Protease Inhibitors from a Water Bloom of the Cyanobacterium *Microcystis aeruginosa*

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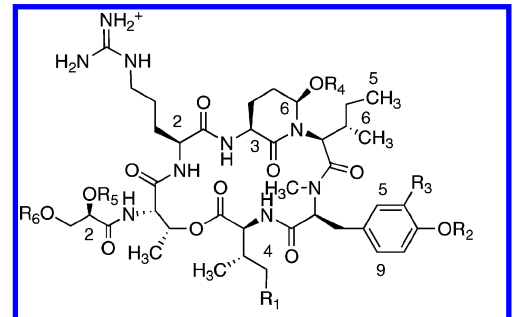
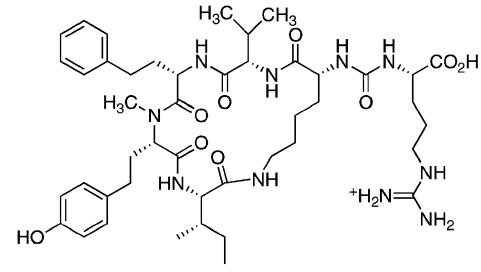
Bioassay-guided fractionation of the polar extract of a *Microcystis aeruginosa* water bloom biomass yielded 10 micropeptins and one anabaenopeptin. Eight of the micropeptins, micropeptins HU1069 (**1**), HU989 (**2**), HU1021 (**3**), HU1041 (**4**), HU975 (**5**), HU895A (**6**), HU909 (**7**), and HU895B (**8**), are new, while two, micropeptins 478-A (**10**) and 478-B (**11**), were previously isolated from a bloom of *M. aeruginosa* from Japan. The new anabaenopeptin HU892 (**9**) belongs to the relatively rare subgroup, presenting an aliphatic amino acid at the carboxylic end of the peptide and *N*-methylhomoaromatic amino acid at the second position. The structures of the compounds were determined by 1D and 2D NMR techniques and mass spectrometric data. The isolated micropeptins inhibited trypsin with IC_{50}'s that varied between 0.7 and 5.2 μM and unexpectedly inhibited chymotrypsin with IC_{50}'s that varied between 2.8 and 72.0 μM. The SAR of these micropeptins is discussed.

The micropeptins are a group of cyclic depsipeptides that are produced by fresh water and marine cyanobacteria.[1] They usually accompany, together with the other four groups of protease inhibitors, the hepatotoxic microcystins.[2] The micropeptins compose the most diverse group of the five known groups of protease inhibitors that are produced by water bloom forming cyanobacteria. Over eighty different micropeptin variants are known from *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, *Microcystis*, *Symploca*, and *Lyngbya* spp. of cyanobacteria.[3] The micropeptins may be divided into three subgroups: (i) those that inhibit trypsin-type serine proteases (i.e., trypsin, plasmin, thrombine); (ii) those that inhibit chymotrypsin-type serine proteases (i.e., chymotrypsin, elastase); and (iii) those that exhibit cytotoxic activity along with inhibition of chymotrypsin-type serine proteases. This selectivity is gained by the nature of the amino acid that occupies the fifth position from the carboxylic end of the peptide chain. Basic amino acids (i.e., arginine and lysine) at this position select for inhibition of trypsin-type serine protease, while aliphatic, aromatic, and other neutral amino acids select for inhibition of chymotrypsin-type serine proteases.[4] Dehydroaminobutyric acid-containing micropeptins exhibit cytotoxic activity and inhibit chymotrypsin-type serine proteases.[5] As part of our continuing interest in the chemical ecology of cyanobacterial water blooms and the search for novel drugs for human diseases, we examined the extracts of a *Microcystis aeruginosa* bloom collected in October 2004 from Hulda Reservoir, Israel. The extract of this bloom (IL-342) afforded 11 protease inhibitors belonging to two groups: 10 micropeptins of which eight are new natural products and two are known from a bloom of *Microcystis aeruginosa* from Japan,[6] together with a novel anabaenopeptin. The structure elucidation and biological activity of the new compounds are presented below.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
micropeptin HU1069 (**1**)	Me	Me	Cl	H	SO_3^-	SO_3^-
micropeptin HU989 (**2**)	Me	Me	Cl	H	H	SO_3^-
micropeptin HU1021 (**3**)	Me	H	H	H	SO_3^-	SO_3^-
micropeptin HU1041 (**4**)	H	H	Cl	H	SO_3^-	SO_3^-
micropeptin HU975 (**5**)	H	Me	Cl	H	H	SO_3^-
micropeptin HU895A (**6**)	H	Me	Cl	H	H	H
micropeptin HU909 (**7**)	H	Me	Cl	Me	H	H
micropeptin HU895B (**8**)	Me	H	Cl	H	H	H
micropeptin 478-A (**10**)	Me	H	Cl	H	H	SO_3^-
micropeptin 478-B (**11**)	Me	H	Cl	H	SO_3^-	SO_3^-



