New Nodularins: A General Method for Structure Assignment

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A general method has been developed for assigning the structures of nodularin, a potent hepatotoxin, tumor promoter, and protein phosphatase inhibitor, and minor components isolated from a cultured and a bloom sample of the cyanobacterium *Nodularia spumigena*. It consists of (1) FABMS analysis (determination of molecular weight and molecular formula), (2) ^{1}H NMR spectroscopy on the parent compound and chiral GC analysis of an acid hydrolyzate (identification and stereochemistry of amino acid components), (3) ozonolysis followed by $NaBH_4$ reduction (conversion to a linear peptide), and (4) FABMS/CID/MS analyses of the linear peptide and the parent compound (sequence analysis). The method has been employed in assigning structures to three new nodularins (**2**–**4**) and can be applied to other cyclic peptides containing α,β-dehydroamino acid unit(s), especially the related microcystins, cyclic heptapeptide hepatotoxins. Two nodularins, [DMAdda^3]nodularin (**2**) and [(6*Z*)-Adda^3]nodularin (**3**), were obtained from a bloom sample collected from Lake Ellesmere (New Zealand), and [D-Asp^1]nodularin (**4**) was isolated from cultured cells (strain L-575). The LD50s of **2** and **4** were 150 and 75 μg/kg (ip, mice), respectively, but **3** did not show apparent toxicity at 2.0 mg/kg.

Nodularin (**1**)[1], a cyclic pentapeptide hepatotoxin produced by a brackish water cyanobacterium (blue-green alga), *Nodularia spumigena*,[2,3] shows strong inhibitory activity to protein phosphatases 1 and 2A[4] and has been reported to be a potent tumor promoter,[5] similar to the related microcystins,[4-7] cyclic heptapeptide hepatotoxins. Both nodularin and microcystins contain the unusual (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and D-*erythro*-β-methylaspartic acid (D-MeAsp), and nodularin has a 2-(methylamino)-2-dehydrobutyric acid (Mdhb) unit while microcystins have a dehydroalanine unit. Recently, a cyclic pentapeptide, motuporin, has been isolated from a sponge, *Theonella swinhoei*, collected from Papua New Guinea, and the structure assigned for motuporin was very similar to **1**,[8] but has a L-Val unit in place of the L-Arg unit in **1**, that is, the structure of motuporin is [L-Val^2]nodularin.

Table 1. Amino Acid Analysis and HRFABMS Data for 1–5

		HRFABMS		
compd	amino acids[a]	observed (M + H)	composition	Δ[b]
1	D-MeAsp, L-Arg, D-Glu	825.4497	$C_{41}H_{61}N_8O_{10}$	+1.4
2	D-MeAsp, L-Arg, D-Glu	811.4349	$C_{40}H_{59}N_8O_{10}$	+0.5
3	D-MeAsp, L-Arg, D-Glu	825.4489	$C_{41}H_{61}N_8O_{10}$	+2.2
4	D-Asp, L-Arg, D-Glu	811.4341	$C_{40}H_{59}N_8O_{10}$	+1.3
5	D-MeAsp, L-Arg, D-Glu	843.4611	$C_{41}H_{63}N_8O_{11}$	+0.5

[a] Based on GC analysis on a chiral capillary column. [b] Difference (mDa) from the calculated value for each composition.

This variation in the L-amino acid unit is similar to the variation in the two L-amino acid units detected in microcystins.[7] Recent progress on these cyclic peptides[20] includes studies on the biosynthesis of nodularin (**1**)[9] and microcystin-LR (LR)[10] and computer molecular modeling of both toxins.[11] We have recently reported the isolation of linear peptides as possible biosynthetic precursors of **1** and microcystins.[9]

During our structure study on minor constituents of *N. spumigena*, an efficient new method was required for assigning the structures with small amounts of compounds. We report here the development of such a general method for structure assignment of nodularins using **1** as the model compound. The method provided a determination of the

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Table 2. ^{1}H NMR Data for 1–4[a]

proton	mult[b]	δ (J in Hz) 1	2	3	4
[MeAsp][c]					
H-2	d	4.22	4.14 (2.5)	4.13 (2.5)	4.34 (dd, 5.0, 3.0)
H-3	m	3.04	2.95	2.95	2.45 (dd, 13.0, 5.0)
					2.76 (dd, 13.0, 3.0)
H_3-5	d	1.21 (6.8)	1.21 (6.8)	1.20 (6.8)	–
[Arg]					
H-2	dd	4.45	4.47	4.47 (10.5, 4.0)	4.45 (10.0, 4.0)
H-3	m	1.47	1.43	1.40	1.46
	m	2.07	2.06	2.09	2.04
H-4	m	1.49	1.46	1.43	1.49
	m	1.61	1.59	1.56	1.60
H_2-5	m	3.18	3.16	3.18	3.17
[Adda][d]					
H-2	m	3.03 (10.5, 6.8)	3.03 (10.5, 6.8)	3.03 (10.5, 7.0)	3.04 (10.5, 6.8)
H-3	dd	4.50 (10.5, 9.0)	4.50 (10.5, 9.0)	4.54 (10.5, 10.0)	4.49 (10.5, 9.0)
H-4	dd	5.60 (15.5, 9.0)	5.65 (15.5, 9.0)	5.75 (15.5, 10.0)	5.63 (15.5, 9.0)
H-5	d	6.21 (15.5)	6.23 (15.5)	6.50 (15.5)	6.21 (15.5)
H-7	d	5.38 (10.0)	5.40 (10.0)	5.25 (9.5)	5.37 (10.0)
H-8	m	2.58 (10.0, 6.8, 6.5)	2.51 (10.0, 6.8)	2.80 (9.5, 6.5)	2.56 (10.0, 6.8)
H-9	m	3.24 (8.0, 6.5, 4.5)	3.56 (8.5, 4.5)	3.23 (8.5, 4.0)	3.24 (7.5, 4.5)
H-10	dd	2.66 (14.0, 8.0)	2.58 (14.0, 8.5)	2.63 (13.5, 8.5)	2.67 (14.0, 7.5)
	dd	2.81 (14.0, 4.5)	2.82 (14.0, 4.5)	2.74 (13.5, 4.0)	2.82 (14.0, 4.5)
H-12, 16	d	7.17 (7.0)	7.18 (7.0)	7.19 (7.0)	7.18 (7.0)
H-13,15	dd	7.24 (7.0, 7.3)	7.24 (7.3, 7.0)	7.26 (7.3, 7.0)	7.23 (7.3, 7.0)
H-14	dd	7.16 (7.3, 7.0)	7.14 (7.0, 7.3)	7.16 (7.0, 7.3)	7.16 (7.0, 7.3)
H_3-17	d	1.02 (6.8)	1.02 (6.8)	0.98 (7.0)	1.03 (6.8)
H_3-18	d	1.62	1.70 (1.0)	1.84 (0.8)	1.62 (1.0)
H_3-19	d	0.98 (6.8)	1.00 (6.8)	1.01 (6.5)	0.98 (6.8)
H_3-20	s	3.23	–	3.18	3.23
[Glu]					
H-2	dd	4.46	4.56 (4.0, 4.0)	4.56	4.55
H-3	m	1.80	1.76	1.76	1.77
	m	2.26	2.35	2.36	2.35
H-4	m	2.05	2.06	2.06	2.07
	m	2.79	2.85	2.82	2.84
[Mdhb]					
H-3	q	6.94 (7.0)	6.90 (7.0)	6.90 (7.0)	6.91 (7.0)
H_3-4	d	1.74 (7.0)	1.75 (7.0)	1.75 (7.0)	1.75 (7.0)
NCH_3	s	3.08	3.08	3.08	3.06

[a] 500 MHz; CD_3OD (δ 3.30). [b] s = singlet, d = doublet, dd = doublet of doublets, q = quartet, m = multiplet. [c] Asp for 4. [d] *O*-Demethyl-Adda for 2.

absolute configuration of Adda in 1. Our previous assignment of the Adda configuration employed LR as its source, but Adda in nodularin was argued to be the same as that in LR from the similarity of the chemical shifts and coupling constants due to the Adda protons in the ^{1}H NMR spectra of both 1 and LR.[1] The new method required much smaller samples and is considerably more general and reliable than the procedure originally used to assign nodularin's structure.[1]

Once developed, the general method was applied to the structure assignment of new nodularins, minor components 2 and 3, obtained from a bloom sample of *N. spumigena* collected from Lake Ellesmere in New Zealand, and 4 from cultured *N. spumigena* cells. Except for motuporin, noted above, these represent the first natural variations on the nodularin structure.

