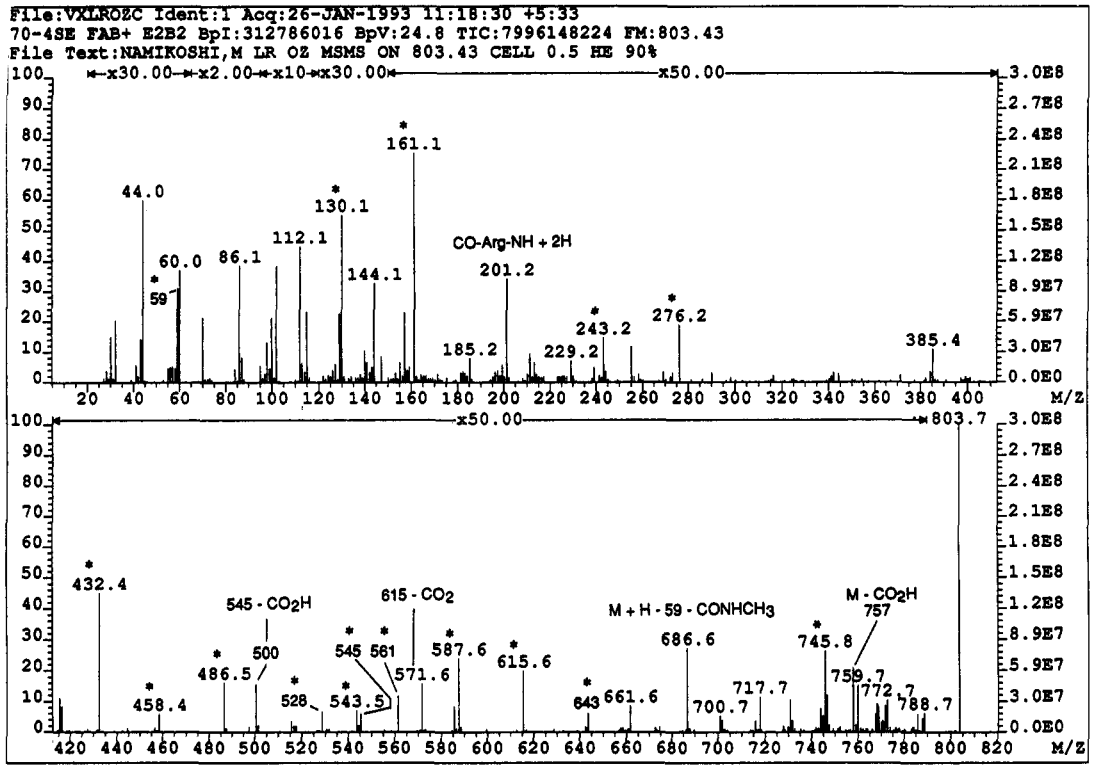
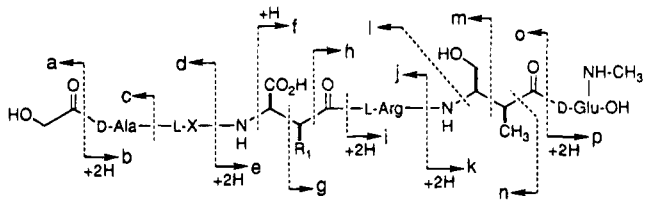
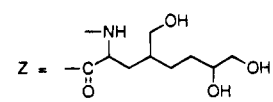


Figure 2. FABMS/CID/MS spectrum of linear peptide **17** obtained from **1** (Scheme 3). (See Scheme 3 for assignment of the peaks labeled "◦").

Scheme 4



	19	20	21	22	23	24
X	Arg	Ala	Phe	Z	Leu	Hil
R_1	CH_3	CH_3	CH_3	CH_3	H	CH_3
(M + H)	846	761	837	893	789	817
a	59	59	59	59	59	59
b	788	703	779	835	731	759
c	130	130	130	130	130	130
d	....	....	277	....	243	257
e	561	....	561	561	547	561
f	....	545	545	545	531	545
g	486	486	....	486	....	486
h	458	458	....	458	458	458
i	432	....	432	432	432	432
j	571	486	562	618	514	542
k	276	276	276	276	276	276
l	586	....	....	....	529	557
m	630	545	621	677	573	601
n	658	573	649	705	601	629
o	686	601	677	733	629	657
p	161	161	161	161	161	161