anabaenopeptin HU892 (**9**)

Results and Discussion

The 10 micropeptins isolated in this research work vary in one amino acid, valine versus isoleucine, in the carboxylic end of the peptide, and in the substitution of three other acid units: *N*Me-tyrosine versus *N*Me-*o*-chlorotyrosine or *N*,*O*-diMe-*o*-chlorotyrosine, amino hydroxy piperidone versus amino methoxy piperidone, and glyceric acid versus its mono- and disulfated derivatives.

Micropeptin HU1069 (**1**) was isolated as a glassy material with a molecular formula of $C_{41}H_{64}ClN_9O_{18}S_2$ based on the HR MALDI TOF quasi-molecular ion at *m*/*z* 910.4486/912.4485 (3:1) corresponding to $[M + H - 2SO_3]^+$. The ^{1}H NMR spectrum, in DMSO-d_6, revealed four doublet NH signals and one triplet proton signal between δ_H 7.0 and 8.5 ppm, pointing to six amino acid residues (taking into account the NMe aromatic amino acid and the Ahp residue that accounts for two amino acids). Analysis of the 1D (^{1}H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data (see Table 5, in the Supporting Information) revealed the six amino acid units and a hydroxy acid unit, namely, isoleucine^{I}, *N*,*O*-diMe-*o*-chlorotyrosine, *N*,*N*-disubstituted isoleucine^{II}, amino-hydroxy-piperidone (Ahp), arginine, threonine, and glyceric acid, that build micropeptin HU1069 (**1**). The structure of the Ahp is suggested on the basis of the COSY and HMQC spectra, the HMBC correlation between Ahp

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Table 1. ^{1}H NMR Data of Compounds **1**−**8** in DMSO-d_6[a]