General Method for Structure Assignment. The general method for structure assignment of nodularin minor components, employing small amounts, was developed using 1. The scheme consists of (1) determination of the molecular weight and assignment of a molecular formula by FABMS and high-resolution (HR) FABMS; (2) identification and stereochemical assignments of amino acid components by ^{1}H NMR spectroscopy and tandem FABMS (FABMS/CID/MS) of the parent compound (identifying Adda and Mdhb), and GC analysis of a derivatized acid hydrolyzate on a chiral capillary column

Table 3. ^{13}C NMR Data for 1[a]

carbon	δ (ppm)	carbon	δ (ppm)	carbon	δ (ppm)
[MeAsp]		[Adda]		C-17	16.4
C-1	177.9	C-1	177.0	C-18	13.0
C-2	60.5	C-2	44.7	C-19	16.6
C-3	41.1	C-3	56.7	C-20	58.7
C-4	178.3	C-4	126.8	[Glu]	
C-5	16.3	C-5	139.0	C-1	176.4
[Arg]		C-6	134.2	C-2	54.5
C-1	172.0	C-7	136.8	C-3	29.0
C-2	52.0	C-8	37.7	C-4	29.9
C-3	29.2	C-9	88.4	C-5	175.6
C-4	25.9	C-10	39.0	[Mdhb]	
C-5	41.9	C-11	140.5	C-1	165.6
C-6	158.6	C-12, 16	130.5	C-2	137.8
		C-13, 15	129.2	C-3	136.6
		C-14	127.0	C-4	13.3
				NCH_3	35.2

[a] 125 MHz; CD_3OD (δ 49.0).

(identifying the other amino acids); (3) ozonolysis followed by $NaBH_4$ reduction and acidic workup to form a linear peptide; and (4) sequence analysis by FABMS/CID/MS of the linear peptide. The absolute stereochemistry of Adda has been determined for LR,[1] and that in 1 was assigned based on the similarity of chemical shifts and coupling constants of this unit in the ^{1}H NMR spectra of 1 and LR.[1] Since the differences in chemical shifts between 1 and LR are larger than those between LR and other microcystins,

the absolute configuration of Adda in 1 was also confirmed during the development of the general method, as discussed below.

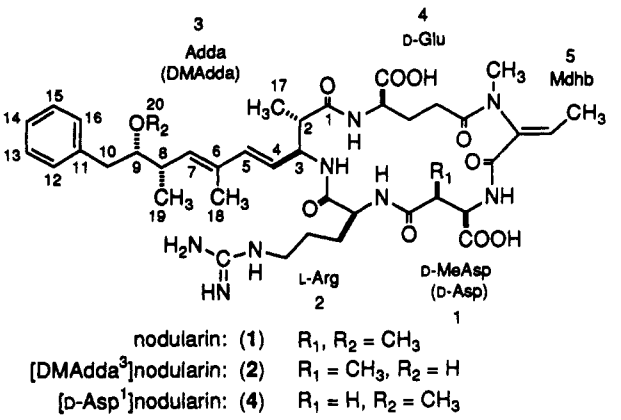
FABMS and HRFABMS of Nodularin (1). The FAB mass spectrum of 1 obtained with a matrix of dithiothreitol/dithioerythritol (magic bullet, M_r = 154)[12] showed a strong $(M + H)^+$ ion together with matrix adducts $(M + 154 + H)^+$ and $(M + 154 \times 2 + H)^+$. HRFAB mass spectral data on the $(M+H)^+$ ion agreed with the molecular formula $C_{41}H_{60}N_8O_{10}$ (Table 1). Amino acid analysis data can be used to support the molecular formula assigned to an unknown nodularin analog from HRFABMS data.

NMR Spectroscopy of Nodularin (1). ^1H NMR data for 1 assigned by the analysis of ^1H–^1H COSY spectra and single-frequency decoupling experiments are listed in Table 2. Table 3 lists the ^{13}C NMR signals of 1, assigned from analysis of ^{13}C–^1H COSY and ^{13}C–^{13}C COSY spectra of ^{13}C-enriched 1.[9] ^1H NMR data allowed identification of the Adda and Mdhb units, which do not appear in amino acid analysis because of decomposition during acid hydrolysis. The presence of the Adda and Mdhb units and any modifications in these units are also observed in FABMS/CID/MS of the parent compound[7] (see below). The stereochemistry of Adda in a new nodularin analog is assigned by comparison of chemical shifts and coupling constants for the Adda protons with those of 1.

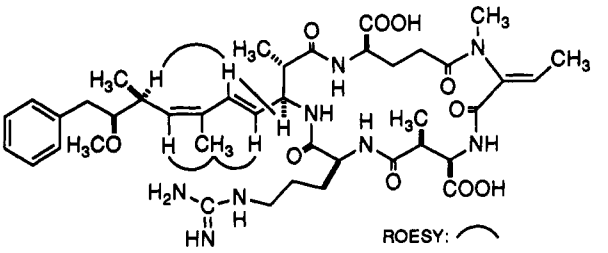
Stereochemistry of Amino Acid Components. Acid hydrolysis of 1 with 6 N HCl at 110 °C for 20 h or at 140 °C for 40 min followed by esterification and acylation of the hydrolyzate gave trifluoroacetyl methyl ester derivatives of amino acid components. GC analysis of the amino acid derivatives on a chiral capillary column (Chirasil Val III)[13] revealed the stereochemistry of each amino acid component (Table 1), except for Adda and Mdhb, which in a new nodularin analog would be assigned by NMR as above. It should be noted that the D-MeAsp unit always gave a mixture of D-*erythro*- and D-*threo*-derivatives, and the acid hydrolysis at 140 °C for 40 min showed a higher ratio of *erythro*:*threo* (ca. 9:1) than the reaction at 110 °C for 20 h (3–4:1).

Ozonolysis of Nodularin (1). Ozonolysis of 1 followed by $NaBH_4$ reduction and HCl workup gave aromatic alcohol 6 [theoretical $(M + H)^+$ for $C_{12}H_{19}O_2$ = 195.1385; found 195.1387 (HRCIMS)] and linear peptide 7 [theoretical $(M + H)^+$ for $C_{24}H_{43}N_8O_{11}$ = 619.3051; found 619.3036 (HRFABMS)] (Scheme 2). When the reduction was quenched with AcOH, a cyclic peptide was obtained, which gave 7 upon HCl treatment and $NaBH_4$ reduction. The structure of this cyclic peptide [*m/z* 617 $(M + H)^+$, FABMS] can be assigned to have a carbinolamine group with a N–C bond between N^ω of the N^ω-methylglutamine unit and C-2 of the reaction product (aldehyde) from the Mdhb unit. The reduction of the ozonide of 1 with $NaB(CN)H_3$ under acidic conditions (pH 3–4 with HCl) gave a poor yield of 7 and complicated products. Ozonation of the Mdhb unit required a longer time (>10 min), and a cyclic peptide with the intact Mdhb unit [*m/z* 627 $(M + H)^+$, FABMS] was obtained when the reaction was quenched within a few minutes. These cyclic and linear peptides showed no hepatotoxicity. Compound 6 was used for comparing the stereochemistry with synthetic compounds, and 7 was hydrolyzed to give amino lactone 8 for determining its stereochemistry (Scheme 2).