6, giving a ^1H NMR spectrum suitable for structure assignment. ^1H NMR data for the Adda unit in **6** are listed in Table 2. The chemical shifts and coupling constants due to the MeSer unit were assigned by the analysis of single-frequency decoupling experiments and a $^1H-^1H$ COSY spectrum of **6**: δ 3.91 (1 H, dd, J = 8.8, 5.0 Hz, H-2), 4.07 (1 H, dd, J = 12.0, 8.8 Hz, H-3), 4.13 (1 H, dd, J = 12.0, 5.0 Hz, H-3), and 3.27 (3 H, s, N-CH3). The chemical shift ascribed to the α-proton (H-2) of the MeSer unit in **6** (δ 3.91) was more similar to that of the L-MeAla unit in [L-MeAla^7]microcystin-LR (δ 3.81) than that of the D-MeAla unit in [D-MeAla^7]microcystin-LR (δ 4.56), which are the two stereoisomers of dehydro derivatives obtained by $NaBH_4$ reduction from **1**.[11] The difference between the chemical shifts for the α-proton of D- and L-MeAla[7] in the corresponding microcystins is presumably due to conformational differences between the two isomers. The stereochemistry of the MeSer unit in **6** was, therefore, assigned as L-, and the structure of **6** as [L-MeSer^7]microcystin-LR as shown in Scheme 1. Attempts to assign the stereochemistry of MeSer[7] by hydrolysis and chiral GC gave only methylamine, presumably from elimination to Mdha followed by hydrolysis.

Alcohol Unit of the Glu Ester Residue in 7. Compound **7** was assigned the structure [D-Glu-OC_3H_6-OH^6]microcystin-LR; however, the alcohol unit was not identified.[2] Alkaline hydrolysis of **7** with NaOH gave **1**, detected by TLC. A $^1H-^1H$ DQF-COSY spectrum of **7** showed unusual correlations between methyl doublets and lower field signals due to the protons on carbons attached to a hydroxyl or ester group. There were three methyl doublets at δ 1.15, 1.17, and 1.20 with a ca. 2:1:1 ratio, respectively. The sum of the integration of these three doublets corresponded to three protons, i.e., one methyl group. Two of these methyl doublets (δ 1.17 and 1.20) were coupled to signals at around δ 4.95 (overlapped with the HDO signal) ascribable to methine groups, each attached to an ester group, which in turn were coupled with the signals of hydroxymethyl protons (δ 3.55–3.65), as $CH_3CH(OCOR)CH_2OH$. The other methyl doublet (δ 1.15) was coupled with a hydroxymethine proton which showed cross peaks with methylene protons (δ 3.94 and 4.03) attached to an ester group, as $CH_3CHOHCH_2$-OCOR. The two methyl doublets at δ 1.17 and 1.20 were observed with a 1:1 ratio and their separation is presumably due to the stereoisomerism at the C-2 position, attached to the large cyclic peptide molecule. The methyl doublet at δ 1.15 also showed a rather broad signal. The alcohol unit of the Glu monoester residue in **7** was, therefore, assigned as 1,2-propanediol and is probably a

Table 2. ^{1}H NMR Data for Adda in 1, 6, 10, and 11[a]

proton (multiplicity)	δ (J, Hz) 1	6	10	11
H-2 (m)	3.05 (10.5, 7.0)	3.12 (10.5, 7.0)	3.06 (10.5, 7.0)	3.03 (10.5, 7.0)
H-3 (dd)	4.56 (10.5, 9.0)	4.55 (10.5, 9.5)	4.56 (10.5, 9.0)	4.58 (10.5, 9.0)
H-4 (dd)	5.48 (15.5, 9.0)	5.54 (15.5, 9.0)	5.48 (15.5, 9.0)	5.54 (15.5, 9.0)
H-5 (d)	6.24 (15.5)	6.23 (15.5)	6.23 (15.5)	6.23 (15.5)
H-7 (d)	5.42 (9.5)	5.40 (9.5)	5.42 (9.5)	5.40 (9.5)
H-8 (m)	2.58 (9.5, 7.0, 7.0)	2.58 (9.5, 7.0, 6.8)	2.58 (9.5, 7.0, 6.8)	2.58 (9.5, 7.0, 6.8)
H-9 (m)	3.27 (7.5, 7.0, 4.5)	3.26 (7.5, 7.0, 4.5)	3.25 (7.5, 7.0, 4.5)	3.25 (7.5, 7.0, 4.5)
H-10 (dd)	2.68 (14.0, 7.5)	2.67 (14.0, 7.5)	2.68 (14.0, 7.5)	2.67 (14.0, 7.5)
(dd)	2.81 (14.0, 4.5)	2.81 (14.0, 4.5)	2.82 (14.0, 4.5)	2.82 (14.0, 4.5)
H-12, 16 (d)	7.18	7.18	7.18	7.18
H-13, 15 (t)	7.24	7.24	7.24	7.24
H-14 (t)	7.15	7.16	7.16	7.16
H_3-17 (d)	1.03 (7.0)	1.04 (7.0)	1.04 (7.0)	1.06 (7.0)
H_3-18 (s)	1.61	1.61	1.61	1.62
H_3-19 (d)	1.00 (7.0)	0.99 (6.8)	1.00 (6.8)	0.99 (6.8)
H_3-20 (s)	3.24	3.24	3.24	3.23

[a] 500 MHz, CD_3OD (δ 3.30 ppm).