compound position	**1**[b]	**2**[c]	**3**[c]	**4**[b]	**5**[b]	**6**[b]	**7**[c]	**8**[c]
Ile/Val 2	4.87, dd	4.78, dd	4.86, dd	4.70, m	4.58, dd	4.51, dd	4.49, dd	4.65, brd
3	1.83, m	1.81, m	1.84, m	2.05, m	2.02, m	1.99, m	1.95, m	1.79, m
4	1.05, m	1.11, m	1.08, m	0.73, d	0.75, d	0.76, d	0.84, d	1.09, m
	1.26, m	1.24, m	1.25, m					1.20, m
5	0.90, t	0.87, t	0.89, t	0.85, d	0.85, d	0.85, d	0.88, d	0.87, t
6	0.69, d	0.70, d	0.69, d					0.71, d
NH	7.78, d	7.56, d	7.43, d	7.54, d	7.64, d	7.75, d	6.99, d	7.60, d
Tyr der. 2	5.05, brd	5.06, dd	5.05, dd	5.02, dd	5.05, dd	5.03, dd	5.07, dd	5.04, dd
3	2.76, dd	2.78, dd	2.68, dd	2.75, m	2.78, dd	2.78, dd	2.79, dd	2.65, dd
	3.24, m	3.23, dd	3.25, dd	3.20, m	3.25, dd	3.21, dd	3.25, dd	3.19, dd
5	7.24, s	7.24, brs	6.99, d	7.12, d	7.24, s	7.24, s	7.25, s	7.13, s
6			6.62, d					
8	7.04, d	7.04, d	6.62, d	6.83, d	7.05, d	7.05, d	7.05, d	6.84, d
9	7.15, d	7.14, d	6.99, d	6.96, d	7.14, d	7.14, d	7.14, d	6.96, d
*N*Me	2.72, s	2.71, s	2.70, s	2.72, s	2.73, s	2.72, s	2.73, s	2.70, s
*O*Me/OH	3.78, s	3.77, s	9.14, s	9.94, s	3.77, s	3.76, s	3.77, s	9.94, brs
Ile 2	4.38, d	4.37, d	4.45, m	4.39, d	4.37, d	4.35, d	4.41, m	4.40, d
3	1.79, m	1.81, m	1.74, m	1.77, m	1.80, m	1.78, m	1.86, m	1.80, m
4	0.63, m	0.62, m	0.63, m	0.64, m	0.61, m	0.64, m	0.61, m	0.64, m
	1.09, m	1.12, m	1.05, m	1.10, m	1.10, m	1.10, m	1.10, m	1.10, m
5	0.63, brd	0.62, brd	0.63, brd	0.64, brd	0.61, brd	0.62, brd	0.61, brd	0.64, brd
6	−0.16, d	−0.15, d	−0.12, d	−0.10, d	−0.15, d	−0.15, d	−0.16, d	−0.08, d
Ahp/Amp 3	4.43, q	4.45, ddd	4.43, m	4.45, m	4.44, q	4.43, m	4.45, m	4.45, m
4	1.73, m	1.73, m	1.73, m	1.74, m	1.73, m	1.75, m	1.73, m	1.75, m
	2.55, brq	2.58, brq	2.60, brq	2.60, brq	2.61, brq	2.60, brq	2.43, brq	2.55, m
5	1.73, m	1.76, m	1.73, m	1.73, m	1.75, m	1.20, m	1.70, m	1.77, m
		1.83, m		1.77, m	1.79, m	1.78, m	2.10, m	
6	4.92, brs	4.91, brs	4.92, brs	4.92, brs	4.92, brs	4.91, brs	4.43, d	4.92, brs
NH	7.37, d	7.35, d	7.35, d	7.27, d	7.28, d	7.33, d	7.21, d	7.39, d
OH/*O*Me	6.04, d	6.09, d	5.96, d	6.06, d	6.16, d	6.23, brs	3.03, s	6.11, d
Arg 2	4.32, m	4.35, m	4.31, m	4.30, m	4.31, m	4.28, m	4.28, m	4.30, m
3	1.44, m	1.42, m	1.44, m	1.44, m	1.43, m	1.48, m	1.47, m	1.48, m
	2.04, m	2.01, m	2.05, m	2.05, m	2.03, m	2.00, m	2.00, m	2.02, m
4	1.44, m	1.42, m	1.44, m	1.44, m	1.43, m	1.46, m	1.50, m	1.46, m
5	3.04, m	3.06, m	3.04, m	3.02, m	3.07, m	3.07, m	3.08, m	3.08, m
6-NH	7.46, brt	7.46, brt	7.43, brt	7.49, brt	7.51, brt	7.61, brt	7.63, brt	7.60, brt
NH	8.55, d	8.59, d	8.53, d	8.53, d	8.63, d	8.68, d	8.69, d	8.62, d
Thr 2	4.63, d	4.64, d	4.63, d	4.63, dd	4.66, d	4.68, d	4.70, d	4.65, d
3	5.43, q	5.46, q	5.42, q	5.45, dq	5.50, q	5.53, brq	5.55, brq	4.59, q
4	1.22, d	1.20, d	1.21, d	1.21, d	1.20, d	1.21, d	1.20, d	1.20, d
NH	7.78, d	7.46, d	7.75, d	7.74, d	7.64, d	7.62, d	7.63, d	7.46, d
GA 2	4.74, dd	4.25, ddd	4.73, t	4.72, t	4.25, ddd	4.06, ddd	4.08, ddd	4.07, ddd
3	3.91, dd	3.85, dd	3.91, dd	3.92, dd	3.82, dd	3.46, ddd	3.49, ddd	3.49, ddd
	3.98, dd	3.97, dd	3.98, dd	3.98, dd	3.97, dd	3.58, ddd	3.60, ddd	3.60, ddd
2-OH		6.07, d			6.09, d	5.81, d	5.79, d	5.74, d
3-OH						4.78, t	4.75, t	4.77, t

[a] Complete NMR data are available in the Supporting Information (coupling constant, HMBC and ROESY correlations). [b] 500 MHz for ^{1}H. [c] 400 MHz for ^{1}H.