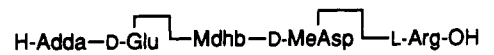
Scheme 1



nodularin: (1) R_1, R_2 = CH_3
[DMAdda^3]nodularin: (2) R_1 = CH_3, R_2 = H
[D-Asp^1]nodularin: (4) R_1 = H, R_2 = CH_3



[(6*Z*)-Adda^3]nodularin (3)



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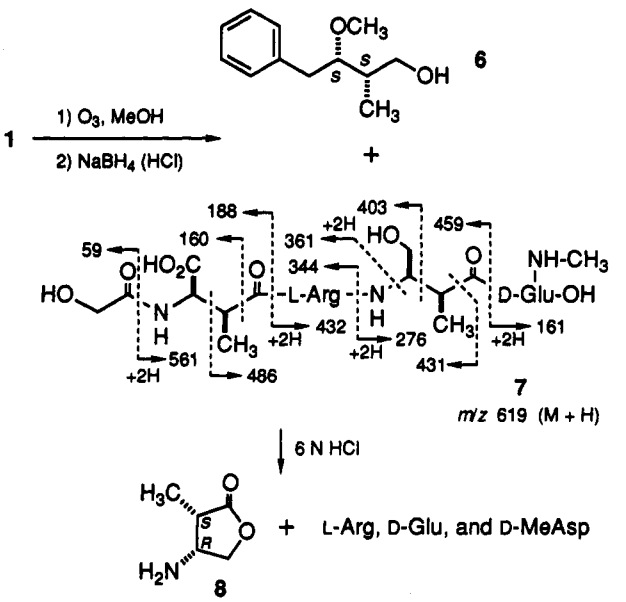
Scheme 2



FABMS/CID/MS Analysis of Nodularin (1). Figure 1 shows the FABMS/CID mass spectrum of 1. The product ion peaks useful for structure assignment are listed in Table 4. The product ion peaks at *m/z* 689 (M – 135) and 135 $[PhCH_2CH(OCH_3)]$ confirmed the presence of the Adda unit, and any modifications in this unit can be detected in these product ions[7,14] as seen in the FABMS/CID/MS spectrum of 2 (see below). The presence of the Mdhb unit

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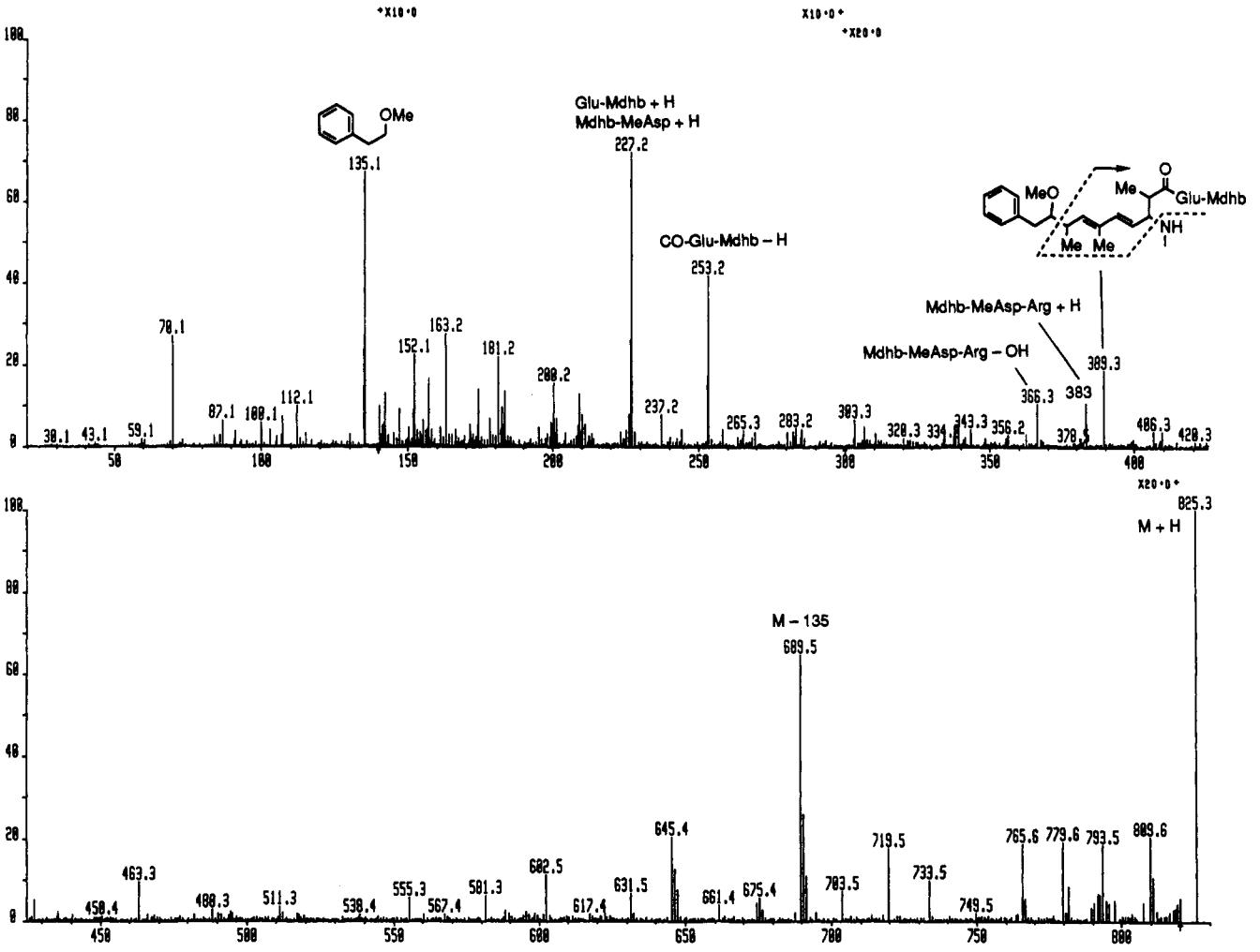

Figure 1. FABMS/CID/MS spectrum of 1.

Table 4. FABMS/CID/MS Data for 1–4

composition	product ion, *m/z* 1	2	3	4
M + H	825	811	825	811
M – 135	689[a]	689[b]	689	675
$C_{11}H_{15}O$-Glu-Mdhb[c]	389	389	389	389
Mdhb-MeAsp-Arg + H	383	383	383	369[d]
Mdhb-MeAsp-Arg + H – OH	366	366	366	352[d]
CO-Glu-Mdhb – H	253	253	253	253
Glu-Mdhb + H	227	227	227	227
Mdhb-MeAsp + H	227	227	227	213[d]
$PhCH_2CH(OCH_3)$	135[a]	–	135	135

[a] Ion formula was confirmed by HRFABMS. [b] (M – 121). [c] $C_{11}H_{15}O$ = Adda – 135 – NH (see Figure 1). [d] Contains Asp instead of MeAsp.

in the product ion peaks at *m/z* 389, 383, 366, 253, and 227 was confirmed by FABMS/CID/MS of dihydro derivatives of this unit [*i.e.* D- and L-2-(methylamino)butyric acid variants] obtained by $NaBH_4$ reduction of 1.[15] Although the product ion peaks at *m/z* 389 ($C_{11}H_{15}O$-Glu-Mdhb), 383 (Mdhb-MeAsp-Arg + H), and 366 (Mdhb-MeAsp-Arg – OH) had rather weak intensities, the whole sequence was detected in the FABMS/CID mass spectrum of 1 (Figure 1), in contrast to those of microcystins.[7]

FABMS/CID/MS of Linear Peptide 7. To confirm the sequence deduced from FABMS/CID/MS of 1, the $(M + H)^+$ ion (*m/z* 619) of linear peptide 7 was subjected to FABMS/CID/MS to give product ions (Figure 2). The product ion peaks obtained by fragmentation in the MeAsp and 3-amino-4-hydroxy-2-butanoic acid units were observed at *m/z* 486, 431, 403, 361, and 160, together with the ions from fragmentation at each peptide bond as shown in Scheme 2, completing the structure assignment of 1.