Table 3. Amino Acid Analysis and HRFABMS Data for 9–15

compd	amino acid[a]	HRFABMS observed[b]	composition	Δ[c]
9	D-Ala, L-Leu, D-Asp, L-Arg, D-Glu	981.5423	$C_{48}H_{73}N_{10}O_{12}$	−1.4
10	D-Ala, L-Hil,[d] D-MeAsp, L-Arg, D-Glu	1009.5698	$C_{50}H_{77}N_{10}O_{12}$	+2.4
11	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu, L-MeLan[e]	1116.5747	$C_{52}H_{82}N_{11}O_{14}S$	+1.6
12	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu	1013.5658	$C_{49}H_{77}N_{10}O_{13}$	+1.4
13	D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu	1013.5661	$C_{49}H_{77}N_{10}O_{13}$	+1.1
14	D-Ala, L-Phe, D-MeAsp, L-Arg, D-Glu	1047.5518	$C_{52}H_{75}N_{10}O_{13}$	−0.3
15	D-Ala, L-Hil,[d] D-MeAsp, L-Arg, D-Glu	1027.5830	$C_{50}H_{79}N_{10}O_{13}$	−0.2

[a] GC on a chiral capillary column (Chirasil Val-III). [b] $(M + H)^+$, *m/z*. [c] Difference (mDa) from the calculated value for each composition. [d] L-Homoisoleucine [(2*S*,4*S*)-2-amino-4-methylhexanoic acid]. [e] L-*N*-Methyllanthionine (stereochemistry assigned by ^{1}H NMR).

mixture of two stereoisomers at C-2, which form monoesters at the C-1 or C-2 hydroxyl groups with the α-carboxylic acid in the Glu unit. Compound **7** is, therefore, assigned the structure [D-Glu-$OC_2H_3(CH_3)OH^6$]-microcystin-LR, a mixture of stereo- and regioisomers of monoesters of 1,2-propanediol with the Glu unit of **1**.

Compound **7** was isolated only once from one batch of freeze dried cell material. However, **7** was not detected in the four batches of lyophilized cell materials used in the present study. This fact and the above evidence that **7** is a mixture of stereo- and regioisomers suggested that **7** is not a natural product but is generated during storage of wet cells (frozen), freeze drying, storage of dried cells (freezer, −20 °C), or MeOH extraction.

Assignment of the Unknown Amino Acid Unit in 8. The amounts of **8** isolated in the previous study were insufficient to assign an unknown amino acid unit in **8**.[2] Larger scale isolation afforded 2.7 mg of **8**, which gave a good ^{1}H NMR spectrum. The ^{1}H NMR signals of the unknown amino acid unit ($C_9H_{13}NO_3$, 167) were assigned by the analysis of single-frequency decoupling experiments and a ^{1}H−^{1}H COSY spectrum of **8**: δ 5.59 (1 H, br d, *J* = 10.0, 1.0, 1.0 Hz, H-6), 5.53 (1 H, br d, *J* = 10.0, 1.0, 1.0 Hz, H-5), 4.28 (1 H, dd, *J* = 12.0, 3.0 Hz, H-2), 4.09 (1 H, m, H-7), 2.33 (1 H, m, H-4), 2.20 (1 H, m, H-3), 1.98 (1 H, m, H-8), 1.88 (1H, m, H-9), 1.58 (1 H, m, H-3), 1.46 (1 H, m, H-8), and 1.20 (1 H, m, H-9). The connectivity of the carbons (2-3-4-9-8-7) was discerned in the ^{1}H−^{1}H COSY spectrum of **8**. The decoupling experiments revealed the connectivity of C-4, 5, 6, and 7 and the coupling constants between H-4 and 5 (1.0 Hz) and H-6 and 7 (1.0 Hz) which suggested that the relative stereochemistry at C-4 and C-7 involved trans-pseudoequatorial substituents. Consequently, the unknown amino acid unit was assigned as 1′,2′,3′,4′-tetrahydrotyrosine. The stereochemistry should be either (2*S*,4*S*,7*R*) or (2*S*,4*R*,7*S*), since this unit is one of the two variable L-amino acid residues of microcystins.

The sequence of **8** was confirmed by the general method. Compound **8** gave linear peptide **22** [theoretical $(M + H)^+$ for $C_{36}H_{65}N_{10}O_{16}$ = 893.4580, found 893.4584 (HRFABMS)] upon ozonolysis followed by $NaBH_4$ reduction. The FABMS/CID/MS spectrum of **22** showed the product ion peaks found in Scheme 4, confirming the structure of **8** as microcystin-(H_4)YR (Scheme 1).

Four stereoisomers [(2*S*,4*S*,7*R*), (2*S*,4*R*,7*S*), (2*R*,4*S*,7*R*), and (2*R*,4*R*,7*S*)] of 1′,2′,3′,4′-tetrahydrotyrosine derivatives were synthesized by Souchet et al. as intermediates for the total synthesis of anticapsin.[16] This is the first instance of the isolation of this amino acid from natural sources.