H-6 and C-2, and comparison of the chemical shifts of the carbon and proton signals with similar moieties in the known micropeptin 478-B (**11**).[6] The glyceric acid residue was established as 2,3-disulfated glyceric acid on the basis of it proton and carbon chemical shifts, which are almost identical with that of micropeptin 478-B (**11**).[6] All proton and carbon signals of the latter residues were fully assigned by the COSY, TOCSY, HMQC, and HMBC data (see Table 5, in the Supporting Information). The amino acid sequence of **1** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Ile-*N*,*O*-diMe-*o*-chloroTyr and Ahp-Arg), the NMe of *N*,*O*-diMe-*o*-chloroTyr with Ile^{II}-carbonyl, and H-2 of Ile^{II} with C-6 of the Ahp residue and through NOE correlations between α-NH of Arg and H-2 and H-3 of Thr and Thr H-2 and H-2 of GA. The ester bond was assigned by a NOE correlation between H_3-4 of threonine and H-2 of isoleucine. Acid hydrolysis of **1** and derivatization with Marfey's reagent,[7] followed by HPLC analysis, demonstrated the L-configuration of the isoleucine residues, *N*,*O*-diMe-*o*-chlorotyrosine (by comparison with synthetic material), arginine, and threonine residues. Jones oxidation[8] of **1**, followed by a similar hydrolysis, derivatization, and HPLC analysis, determined the 3*S*-configuration for the Ahp residue (oxidation and subsequent hydrolysis liberated L-glutamic acid from Ahp). The configuration of C-6 of the Ahp was determined as *R* on the basis of the *J*-values of H-6, <1 Hz, which points to an equatorial orientation of this proton, the chemical shift of the pseudoaxial H-4, δ_H 2.54 brq, which is downfield shifted by the axial hydroxy group, and NOE correlation between H-4pax and the 6-OH. The configuration of the glyceric acid was determined to be D (*R*), by comparison of the retention time of the glyceric acid from the hydrolysate with the retention time of authentic samples of D- and L-glyceric acid on a chiral HPLC column.

Micropeptin HU989 (**2**) was isolated as a glassy material. It presented ^{1}H and ^{13}C NMR data similar to those of micropeptin HU1069 (**1**). The only notable difference was located in the glyceric acid residue; H-2 in **2** is upfield shifted by 0.5 ppm relative to that of **1** and coupled with an acidic proton resonating at 6.07 ppm, while C-2 in **2** is upfield shifted by 3.8 ppm relative to that of **1**. These differences are in accordance with a 3-sulfated glyceric acid. The molecular formula of **2**, $C_{41}H_{64}ClN_9O_{15}S$, was deduced from the high-resolution MALDI TOF quasi-molecular ion, $[M + Na]^+$, at *m*/*z* 1012.3863/1014.3863 (3:1). As for **1**, analysis of the 1D (^{1}H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **2** (see Table 6, in the Supporting Information) allowed the full assignment of the following acid residues: isoleucine^I, *N*,*O*-diMe-*o*-chlorotyrosine, *N*,*N*-

Table 2. ^{13}C NMR Data of Compounds **1**−**8**, **10**, and **11** in DMSO-d_6