Absolute Stereochemistry of Adda in Nodularin (1). Amino Lactone 8: The four possible stereoisomers of the Cbz-protected amino lactones were synthesized according to the reported method[16] as shown in Scheme 3.[17] The *trans*- and *cis*-isomers were separated by silica gel chromatography (benzene–EtOAc, 65:35),[17] and the Cbz-group was removed by HBr/AcOH to give the free amino lactones, which were acylated with trifluoroacetic anhydride (TFAA) and analyzed on a chiral GC capillary column (Chirasil Val III), the same as for the separation of amino acid derivatives. The four synthetic TFA-amino lactones showed retention times of 14.0 (TFA-8), 14.7 (TFA-11), 14.9 (TFA-9), and 15.3 min (TFA-10) by isothermal chromatography at 160 °C. Linear peptide 7 gave a mixture of two amino lactones together with L-Arg, D-MeAsp, and D-Glu upon acid hydrolysis. The TFA derivatives of natural amino lactones obtained by acid

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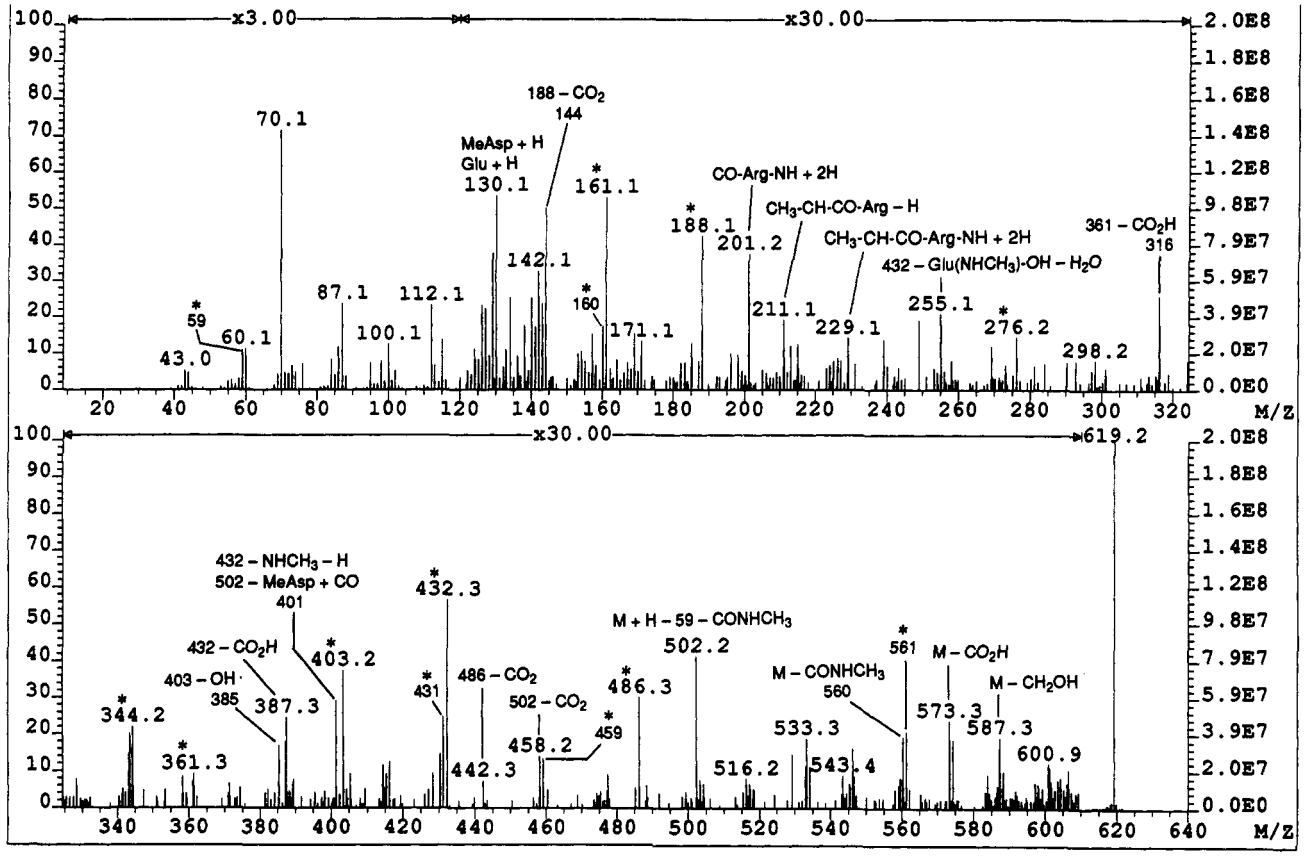
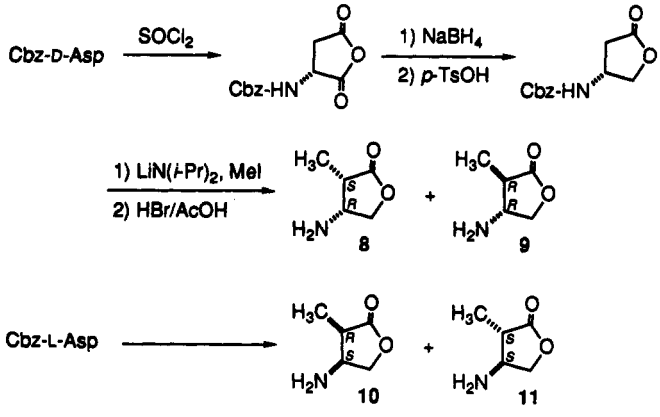


Figure 2. FABMS/CID/MS spectrum of linear peptide 7 obtained from 1 (Scheme 2). (See Scheme 2 for assignment of the peaks labeled with *.)

Scheme 3



hydrolysis of 7 at 140 °C for 40 min showed a ca. 9:1 ratio of TFA-8:TFA-9, and the ratio was changed to ca. 3:2 when 7 was hydrolyzed at 110 °C for 19 h, which is a similar epimerization to that observed for the hydrolysis products from the MeAsp unit. Thus, the stereochemistry of the two chiral centers at C-2 and -3 in the Adda unit of 1 was confirmed as (2*S*,3*S*).

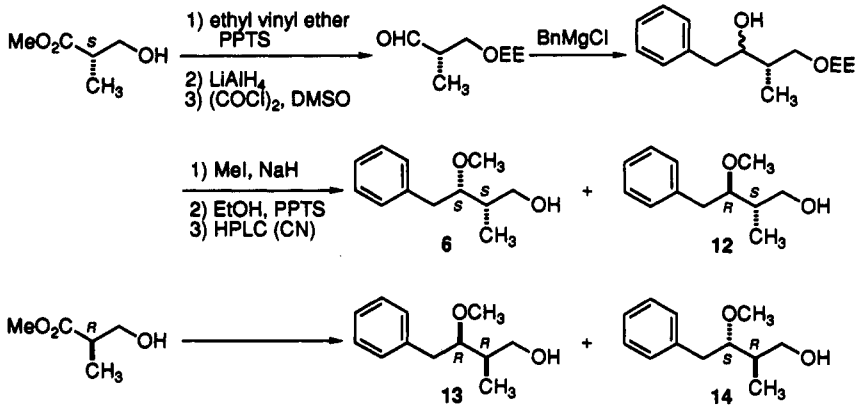
Aromatic Alcohol 6. The four stereoisomers of the aromatic alcohols which might be obtained from Adda were synthesized (Scheme 4) as reported in the preliminary communication[1] (also see Experimental Section). Compounds 12 and 14 were distinguished from the natural compound by HPLC on a cyanoethyl bonded silica gel (CN) column with hexane–2-PrOH (15:1, t_R, 6 and 13 = 6.3 min, 12 and 14 = 6.6 min) and ^{1}H NMR spectroscopy (see Experimental Section). Distinction between 6 and 13 was accomplished by ^{1}H NMR using a chiral solvating reagent, (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol,[18] as shown in Figure 3.