Structure of 9. The molecular formula $C_{48}H_{72}N_{10}O_{12}$ and amino acid components of **9** (Table 3) were identical to those of [D-Asp^3]microcystin-LR.[17] The direct comparison of TLC and HPLC of **9** and [D-Asp^3]microcystin-LR, isolated from *Anabaena flos-aquae* CYA 83/1,[18] showed that the two compounds were identical. Since the structure of **9** obtained from *A. flos-aquae* was assigned based on FABMS/CID/MS data for the intact molecule, the general method was applied to confirm the structure. Ozonolysis followed by $NaBH_4$ reduction of **9** gave linear peptide **23** [theoretical $(M + H)^+$ for $C_{32}H_{57}N_{10}O_{13}$ = 789.4107, found 789.4108 (HRFABMS)]. The FABMS/CID/MS spectrum of **23** revealed the se-

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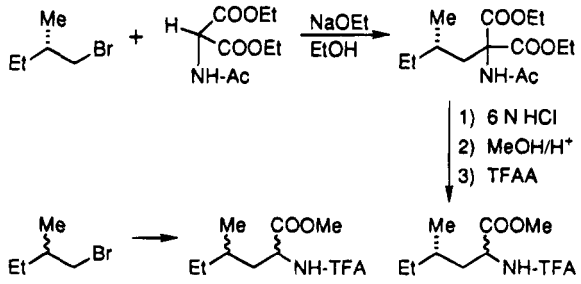
(17) Krishnamurthy, T.; Szatraniec, L.; Hunt, D. F.; Shabanowitz, J.; Yates, J. R., III; Hauer, C. R.; Carmichael, W. W.; Skulberg, O.; Codd, G. A.; Missler, S. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 770–774.
(18) Sivonen, K.; Namikoshi, M.; Evans, W. R.; Carmichael, W. W.; Sun, F.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. *Appl. Environ. Microbiol.* **1992**, *58*, 2495–2500.

Scheme 5



quence of **9** by the product ion peaks shown in Scheme 4. Thus, the structure of **9** was confirmed as [D-Asp^3]-microcystin-LR (Scheme 1).

Structure of 10. The molecular ion peak 1009.5698 [(M + H)$^-$] detected in the HRFABMS of **10** agreed with the molecular formula $C_{50}H_{76}N_{10}O_{12}$ (Table 3), which was not identical to that of known microcystins. Amino acid analysis data for **10** showed an unknown amino acid together with D-Ala, D-MeAsp, L-Arg, and D-Glu. The ^{1}H NMR spectrum of **10** revealed the presence of the Adda unit as listed in Table 2. The chemical shifts and coupling constants due to the Adda protons in **10** closely resembled those in **1**, confirming the relative stereochemistry of this unit in **10**. The ^{1}H signals ascribable to the Mdha unit were detected at δ 5.89 (1 H, s, H-3), 5.43 (1 H, s, H-3), and 3.33 (3 H, s, *N*-CH3) in the ^{1}H NMR spectrum of **10**. FABMS/CID/MS data for **10** also showed the presence of the Adda and Mdha units (Table 1).

The unknown amino acid unit was assigned by the analysis of the ^{1}H−^{1}H COSY spectrum of **10**. The ^{1}H signal ascribed to the α-proton (H-2) of the unknown amino acid unit (δ 4.27, 1 H, dd J = 11.0, 4.0 Hz) was coupled to geminal proton signals at δ 1.94 (1 H, m, H-3) and 1.75 (1 H, m, H-3), which showed cross peaks with a methine proton signal at δ 1.60 (1 H, m, H-4) coupled with the methyl doublet at δ 0.88 (J = 6.8 Hz, 4-CH_3). A methyl triplet was detected at δ 0.87 (J = 7.2 Hz, H_3-6), which was coupled with geminal proton signals at δ 1.44 (1 H, m, H-5) and 1.08 (1 H, m, H-5). These data revealed the structure of the unknown amino acid as 2-amino-4-methylhexanoic acid (homoisoleucine, Hil)[19] as shown in Scheme 1.

The stereochemistry of natural Hil was assigned by chemical synthesis and GC analysis. The mixtures of (2*S*,4*S*)- and (2*R*,4*S*)-Hil and of all four isomers were synthesized starting from *S*-(+)- and (±)-1-bromo-2-methylbutane, respectively, by the method described by Han and Pascal[20] (Scheme 5). The four derivatized stereoisomers of Hil were separated on a chiral GC column, and the coelution with the mixture of (2*S*,4*S*)- and (2*R*,4*S*)-isomers[21] assigned the elution order [(2*R*,4*S*), (2*R*,4*R*), (2*S*,4*S*), and (2*S*,4*R*)]. The natural Hil derivative eluted at the third peak assigning the stereochemistry as (2*S*,4*S*).

Thus, the seven amino acid residues were assigned. FABMS/CID/MS data for **10** listed in Table 1 showed the sequence of Adda-Glu-Mdha-Ala. The structure of **10** would be assigned as microcystin-HilR by the similarity of the ^{1}H NMR spectrum of **10** to that of **1** and by analogy to the other components.

The sequence of **10** and the absolute stereochemistry of Adda were confirmed by the general method. Compound **10** gave linear peptide **24** [theoretical (M + H)$^+$ for $C_{34}H_{61}N_{10}O_{13}$ = 817.4420, found 817.4423 (HR-FABMS)] upon ozonolysis and $NaBH_4$ reduction. The FABMS/CID/MS spectrum of **24** showed the product ion peaks listed in Scheme 4, which confirmed the sequence of **10**. Acid hydrolysis of **24** afforded amino lactone **18**, detected on capillary GC as its TFA-derivative. Consequently, the structure of **10** was assigned as microcystin-HilR (Scheme 1).