compound position	mult.[c]	**1**[a]	**2**[b]	**3**[b]	**4**[a]	**5**[a]	**6**[a]	**7**[b]	**8**[b]	**10**[b]	**11**[b]
Ile/Val 1	qC	172.9	173.0	172.9	172.6	173.0	172.6	172.4	172.8	172.5	172.5
2	CH	54.3	54.5	54.3	56.2	54.5	56.5	56.5	54.4	55.3	55.2
3	CH	37.4	37.4	37.4	31.1	37.4	30.7	31.1	37.1	37.7	37.7
4	CH_2/CH_3	26.2	26.4	26.2	17.7	26.4	18.0	18.1	26.1	24.5	24.5
5	CH_3	11.8	11.8	11.7	19.4	11.8	19.3	19.1	11.5	11.1	11.1
6	CH_3	14.8	15.0	14.8		15.0			14.9	16.0	16.0
Tyr-der. 1	qC	169.5	169.5	169.4	169.6	169.5	169.2	169.0	169.2	168.9	168.9
2	CH	60.7	60.9	60.9	60.6	60.9	60.5	60.6	60.7	60.4	60.4
3	CH_2	33.2	33.2	33.6	33.2	33.2	32.9	32.8	33.2	34.8	34.8
4	qC	131.5	131.2	127.6	129.3	131.2	130.9	130.7	131.8	129.1	129.1
5	CH	130.9	131.0	130.6	130.8	131.0	130.6	130.5	130.6	130.6	130.6
6	qC/CH	121.5	121.7	115.5	120.0	121.7	121.4	121.4	120.0	119.8	119.8
7	qC	153.8	154.0	156.4	152.2	154.0	153.7	153.6	152.3	152.0	152.0
8	CH	113.3	113.5	115.5	116.8	113.5	113.2	113.2	116.8	116.7	116.7
9	CH	129.7	129.8	130.6	129.4	129.8	129.4	129.4	129.2	129.0	129.0
*N*Me	CH_3	29.9	30.7	30.4	30.4	30.7	30.4	30.3	30.4	30.1	30.1
*O*Me	CH_3	56.4	56.6			56.6	56.2	56.2			
Ile 1	qC	169.9	170.1	170.0	169.9	170.1	169.7	169.9	169.9	169.7	169.7
2	CH	54.3	54.5	54.3	54.4	54.5	54.2	53.9	54.4	54.2	54.2
3	CH	33.1	33.3	33.9	33.2	33.3	32.9	32.5	33.3	32.9	32.9
4	CH_3	23.9	24.0	23.9	24.0	24.0	23.7	23.6	23.9	23.7	23.7
5	CH_2	10.4	10.6	10.5	10.5	10.6	10.2	10.1	10.4	10.2	10.2
6	CH_3	13.9	14.1	14.0	13.9	14.1	13.9	13.5	13.9	13.8	13.8
Ahp/Amp2	qC	169.1	169.6	169.5	169.2	169.6	169.4	169.0	169.5	169.2	169.2
3	CH	48.9	49.3	48.9	48.9	49.3	49.1	49.2	49.2	48.8	48.8
4	CH_2	22.0	22.0	21.9	21.8	22.0	21.6	21.5	21.8	21.8	21.8
5	CH_2	30.5	30.1	29.8	29.9	30.1	29.8	23.6	29.9	29.7	29.7
6	CH	74.2	74.4	74.1	74.2	74.4	74.1	83.1	74.2	73.9	73.9
*O*Me	CH_3							55.3			
Arg 1	qC	170.2	170.3	169.6	170.3	170.3	170.1	170.2	170.1	170.3	170.3
2	CH	51.3	52.0	51.2	51.8	52.0	52.3	52.2	52.1	51.2	51.2
3	CH_2	24.8	26.4	24.8	31.0	26.4	27.5	27.6	26.5	26.7	26.7
4	CH_2	24.8	25.4	24.8	24.8	25.4	25.2	25.1	25.3	24.6	24.7
5	CH_2	43.0	41.0	45.9	48.8	41.0	45.6	45.5	40.5	40.3	40.3
7	qC	156.7	157.0	156.7	156.7	157.0	156.8	156.7	156.9	156.5	156.5
Thr 1	qC	169.7	169.4	169.7	169.4	169.4	169.2	169.0	169.4	168.8	168.8
2	CH	55.1	54.6	55.0	55.1	54.6	54.2	54.2	54.4	54.4	54.3
3	CH	72.1	72.7	72.1	71.9	72.2	72.3	72.3	72.6	72.1	71.7
4	CH_3	17.5	18.1	17.4	17.7	18.1	18.1	17.8	18.0	17.6	17.7
GA 1	qC	169.6	172.2	172.9	169.8	172.2	172.7	172.6	169.2	171.7	171.1
2	CH	74.6	70.8	74.6	74.7	70.8	72.7	72.6	72.7	70.4	74.5
3	CH_2	66.5	68.6	66.5	66.5	68.6	63.8	63.7	63.9	68.2	66.3

[a] 125 MHz for ^{13}C. [b] 100 MHz for ^{13}C. [c] Multiplicity and assignment from a HSQC experiment.