Since the amount of natural 6 obtained from LR[1] was limited (0.6 mg), a suitable solvent and concentration of the reagent were sought using 0.6 mg of a racemic mixture (6:13, 1:1), and C_6D_6 was found to give the best result. A 6% solution of the reagent in C_6D_6 (27.0 mg in 0.45 mL) was the most practical concentration and was used for the analysis of natural and synthetic 6 and 13 (Figure 3). Solutions of synthetic 6 and 13 contained, respectively, 10 and 5% of the opposite antipode (Figures 3B and 3C, respectively). The solution of natural 6 and synthetic 13 gave two nearly equal singlets for the methoxy methyl signal (Figure 3E) when each was present in the same amount (each 0.3 mg), while the mixture of natural and synthetic 6 showed essentially one signal for the methoxy methyl group (Figure 3D). Consequently, the stereochemistry at the C-8 and C-9 positions of Adda in LR was determined as (8*S*,9*S*). The mixture of synthetic 13 and natural 6 obtained from 1 (each 0.1 mg) with 6% of the chiral solvating reagent in C_6D_6 showed essentially the same spectrum as Figure 3E, confirming the stereochemistry of C-8 and -9 of the Adda unit in 1.

Isolation of Minor Components 2-5. Dried cells of *N. spumigena* obtained both from a water bloom and from a cell culture were extracted separately with MeOH. In each case the extract was subjected to solid-phase extraction with ODS followed by LH-20 column chromatography. The toxin fraction thus obtained was separated by repeated HPLC and TLC, and compounds 1 (50.0 mg), 2 (1.3 mg), and 3 (2.0 mg) were isolated from the bloom sample (50

(18) Pirkle, W. H.; Rinaldi, P. L. *J. Org. Chem.* **1978**, *43*, 4475–4480.

Scheme 4



PPTS = pyridinium *p*-toluenesulfonate. EE = 1-(ethoxy)ethyl.

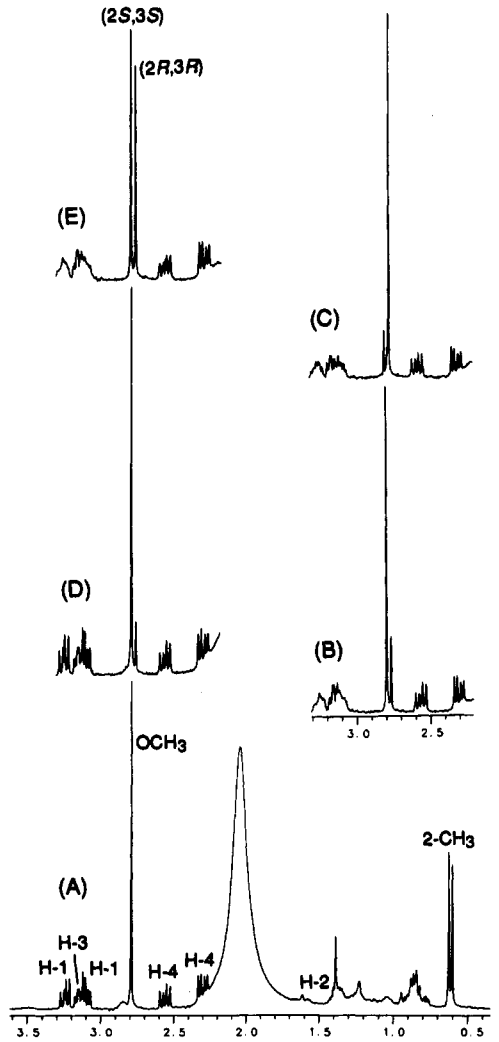


Figure 3. ^{1}H NMR spectra of (A) natural **6**, (B) synthetic **6**, (C) synthetic **13**, (D) mixture of natural **6** and synthetic **6**, and (E) mixture of natural **6** and synthetic **13** observed with chiral solvating reagent (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol[18] (6% solution).

g). Cultured cells (32 g) gave **1** (19.3 mg), **4** (0.4 mg), and **5** (0.1 mg), but **2** and **3** were not detected in any fraction. R_f values on TLC and HPLC retention times of the isolated compounds are listed in the Experimental Section. The structure of **5** was assigned based on the analysis of FABMS/CID/MS data as reported elsewhere.[9] The general method developed with **1** above was utilized for the structure assignment of the new cyclic peptides **2**–**4**.

Structure of 2. The molecular formula $C_{40}H_{58}N_8O_{10}$ for **2** was assigned from HRFABMS data combined with amino acid analysis data on a chiral GC capillary column, which showed three amino acid components: D-MeAsp, L-Arg, and D-Glu (Table 1). The difference in the molecular weight (formula) between **2** and **1**, 14 Da (CH_2), was not detected in the three amino acid components appearing on GC. The ^{1}H NMR spectrum of **2** revealed the presence of the Mdhb unit with signals at δ 6.90 (1H, q, J = 7.0 Hz), 3.08 (3H, s), and 1.75 (3H, d, J = 7.0 Hz) (Table 2), which are ascribable to the olefinic proton (H-3), NCH_3, and the methyl group on the double bond (H_3-4), respectively. FABMS/CID/MS data for **2** showed fragment ion peaks at m/z 389, 383, 366, 253, and 227, the same as for **1**, confirming the presence of the Mdhb unit (Table 4). However, a fragment ion peak at m/z 135 [$PhCH_2CH(OCH_3)$], which is the base peak among the product ions of ordinary Adda-containing compounds, was not detected; instead an $(M - 121)^+$ peak was observed at m/z 689, suggesting that the difference (14 Da, CH_2) is ascribable to a demethyl variant of Adda. ^{1}H NMR signals due to the Adda unit in **2** were similar to those in **1**, except for the lack of the C-9 methoxy methyl (H_3-20) signal, which was observed at δ 3.23 in the spectrum of **1** (Table 2). The H-9 signal of the modified Adda unit in **2** was detected at lower field (δ 3.56) than that in **1** (δ 3.24). These data clearly showed that **2** has an *O*-demethyl-Adda (DMAdda) unit.[7]

The stereochemistry of the DMAdda unit in **2** was assigned as the same as that of the Adda unit in **1** from the ^{1}H NMR data for these units.[1,7,15] The chemical shifts of all DMAdda protons in **2** and all Adda protons in **1** are very close to each other, except for those near the 9-OH group (H-8, H-10, H_3-18, and especially H-9). The shifts in resonance for **2** vs **1** for H-4 (+0.05 ppm), H-8 (−0.07), H-9 (+0.32), H-10a (−0.08), and H_3-18 (+0.08) were very similar to those detected between [DMAdda^5]microcystin-LR and LR.[7] Moreover, the coupling constants for all DMAdda protons in **2** are also very close to those for Adda in **1**, confirming the relative stereochemistry in DMAdda in **2**.

Thus, the five amino acid components were assigned. The FABMS/CID/MS data for **2** suggested the sequence of the five amino acids to be the same as that in **1** since the same fragment ion peaks (m/z 389, 383, 366, 253, and 227) were observed in the spectra of **2** and **1** (Table 4).

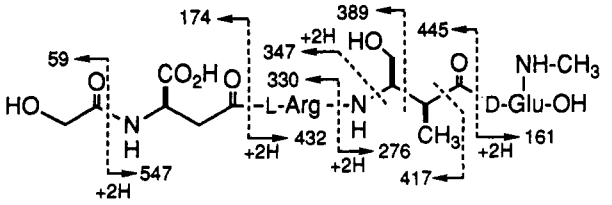
The absolute stereochemistry of DMAdda and the sequence of **2** were confirmed with an ozonolysis product. Compound **2** gave linear peptide **7** [theoretical $(M + H)^+$ for $C_{24}H_{43}N_8O_{11} = 619.3051$; found 619.3059 (HRFABMS)] upon ozonolysis followed by $NaBH_4$ reduction. The FABMS/CID/MS spectrum of **7** was identical to that of **7** obtained from **1**. Acid hydrolysis (at 140 °C for 40 min) of **7** and derivatization of the hydrolyzate showed TFA-8 and a small amount of TFA-9. Thus, the structure of **2** was assigned as [DMAdda^3]nodularin as shown in Scheme 1.