Structure of 11. Compound **11** had a rather large molecular weight (1115). HRFABMS data for **11** agreed with the molecular formula $C_{52}H_{82}N_{11}O_{14}S$ (Table 3). Amino acid analysis data for **11** (Table 3) revealed D-Ala, L-Leu, D-MeAsp, L-Arg, D-Glu, and an unknown peak which eluted at higher temperature on GC. The ^{1}H NMR spectrum of **11** showed the presence of the Adda unit and the relative stereochemistry of this unit by the signals listed in Table 2. The presence of the Adda unit in **11** was also confirmed by the product ion peaks at *m/z* 135 and 980 (M − 135) in the FABMS/CID/MS spectrum of **11**. The product ion peaks at *m/z* 375, 239, 213, and 155 were detected in the spectrum of **11**, which were observed at the same positions as those of **1**. Although these peaks suggested the presence of a Mdha unit, the ^{1}H NMR spectrum of **11** did not show signals ascribable to this unit.

The product ion peak at *m/z* 995 in the FABMS/CID/MS spectrum of **11**, which was observed at the same position as the molecular ion peak of **1**, suggested that the peak was generated by an elimination of a 121-Da ($C_3H_7NO_2S$) unit from the molecular ion of **11** and that the peaks at *m/z* 375, 239, 213, and 155 would be produced from this peak (*m/z* 995) or from the molecular ion by fragmentation followed by elimination of the above unit. This unit ($C_3H_7NO_2S$, 121 Da) was the same as the difference between **11** and **1** and had the molecular formula (weight) of Cys. Compound **11** was ninhydrin positive (**1** was negative), suggesting that **11** had a free amino group.

These data suggested that the unknown amino acid unit ($C_7H_{12}N_2O_3S$) in **11** is an *N*-methyl derivative of lanthionine[22] (*N*-methyllanthionine = MeLan), that is, the structure of **11** would be explained by an addition of Cys to the Mdha unit of **1**. The reaction of **1** with L-Cys in alkaline solution gave semisynthetic **11** [theoretical (M + H)$^+$ for $C_{52}H_{82}N_{11}O_{14}S$ = 1116.5763, found 1116.5779 (HRFABMS)] and a very minor product [found 1116.5768 (HRFABMS)] (ca. 20:1). Similar treatment of **1** with D-Cys afforded synthetic **11**-(D-Cys) [found 1116.5768 (HRFABMS)] and a minor product [found 1116.5768 (HRFABMS)]. L-Ala did not react with **1** under the same conditions, confirming that the addition occurred at the thiol group in Cys. FABMS/CID/MS of natural and synthetic **11**, synthetic **11**-(D-Cys), and both minor products were identical. The MeLan derivative in the hydrolysates of these natural and synthetic compounds showed the same retention time on chiral capillary GC, which might be ascribable to a racemization of the amino acid during acid hydrolysis,[23] or to a failure to separate the isomers. Synthetic **11** and **11**-(D-Cys) had the same R_f value on TLC and the same retention time on HPLC as natural **11**, but the minor products were separated

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(20) Han, H.; Pascal, R. A., Jr. *J. Org. Chem.* **1990**, *55*, 5173−5176.

(21) The (2*S*,4*S*)-isomer was slightly predominant over the (2*R*,4*S*)-isomer; Bernasconi, S.; Corbella, A.; Gariboldi, P.; Jommi, G. *Gazz. Chim. Ital.* **1977**, *107*, 95−99.

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by TLC and HPLC from natural **11**. The ^{1}H NMR spectrum of synthetic **11** was superimposable on that of natural **11**, while synthetic **11**-(D-Cys) gave a slightly different ^{1}H NMR spectrum. ^{1}H NMR signals ascribed to the MeLan unit in **11** were observed at δ 3.78 (1 H, dd, J = 9.0, 3.5 Hz, H-2), 3.27 (1 H, dd, J = 15.0, 3.5 Hz, H-3), 3.23 (3 H, s, N-CH3), and 2.88 (1 H, dd, J = 15.0, 9.0 Hz, H-3) for the MeAla unit (inside amino acid residue) and at δ 4.45 (1 H, H-2), 3.53 (1 H, H-3), and 3.19 (1 H, H-3) for the outside Cys residue. The chemical shift due to the α-proton (H-2) of the MeLan unit in natural (and synthetic) **11** (δ 3.78) and synthetic **11**-(D-Cys) (δ 3.75) resembled that of the L-MeAla unit in [L-MeAla^7]microcystin-LR[11] (δ 3.81). The ratio of [L-MeAla^7]microcystin-LR and [D-MeAla^7]microcystin-LR in the $NaBH_4$ reaction mixture of **1** was *ca.* 4:1.[11] The main product [synthetic **11** and **11**-(D-Cys)] was formed in *ca.* 20 times the amount of the minor product in the reaction of **1** with Cys. These observations argued the stereochemistry of the MeLan unit in **11** as the (2*R*,6*R*)-configuration, and accordingly the structure of **11** is [L-MeLan^7]microcystin-LR as shown in Scheme 1.

Lanthionine[22] has been detected in "lantibiotics" such as nisin, subtilin, and epidermine as the *meso*-form.[24] L-MeLan in **11** is the first instance of *N*-methylated lanthionine as a natural product. The biosynthesis of lanthionine has been proposed to involve addition of the thiol group of a Cys residue to a double bond of Dha, generated by dehydration from Ser, forming a sulfide bridge.[25] Therefore, **11** should be biosynthetically formed from **1**.

Structure of 15. The structures of three linear peptides **12**–**14** have been reported in the previous report.[12] Compound **13** was obtained in sufficient amount (3.0 mg) from 950 g of cell materials to assign the structure.[12] Although the ^{1}H NMR spectrum of **13** showed broad signals not helpful for structure assignment, FABMS/CID/MS of **13** revealed the amino acid sequence. The *N*-terminal amino acid (L-Leu) was confirmed by dansylation of **13** and acid hydrolysis of the dansylated peptide to compare the dansyl-Leu with an authentic sample.[12]

Compound **15** was positive to ninhydrin, the same as **12**–**14**. The molecular formula $C_{50}H_{79}N_{10}O_{13}$ was deduced from HRFABMS data for **15** (Table 3). The amino acid analysis of **15** showed D-Ala, D-MeAsp, L-Arg, D-Glu, and an unknown amino acid which was the same amino acid (Hil) detected in **10** (Table 3). The amino acid components showed the difference in the molecular weight (formula) between **15** and **13** to be 14 Da, CH_2, that is, **15** has an Hil unit in place of the Leu unit in **13**. Compound **15** gave a poor ^{1}H NMR spectrum since the amounts obtained were inadequate, and the spectrum was not helpful for assigning the structure. The FABMS/CID/MS spectrum of **15** was similar to that of **13**, and the useful product ion peaks were detected as shown in Scheme 2. The product ion peaks at m/z 900, 771, 302, 173, and 135 were observed in the FABMS/CID/MS spectrum of **15** at the same positions as those of **13**, while the peaks at m/z 855, 670, 536, 430, 413, and 100 were each detected 14 Da higher than the corresponding peaks of **13**. Thus, the structure of **15** was assigned as shown in Scheme 2.

Toxicity data for **1**–**5**, **7**, and **8** were reported in the previous papers.[2,26] The LD_{50}'s of **6** and new microcystins were 150 (**6**), 100 (**10**), and 1000 (**11**) μg/kg (ip, mice). Although the amounts of linear peptides **14** and **15** were not sufficient to examine their toxicity to mice, these linear peptides should be nontoxic since **12** and **13** showed no toxicity to mice at 1.1 and 2.25 mg/kg, respectively.[5]

The general method for assigning the structures of cyclic peptides possessing α,β-unsaturated amino acid unit(s) developed with nodularin[5] was also successfully applied to the structure assignment of microcystins. This method is, therefore, useful and can be applied to other cyclic peptides containing α,β-dehydroamino acid(s) to make linear peptide(s). Linear peptides with such unit-(s) can also be examined by this method to generate any peptide fragments. It should be noted that a mild acidic hydrolysis to cleave the dehydroamino acid unit of microcystins gave a poor yield of the corresponding linear peptides and similar treatment of nodularin resulted in decomposition. Nodularins were converted to linear peptides satisfactorily only by the present method for assigning the sequences.

Experimental Section

General. FAB mass spectra were run on either a ZAB-SE or a 70-SE4F mass spectrometer using Xe atoms (8 keV energy) and a matrix of dithiothreitol/dithioerythritol ("magic bullet").[15] Tandem mass spectra (linked scan at constant B/E) in the FAB mode were obtained on a four-sector tandem mass spectrometer (70-SE4F) using He as a collision gas: resolution of the first and second mass spectrometers, both 1000; accelerating potential, 8 keV; collision energy, 4 keV; attenuation, 90%. HRFAB mass spectra were acquired at a resolving power of 10000 (10% valley). ^{1}H NMR spectra were recorded on a GN-500 FT NMR spectrometer using CD_3OD (δ 3.30 ppm) as solvent. The ^{1}H NMR signals were assigned based on the analysis of ^{1}H–^{1}H COSY spectra and single-frequency decoupling experiments. Specific rotations were obtained on a DIP-370 digital polarimeter using a 3.5 × 10 mm cell. Since the sample amounts (isolated and) used for determining the specific rotations were small, the measurements were repeated at least 6 times for samples of higher concentration and 20 times for those of lower concentration to obtain more precise data.