Structure of 3. Compound **3** had the same molecular formula ($C_{41}H_{60}N_8O_{10}$) as that of **1**, assigned from HR-FABMS data (Table 1). Although TLC did not separate **3** from **1**, HPLC with solvent (A) showed separation of the two compounds. Amino acid analysis (Table 1) and FABMS/CID/MS data (Table 4) for **3** were identical to those for **1**, suggesting that **3** is a stereoisomer of **1**. Ozonolysis and $NaBH_4$ reduction of **3** gave linear peptide **7** [found 619.3048 (HRFABMS)], and GC analysis of the acid hydrolyzate of **7** showed that the stereochemistry at the C-2 and C-3 positions of Adda in **3** was the same as that in **1**. The difference between **3** and **1** was detected in their ^1H NMR spectra. The lower-field shift of the signals due to H-4 (+0.15 ppm), H-5 (+0.29), H-8 (+0.22), and H_3-18 (+0.22) and higher-field shift of the H-7 signal (−0.13) of **3** compared to the chemical shifts due to corresponding protons of **1** closely resembled those observed between [(6*Z*)-Adda^3]microcystin-LR and LR.[19] The other, smaller differences in the chemical shifts of the Adda protons between **3** and **1** were also very similar to those between LR and its (6*Z*)-isomer. Moreover, NOE's were observed between H-3 and H-5, H-4 and H_3-18, H-5 and H-8, and H_3-18 and H-7 in the ROESY spectrum of **3** (Scheme 1), which were identical to those observed in the ROESY spectrum of [(6*Z*)-Adda^3]microcystin-LR.[19] These data revealed that **3** is a stereoisomer of **1** at the Δ^6 double bond in the Adda unit, that is, the structure of **3** is [(6*Z*)-Adda^3]nodularin, as shown in Scheme 1.

Compound **3** was not detected in any fractions separated from cultured cells (strain L-575) obtained in several different cultivations. The strain L-575 was isolated from a water bloom collected from the same lake as the bloom sample used in the present study.[2] The formation of the (6*Z*)-isomer of Adda is not associated with that of the dominant (6*E*)-isomer in the cell culture. It suggests that (6*Z*)-isomers of nodularin and microcystins might be formed by sunlight rather than by biosynthesis as argued.[19]

Structure of 4. The molecular formula $C_{40}H_{58}N_8O_{10}$ of **4** was deduced from its HRFABMS data (Table 1). The differences in molecular weight and molecular formula (14 Da, CH_2) between **4** and **1** were detected in the amino acid analysis, that is, **4** has D-Asp in place of D-MeAsp in **1** (Table 1). The ^1H NMR spectrum of **4** revealed the presence of the Mdhb and Adda units, as listed in Table 2. Comparison of the chemical shifts and coupling constants due to the Adda protons in **4** and **1** allowed assignment of the relative stereochemistry of Adda in **4**. FABMS/CID/MS of **4** confirmed the presence of the Mdhb and Adda units and showed the position of the Asp unit by the fragment ion peaks at *m/z* 369 (Mdhb-Asp-Arg + H), 352 (Mdhb-Asp-Arg − OH), and 213 (Mdhb-Asp + H),

Scheme 5



15 *m/z* 605 (M + H)

which were each 14 Da less than the corresponding peaks of **1** (*m/z* 383, 366, and 227) (Table 4), while the fragment ion peaks of **4** containing the Glu unit were detected at the same positions as those of **1** (*m/z* 389, 253, and 227). These data indicated the structure of **4** to be [D-Asp^1]nodularin.

Compound **4** gave linear peptide **15** [theoretical $(M + H)^+$ for $C_{23}H_{41}N_8O_{11} = 605.2895$; found 605.2902 (HR-FABMS)] upon ozonolysis followed by $NaBH_4$ reduction. FABMS/CID/MS of **15** confirmed the sequence by the fragment ion peaks shown in Scheme 5. A prominent peak due to fragmentation in the Asp unit was not detected in the FABMS/CID/MS spectrum of **15**, in contrast with the peaks at *m/z* 160 and 486 observed in that of **7** generated by fragmentation in the MeAsp unit. Acid hydrolysis of **15** afforded the same amino lactone **8** as that obtained from **7**, confirming the absolute stereochemistry of Adda in **4**. Thus, the structure of **4** was assigned as shown in Scheme 1.

Conclusion and Bioactivities. In summary, a general method for assigning the structures of nodularins has been developed using **1** as the lead compound and applied to the minor components **2**–**4** isolated from cultured and bloom samples of *N. spumigena* to examine its utility. The method can be used for the structure assignment of other cyclic peptides containing α,β-dehydroamino acid(s), especially the related cyclic heptapeptides, microcystins.[20]

The LD50s to mice (ip) of the minor components were 150 (**2**) and 75 μg/kg (**4**) (LD50 of **1** = 60 μg/kg). Compounds **3** and **5** did not show apparent toxicity to mice at 2.0 and 1.0 mg/kg, respectively, which shows that the (4*E*,6*E*) geometry of the diene in the Adda unit and the cyclic structure are essential for the activity of nodularins and microcystins.[9,19]

It is of interest that motuporin showed *in vitro* cytotoxicity to several cancer cell lines.[8] On the other hand, **1** and LR did not show *in vitro* cytotoxicity against L1210 murine leukemia cells at a concentration of 100 μg/mL, which is presumably ascribable to the replacement of the polar Arg unit in **1** by the hydrophobic Val unit in motuporin.[20]

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").[12] Tandem mass spectra (MS/CID/MS, 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 10 000 (10% valley). EI and CI mass spectra were measured on CH-5 and 70-VSE mass spectrometers, respectively, and

(19) Harada, K.-I.; Ogawa, K.; Matsuura, K.; Murata, H.; Suzuki, M.; Watanabe, M. F.; Itezono, Y.; Nakayama, N. *Chem. Res. Toxicol.* **1990**, *3*, 473–481.

(20) Rinehart, K. L.; Namikoshi, M.; Choi, B. W. *J. Appl. Phycol.* **1994**, in press.

methane was used as reagent gas for CIMS. ^{1}H NMR spectra were recorded on either an XL-200, a QE-300, or a GN-500 FT NMR spectrometer using $CDCl_3$, C_6D_6 (TMS = 0.0 ppm), or 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 Jasco DIP-370 digital polarimeter using a 3.5 × 100-mm cell or a 3.5 × 10-mm cell.

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

	1	2	3	4	5
(A)	0.35	0.21	0.36	0.28	0.23
(B)	0.13	0.11	0.13	0.11	0.07
(C)	0.41	0.36	0.42	0.34	0.22
(D)	0.49	0.48	0.49	0.42	0.31

HPLC was carried out on a Nucleosil 7 C_{18} column (10 × 250 mm) for preparative separation (2.0 mL/min) and a Nucleosil 5 C_{18} column (4.6 × 250 mm) for analytical separation (1.0 mL/min) with solvents (A) MeOH–0.7% Na_2SO_4 (6:4, preparative; 55:45, analytical), (B) CH_3CN–0.1% NH_4OAc (27:73, preparative; 25:75, analytical), and (C) MeOH–0.05% TFA (6:4, analytical and preparative). Retention times (min, analytical) for isolated compounds were as follows:

	1	2	3	4	5
(A)	9.4	4.6	12.2	8.9	9.0
(B)	8.0	3.6	8.2	8.0	7.3
(C)	8.8	4.6	10.2	7.8	5.7

Nodularia spumigena. A field sample was collected from Lake Ellesmere (New Zealand) in 1983. The cells were lyophilized and stored in a freezer pending extraction. Laboratory cell cultures of *N. spumigena* L-575 were grown according to the procedure described by Carmichael et al.[2] Log to stationary growth phase cultures were harvested by tangential flow centrifugation, lyophilized, and stored at −80 °C until used for toxin extraction.

Isolation of Nodularins. Dried cells (field sample, 50 g) were extracted with MeOH (1500 mL × 3, 16 h each). Each extract was filtered from cells and evaporated in vacuo. The residue was suspended in H_2O (500 mL) and filtered. The filtrate was applied on an ODS silica gel column (100–200 mesh, 60 g), and the column was washed with H_2O (500 mL) and 5% MeOH in H_2O (400 mL) and then eluted with MeOH (300 mL). The toxin-containing fractions detected by TLC were collected and evaporated to dryness, redissolved in MeOH, and chromatographed on an LH-20 column (25-100 mesh, 470 mL) to give a toxin fraction (328 g). The toxin fraction was separated by HPLC with solvent B (27:73) to give fractions 1 (43 mg), 2 (107 mg), and 3 (141 mg). Fraction 1 was subjected to preparative TLC with solvent A to separate a crude sample of 2, which was purified by HPLC (solvent A, 6:4) to afford 1.3 mg, $[\alpha]^{23}_D$ −52.0° (*c* 0.009, MeOH). HPLC separation (solvent A, 6:4) of fraction 2 yielded pure 1 (50.0 mg), $[\alpha]^{25}_D$ −86.1° (*c* 0.18, MeOH), and 2.0 mg of 3, $[\alpha]^{23}_D$ −63.8° (*c* 0.013, MeOH).

The toxin fraction (170 mg) obtained from cultured cells (32 g) as above was chromatographed on HPLC with solvent B (27:73) to give 1 (15.2 mg) and fractions 1 (12.3 mg) and 2 (1.2 mg). Fraction 1 was further separated by TLC (solvent A) to afford 1 (4.1 mg) and crude samples of 4 and 5, which were purified separately by HPLC with solvents C and A (6:4), respectively, yielding 0.4 mg of 4, $[\alpha]^{25}_D$ −87.6° (*c* 0.011, MeOH), and 0.1 mg of 5, $[\alpha]^{25}_D$ −58.2° (*c* 0.0015, MeOH).

For the purified compounds 1–5, amino acid analyses and HRFABMS data for 1–5, ^{1}H NMR data for 1–4, ^{13}C NMR data for 1, and FABMS/CID/MS data for 1–5 are listed in Tables 1, 2, 3, and 4, respectively.

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

Hydrolysis of Nodularins and Derivatization of Amino Acids. Each sample (ca. 100 μg) was hydrolyzed with 6 N HCl (200 μL) at 110 °C for 19-23 h or 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.

Ozonolysis of Nodularins. Nodularin (1, 5.3 mg) in MeOH (0.8 mL) was treated with a stream of O_3/O_2 at −78 °C for 10 min. A solution of $NaBH_4$ (5 mg) in H_2O (0.3 mL) was added to the reaction mixture and the mixture was stirred for 10 min at 0 °C and for 15 min at rt. During the reduction, MeOH was removed by N_2, the mixture was diluted with H_2O (0.5 mL) and acidified with 1 N HCl (pH 2–3), and then $NaBH_4$ (5 mg) in H_2O (0.2 mL) was added. After being stirred for 20 min at rt, the reaction mixture was acidified (pH 3–4) with 1 N HCl and extracted with EtOAc (1 mL × 3). The EtOAc extract was evaporated and separated by TLC (benzene–acetone, 9:1) to give 0.1 mg of **6**. The aqueous layer was passed through an ODS cartridge, and the cartridge was rinsed with H_2O and eluted with MeOH. The MeOH eluate was evaporated to afford **7**. The H_2O eluate and washings from the cartridge were combined, evaporated to dryness, and extracted with MeOH. The MeOH extract was passed through an ODS cartridge and evaporated. The residue was dissolved in H_2O and adsorbed on an ODS cartridge, the cartridge was washed with H_2O, and **7** was eluted with MeOH. The H_2O eluate and washings were subjected to the same workup again to recover more **7** (total 1.7 mg).

Compounds 2 (0.7 mg), **3** (0.8 mg), and **4** (0.6 mg) were treated separately as above, except the EtOAc extraction was omitted.

Hydrolysis of Linear Peptides and Derivatization of Hydrolyzates for GC. Each linear peptide (60–100 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 chiral GC capillary column.

Synthesis of Amino Lactones 8–11. Cbz-Protected amino lactones were synthesized from Cbz-D-Asp (for **8** and **9**) and Cbz-L-Asp (for **10** and **11**) according to the procedures reported by McGarvey *et al.*[16a] and Takahashi *et al.*[16b] The reaction mixture was separated by silica gel column chromatography with benzene–EtOAc (65:35).[17] The *trans*-isomer eluted faster than the *cis*-isomer, and the ratio was about 4:1 (Cbz-**8**:Cbz-**9**; Cbz-**10**:Cbz-**11**). Each product was recrystallized from benzene–hexane.

Cbz-8: mp 115–116 °C; $[\alpha]^{26}_D$ +53.7° (*c*, 0.019, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.35 (5H, Ph), 5.20 (1H, br, NH), 5.09 (2H, s, OCH_2Ph), 4.60 (1H, m, J = 7.1, 5.0, 1.0 Hz, H-3), 4.41 (1H, dd, J = 10.0, 5.0 Hz, H-4), 4.22 (1H, dd, J = 10.0, 1.0 Hz, H-4), 2.81 (1H, m, J = 7.3, 7.1 Hz, H-2), 1.19 (3H, d, J = 7.3 Hz, 2-CH_3). NOE's were observed between H-2 and H-3 and H-3 and H-4 (δ 4.41). HRFABMS Calcd for $C_{13}H_{16}NO_4$: M_r 250.1079 (M + H). Found: M_r 250.1084.

Cbz-**9**: mp 89–90 °C; $[\alpha]^{26}_D$ +40.4° (*c* 0.015, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.36 (5H, Ph), 5.15 (1H, br, NH), 5.14 (2H, s, OCH_2Ph), 4.53 (1H, dd, J = 8.5, 7.7 Hz, H-4), 4.13 (1H, m, J = 8.4, 8.2, 7.7 Hz, H-3), 3.93 (1H, dd, J = 8.5, 8.2 Hz, H-4), 2.49 (1H, m, J = 8.4, 7.1 Hz, H-2), 1.31 (3H, d, J = 7.1 Hz, 2-CH_3). An NOE was observed between the 2-CH_3 and H-3. HRFABMS Found: M_r 250.1084 (M + H).

Cbz-10: mp 115–116 °C; $[\alpha]^{26}_D$ –53.8° (*c*, 0.015, $CHCl_3$). HRFABMS Found: M_r 250.1084 (M + H).

Cbz-11: mp 90–91 °C; $[\alpha]^{26}_D$ –41.0° (*c* 0.016, $CHCl_3$). HRFABMS Found: M_r 250.1086 (M + H).

Cbz-8 (50.0 mg) was dissolved in 30% HBr/AcOH (0.5 mL), and the mixture was allowed to stand at rt for 30 min. The crystals precipitated, were collected, washed with acetone on a filter paper, and dried in vacuo (P_2O_5) to give 37.3 mg (95%) of 8 hydrobromide, which was treated with CH_2Cl_2 (2 mL), TFAA (140 μL), and pyridine (200 μL) at 0 °C for 2 h and at rt for 16 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL), washed with 1 N HCl (5 mL × 2), saturated $NaHCO_3$ (10 mL), and saturated NaCl (10 mL), dried (Na_2SO_4), evaporated, and recrystallized from Et_2O–hexane to give 39.4 mg (98%) of TFA-8 [mp 78–79 °C, $[\alpha]^{25}_D$ +3.8° (*c* 0.09, $CHCl_3$): ^1H NMR (200 MHz, $CDCl_3$) δ 7.48 (1H, br, NH), 4.93 (1H, ddd, *J* = 7.0, 5.2, 1.0 Hz, H-3), 4.50 (1H, dd, *J* = 10.5, 5.2 Hz, H-4), 4.28 (1H, dd, *J* = 10.5, 1.0 Hz, H-4), 2.92 (1H, m, *J* = 7.3, 7.0 Hz, H-2), 1.23 (3H, d, *J* = 7.3 Hz, 2-CH_3)]. HRCIMS Calcd for $C_7H_9F_3NO_3$: M_r 212.0535 (M + H). Found: M_r 212.0536.

Cbz-9-11 were treated separately like Cbz-8, but 9 and 11 hydrobromides were not precipitated from the reaction mixture (HBr/AcOH), which were evaporated and triturated with acetone to give crystals.