TLC was performed on precoated silica gel plates (Kieselgel 60 F_{254}), 0.25 mm thick for analytical and preparative separation, and 1 mm thick for preparative separation. Solvents (A) $CHCl_3$–MeOH–H_2O, 26:15:3, (B) EtOAc–2-PrOH–H_2O, 8:4:3, (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 and then heating for analytical samples. R_f values for isolated compounds were as follows:[27]

	1	**6**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
(A)	0.26	0.20	0.26	0.29	0.09	0.11	0.14	0.15	0.17
(B)	0.15	0.14	0.13	0.18	0.05	0.05	0.05	0.05	0.05
(C)	0.40	0.39	0.35	0.42	0.19	0.19	0.16	0.17	0.19
(D)	0.42	0.42	0.40	0.50	0.17	0.24	0.28	0.27	0.30

HPLC was carried out on a Nucleosil 7 C_{18} column (10 × 250 mm) for preparative separation and a Nucleosil 5 C_{18} column (4.6 × 250 mm) for analytical separation with solvents (A) MeOH–0.7% Na_2SO_4 (6:4) and (B) CH_3CN–0.1% NH_4OAc

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(27) R_f values reported in the previous paper[2] for [L-MeSer^7]-microcystin-LR (**6**) were accidentally interchanged by those for **13**.

(27:73) at 2.0 (preparative) and 1.0 mL/min (analytical). Retention times (min, analytical) for isolated compounds were as follows:

	1	6	9	10	11	12	13	14	15
(A)	6.4	5.7	6.6	7.0	6.1	8.1	6.8	6.5	8.0
(B)	7.7	6.7	9.7	9.0	6.4	8.0	5.2	6.9	8.0

Cell Material. Field samples used in the present study were from the same source as that reported in the previous paper.[2] The cells collected from Homer Lake (Illinois) on August 13, 1988, consisted of *M. aeruginosa* (dominant) plus smaller quantities of *M. viridis* and *M. >wesenbergii*,[2] which were lyophilized in several batches separately and stored in a freezer (−20 °C) pending extraction.

Isolation of Microcystins. The remaining fractions from the previous isolation[2] were subjected to repeated HPLC and TLC separation to give **10** and **13**. The dried cells (200 g) were separated in a manner similar to that reported previously.[2] The MeOH extract was adsorbed on an ODS column (150 g), and the column was washed successively with H_2O (3.0 L) and 10% MeOH in H_2O (2.0 L); the microcystins were eluted with 80% MeOH (1.0 L). The toxin fraction was chromatographed on an LH-20 column (470 mL) with MeOH and then on a silica gel column (150 g) with $CHCl_3$:MeOH:H_2O (26:15:3) to afford a crude sample of **1** (298 mg) and fraction 2. Similar procedures were repeated four times with a total of 950 g of cells. Fraction 2 (682 mg) was separated by HPLC with solvent (B) into ten fractions. Each fraction was repeatedly chromatographed on an ODS HPLC column and on silica gel TLC to give **1**−**15**. Crude samples of **6** and **8**−**15**, thus obtained, were purified separately by TLC [solvent (A) for **9**-**12** and **14**, (D) for **6**, **8**, **13**, and **15**], and then by HPLC [solvent (A) for **6**, **8**, and **11**−**15**, (B) for **9** and **10**] to afford **6** [1.4 mg, $[\alpha]^{26}_D$ −86.0° (*c* 0.009, MeOH), **8** [2.7 mg, $[\alpha]^{26}_D$ − 53.9° (*c* 0.018, MeOH), **9** (0.7 mg), **10** [1.7 mg, $[\alpha]^{26}_D$ −84.1° (*c* 0.0011, MeOH)], **11** [0.5 mg, $[\alpha]^{26}_D$ −87.9° (*c* 0.003, MeOH)], **12** [0.2 mg, $[\alpha]^{25}_D$ −46.2° (*c* 0.001, MeOH)], **13** [3.0 mg, $[\alpha]^{26}_D$ −34.0° (*c* 0.020, MeOH)], **14** (0.1 mg), and **15** (0.1 mg).

Ozonolysis of Microcystins (Linear Peptides). A solution of each microcystin (0.3−0.6 mg) in MeOH (0.5 mL) was treated with O_3/O_2 at −78 °C for 3−4 min. The reaction mixture was treated with $NaBH_4$ (2 mg) in H_2O (0.2 mL) and stirred at 0 °C for 10 min and at rt for 15 min. During the reduction MeOH was removed by N_2, and H_2O (0.3 mL) was added to the reaction mixture, which was acidified with 1 N HCl (pH 2−3), and then $NaBH_4$ (3 mg) in H_2O (0.1 mL) was added. After being stirred at rt for 15 min, the reaction mixture was acidified with 1 N HCl (pH 3−4) and passed through a preconditioned ODS cartridge. The cartridge was rinsed with H_2O and eluted with MeOH to give a linear peptide.

17: Anal. Calcd for $C_{33}H_{59}N_{10}O_{13}$: M_r 803.4263(M + H). Found: M_r 803.4276 (HRFABMS).

19: Anal. Calcd for $C_{33}H_{60}N_{13}O_{13}$: M_r 846.4434 (M + H). Found: M_r 846.4445 (HRFABMS).

20: Anal. Calcd for $C_{30}H_{53}N_{10}O_{13}$: M_r 761.3794 (M + H). Found: M_r 761.3792 (HRFABMS).