TFA-9: mp 65–66 °C; $[\alpha]^{25}_D$ +36.2° (*c* 0.09, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.19 (1H, br, NH), 4.63 (1H, dd, *J* = 9.8, 7.0 Hz, H-4), 4.45 (1H, ddd, *J* = 7.0, 6.6, 5.8 Hz, H-3), 4.12 (1H, dd, *J* = 9.8, 5.8 Hz, H-4), 2.68 (1H, m, *J* = 7.2, 6.6 Hz, H-2), 1.39 (3H, d, *J* = 7.2 Hz, 2-CH_3). HRCIMS Found: M_r 212.0536 (M + H).

TFA-10: mp 78–79 °C; $[\alpha]^{25}_D$ –3.6° (*c* 0.12, $CHCl_3$). HRCIMS Found: M_r 212.0536 (M + H).

TFA-11: mp 64–65 °C; $[\alpha]^{25}_D$ –36.6° (*c* 0.15, $CHCl_3$). HRCIMS Found: M_r 212.0540 (M + H).

Synthesis of Aromatic Alcohols 6 and 12-14. (*S*)-3-(1′-Ethoxyethoxy)-2-methylpropan-1-ol [bp 85–88 °C (4.8 mmHg), $[\alpha]^{26}_D$ +9.3° (*c* 0.04, $CHCl_3$). Anal. Calcd for $C_6H_{13}O_2$: M_r 117.0916 (M – OEt). Found: M_r 117.0919 (M – OEt) (HREIMS)] and the antipode[21] were synthesized from commercial methyl (*S*)-3-hydroxy-2-methylpropanoate and the (*R*)-isomer, respectively, by a reported procedure.[21]

A solution of DMSO (7.23 g, 92.6 mmol) in CH_2Cl_2 (23 mL) was added dropwise to a cooled (–78 °C) solution of $(COCl)_2$ (8.80 g, 69.4 mmol) in CH_2Cl_2 (110 mL) under argon, and the mixture was stirred for 2 min. The (*S*)-alcohol (7.50 g, 46.3 mmol) above in CH_2Cl_2 (28 mL) was added dropwise to the mixture, and the milky suspension was stirred at –78 °C for 1 h, treated with Et_3N (32.4 mL, 231.5 mmol), stirred for 15 min at –78 °C, and then allowed to warm to 0 °C (over 20 min). After being stirred at 0 °C for 20 min, the reaction mixture was partitioned between benzene–Et_2O (4:1, 160 mL) and H_2O (160 mL), and the organic layer was washed with saturated NaCl, dried (Na_2SO_4), and evaporated. The residue was dissolved in Et_2O, and insoluble materials were filtered off. The filtrate was evaporated, and the residue was used for the next reaction immediately.

Benzylmagnesium bromide in THF (2.0 M, 28 mL, 56 mmol) was added dropwise to a solution of the above residue in THF (30 mL) at –20 °C under argon, and the mixture was stirred at rt for 18 h, poured into ice-cooled saturated NH_4Cl, and extracted with Et_2O (200 mL × 4). The Et_2O extract showed mainly two spots on TLC (hexane–EtOAc, 4:1), and the lower spot corresponded to benzyl alcohol, but the products were not stable to separation by silica gel. The reaction mixture after evaporation of Et_2O was used for the next step.

Sodium hydride (60% dispersion in mineral oil, 3.36 g, 83 mmol) was added in portions to a solution of the above reaction residue in DMF (80 mL) at 0 °C, and the mixture was stirred at rt for 1 h, treated with MeI (10.5 mL, 168 mmol), and stirred for 1 h at 45–50 °C. Water was added to the reaction mixture at 0 °C to dissolve any precipitates, and the mixture was extracted with Et_2O (200 mL and 150 mL × 2). The organic extract was washed with saturated NaCl, dried (Na_2SO_4), and evaporated. The residue was treated with EtOH (150 mL) and PPTS (1.60 g, 6.37 mmol) at rt for 16 h, and the product was diluted with Et_2O (150 mL), washed with saturated $NaHCO_3$ and NaCl, dried (Na_2SO_4), and evaporated. The residue was chromatographed on silica gel (180 g) with benzene–acetone (94:6) to give 2.43 g (27%, overall) of the mixture of 6 and 12.

The diastereomeric mixture of 6 and 12 was separated by preparative HPLC (CN column) with hexane–2-PrOH (10:1, 2.0 mL/min).

Compound 6 (t_R = 20.5 min): $[\alpha]^{26}_D$ +10.3° (*c* 0.003, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.28 (5H, m, Ph), 3.67 (1H, dd, *J* = 10.7, 7.3 Hz, H-1), 3.60 (1H, dd, *J* = 10.7, 4.5 Hz, H-1), 3.56 (1H, ddd, *J* = 7.3, 6.3, 2.9 Hz, H-3), 3.31 (3H, s, OCH_3), 2.92 (1H, dd, *J* = 13.7, 7.3 Hz, H-4), 2.70 (1H, dd, *J* = 13.7, 6.3 Hz, H-4), 1.92 (1H, m, *J* = 7.3, 6.8, 4.5, 2.9 Hz, H-2), 0.99 (3H, d, *J* = 6.8 Hz, 2-CH_3). HRCIMS Calcd for $C_{12}H_{19}O_2$: M_r 195.1385 (M + H). Found: M_r 195.1395.

Compound 12 (t_R = 21.1 min): $[\alpha]^{26}_D$ –5.0° (*c* 0.006, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.25 (5H, m, Ph), 3.73 (1H, dd, *J* = 11.0, 3.5 Hz, H-1), 3.57 (1H, dd, *J* = 11.0, 6.4 Hz, H-1), 3.39 (1H, ddd, *J* = 6.5, 6.4, 5.2 Hz, H-3), 3.31 (3H, s, OCH_3), 2.92 (1H, dd, *J* = 14.2, 5.2 Hz, H-4), 2.82 (1H, dd, *J* = 14.2, 6.4 Hz, H-4), 1.77 (1H, m, *J* = 7.0, 6.5, 6.4, 3.5 Hz, H-2), 0.98 (3H, d, *J* = 7.0 Hz). HRCIMS Found: M_r 195.1395 (M + H).

Compounds 13 {$[\alpha]^{26}_D$ –10.1° (*c* 0.005, $CHCl_3$). HRCIMS Found: M_r 195.1395 (M + H)} and 14 {$[\alpha]^{26}_D$ +5.4° (*c* 0.006, $CHCl_3$). HRCIMS Found: M_r 195.1393 (M + H)} were synthesized as above.

^1H NMR Spectroscopy of Aromatic Alcohols 6 and 12–14 with a Chiral Solvating Reagent. ^1H NMR spectra were obtained on a QE-300 FT NMR spectrometer with C_6D_6 as solvent, TMS (0.0 ppm) as a standard, and the following conditions: number of acquisitions, 48; data size, 16 K; pulse width, 6.28 μs (44°); pulse interval, 3.71 s; spectral width, 6024 Hz; frequency, 300.15 MHz. Each sample (0.6 mg) was dissolved in 0.45 mL of C_6D_6 containing 6% (27.0 mg) of (*R*)-(–)-2,2,2-trifluoro-1-(9-anthryl)ethanol[18] (for Figures 3A, 3B, and 3C). The above solution of natural 6 (obtained from LR) was divided into two aliquots. One portion was mixed with half of the solution of synthetic 6 (Figure 3D), and another portion was mixed with half of synthetic 13 (Figure 3E) to determine the stereochemistry of natural 6 obtained from LR. Natural 6 (0.1 mg) obtained from 1 and 0.1 mg of synthetic 13 were dissolved in 0.45 mL of C_6D_6 with the reagent (6%) for ^1H NMR spectrometry (254 acquisitions).

Toxicity Testing. The compounds were dissolved in water and injected intraperitoneally into four ICR-Swiss male mice (15–25 g) at each of 4–6 concentrations. An estimate of LD50, signs of poisoning, survival times, and body and liver weights were recorded and compared to the effects of known cyanobacterial peptide hepatotoxins.

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Supplementary Material Available: Copies of ^1H NMR spectra of 1–4, 6, Cbz 8–11, TFA 8–11, 12–14, (*S*)- and (*R*)-3-(1′-ethoxyethoxy)-2-methylpropan-1-ols; chiral capillary gas chromatograms of TFA 8–11 and natural amino lactones (30 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.

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