21: Anal. Calcd for $C_{36}H_{57}N_{10}O_{13}$: M_r 837.4107 (M + H). Found: M_r 837.4121 (HRFABMS).

22: Anal. Calcd for $C_{36}H_{65}N_{10}O_{16}$: M_r 893.4580 (M + H). Found: M_r 893.4584 (HRFABMS).

23: Anal. Calcd for $C_{32}H_{57}N_{10}O_{13}$: M_r 789.4107 (M + H). Found: M_r 789.4108 (HRFABMS).

24: Anal. Calcd for $C_{34}H_{61}N_{10}O_{13}$: M_r 817.4420 (M + H). Found: M_r 817.4423 (HRFABMS).

Gas Chromatography. Capillary GC analyses were carried out on a Chirasil Val-III column (0.32 mm x 25 m) with He as a carrier gas (flow rate, 38 mL/min; sprit ratio, 18:1). The program rate for the analysis of amino acid derivatives was 90 °C (2 min), then 8 °C/min to 190 °C (10 min). The MeLan derivative was detected by isothermal chromatography at 190 °C.

Hydrolysis of Microcystins and Derivatization of Amino Acids. Each sample (60−100 μg) was hydrolyzed with 6 N HCl (200 μL) at 110 °C for 19−21 h or at 140 °C for 40 min in a screw-capped vial. The reaction mixture was evaporated to dryness by N_2, treated with 4 N HCl−MeOH (200 μL) at 110 °C for 15 min and evaporated by N_2. The residue was heated with CH_2Cl_2 and TFAA (each 100 μL) at 150 °C for 10 min and evaporated by N_2. The residue was dissolved in CH_2Cl_2 for GC analysis.

Hydrolysis of Linear Peptides and Derivatization of Hydrolysates for GC. Each linear peptide (50−100 μg) was hydrolyzed as above. The residue obtained after evaporation by N_2 was treated with CH_2Cl_2 and TFAA (each 100 μL) at 110 °C for 5 min, evaporated by N_2, and redissolved in H_2O (300 μL), and the amino lactone derivatives were extracted with EtOAc (200 μL × 2). The EtOAc extract was evaporated and dissolved in CH_2Cl_2 for GC analysis. The aqueous layer was evaporated to dryness by N_2, esterified with 4 N HCl−MeOH, treated with CH_2Cl_2 and TFAA (each 100 μL) at 150 °C for 10 min, and evaporated. The residue was dissolved in CH_2Cl_2 for GC analysis. The TFA-amino lactones were detected by isothermal chromatography at 160 °C on a Chirasil Val-III column.

Synthesis of Hil. The mixture of (2*S*,4*S*)- and (2*R*,4*S*)-isomers of Hil was prepared from *S*-(+)-1-bromo-2-methylbutane and diethyl acetamidomalonate by the method described by Han and Pascal.[20] Similarly, the mixture of four stereoisomers of Hil was synthesized from (±)-1-bromo-2-methylbutane (commercial). The reaction mixture, after 6 N HCl treatment, was directly methylated and trifluoroacetylated for GC.

Reaction of 1 with Cys. A solution of **1** (5.0 mg), $NaHCO_3$ (84 mg), and L-Cys hydrochloride monohydrate (18 mg) in H_2O (1.5 mL) was stirred at rt for 18 h. The reaction mixture was diluted with H_2O (1.0 mL) and passed through an ODS cartridge. The cartridge was rinsed with H_2O and eluted with MeOH. The MeOH eluate was evaporated, and the residue was separated by TLC [0.25-mm thick, solvent (C)] followed by HPLC [solvent (A)] to give **11** [2.5 mg, $[\alpha]^{25}_D$ −85.6° (*c* 0.017, MeOH)] and a minor product (0.1 mg).

Similarly, **1** (5.2 mg) reacted with D-Cys to afford **11**-(D-Cys) [1.7 mg, $[\alpha]^{24}_D$ −91.0° (*c* 0.010, MeOH)] and a minor product (0.1 mg).

Toxicity Testing. The compounds were dissolved in H_2O and injected intraperitoneally into four ICR-Swiss male mice (15−25 g) at each of 4−6 concentrations. An estimate of LD_{50}, signs of poisoning, survival times, and body and liver weights were recorded and compared to the effects of known cyanobacterial peptide hepatotoxins.

Acknowledgment. This study was supported in part by grants from the National Institutes of General Medical Sciences (GM 27029) and of Allergy and Infectious Diseases (AI 04769) to K.L.R. and by a subcontract from the latter grant to W.W.C. We thank Drs. A. M. Dahlem and R. R. Stotts for collection and lyophilization of *Microcystis*.

Supplementary Material Available: ^{1}H NMR spectra of **6**, **8**, **10**, natural **11**, synthetic **11**, and synthetic **11**-(D-Cys); ^{1}H-^{1}H COSY spectra of **6**, **8**, **10**, and synthetic **11**; ^{1}H-^{1}H DQF-COSY spectrum of **7** (11 pages). This material is contained in 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.

JO9417277