disubstituted isoleucine^{II}, Ahp, arginine, threonine, and 3-sulfoglyceric acid. The amino acid sequence of **2** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Ile^{I}- *N*,*O*-diMe-*o*-chloroTyr, Ahp-Arg, and Thr-GA and of the NMe of *N*,*O*-diMe-*o*-chloroTyr with Ile^{II}-carbonyl and by NOE correlations between 6-OH of Ahp and H-2 of isoleucine^{II}, α-NH of Arg and H-2 of Thr, and Thr H-2 and H-2 of GA. The ester linkage between the carbonyl of isoleucine^{I} and the oxygen of threonine was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of isoleucine^{I}. The configurations of the stereogenic centers of **2** were assigned as L-isoleucine residues, L-*N*,*O*-diMe-*o*-chlorotyrosine, L-arginine, L-threonine, 3*S*,6*R*-Ahp, and D-glyceric acid by the same procedure described for **1**.

Micropeptin HU1021 (**3**) was isolated as an amorphous, white solid. HR MALDI-TOF MS measurements for the compound (*m*/*z* 964.4083 $[M + Na - SO_3]^+$), coupled with the NMR data, furnished a molecular formula of $C_{40}H_{63}N_9O_{18}S_2$ for **3**. The ^1H and ^{13}C NMR data of **3** differed from that of **1** only in the signals of the aromatic residue. These data suggested that **3** contains a *p*-substituted phenol moiety instead of the *o*-chloro-*p*-methoxy phenyl moiety in **1**. Analysis of the 1D (^1H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **3** (see Table 7, in the Supporting Information) allowed the full assignment of the following acid residues: isoleucine^{I}, *N*Me-tyrosine, *N,N*-disubstituted isoleucine^{II}, Ahp, arginine, threonine, and disulfated glyceric acid. HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Ile^{I}-*N*Me-Tyr with Thr-GA, the NMe of *N*Me-Tyr with Ile^{II}-carbonyl, C-6 of Ahp with H-2 of isoleucine^{II}, and the carbonyl of isoleucine^{I} with H-3 of threonine, as well as NOE correlations between α-NH of Ahp and H-2 of Arg and between α-NH of Arg and H-2 of Thr, established the connectivity between all of the acid residues that build **3**. The nature of all of the chiral centers of **3** was established as described above for **1**, namely, two L-isoleucine residues, L-*N*Me-tyrosine, L-arginine, L-threonine, 3*S*,6*R*-Ahp, and D-glyceric acid.

Micropeptin HU1041 (**4**) presented ^1H NMR data similar to those of micropeptin 478-B (**11**).[6] The only notable difference was present in the aliphatic region, where the triplet methyl resonating at δ_H 0.89 ppm and the methylene resonating at δ_H 1.01 and 1.22 ppm, in **11**, are substituted with a doublet methyl at δ_H 0.84 ppm, in **4**. The molecular formula of **4**, $C_{39}H_{60}ClN_9O_{18}S_2$, was determined from its high-resolution MALDI TOF quasi-molecular ion, $[M + Na - SO_3]^+$, at *m*/*z* 984.3421/986.3420 (3:1). Thorough analysis of the 1D (^1H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **4** (see Table 8, in the Supporting Information) allowed the full assignment of the following acid residues: valine, *N*Me-*o*-chlorotyrosine, *N,N*-disubstituted isoleucine, Ahp, arginine, threonine, and disulfated glyceric acid. The sequence and the chirality of the stereocenters of the cyclic depsipeptide were established in a similar way to that described above, for **1**−**3**, to establish structure **4** for micropeptin HU1041.

Table 3. NMR Data of Anabaenopeptin HU892 (**9**) in DMSO-d_6[a]

position	δ_C, mult.[b]	δ_H, mult., *J* (Hz)	LR C−H correlations[c]	ROESY correlations[d]
Ile 1	170.7, qC		Ile-2, Lys-ε-NH	
2	58.1, CH	4.18, dd (9.7, 6.0)	Ile-3,4a,4b,5,6,NH	Lys-ε-NH,Ile-3,4a,5,6,NH
3	36.0, CH	1.99, m	Ile-2,4a,5,6	Ile-2,4a,4b,5,6,NH
4	24.2, CH_2	0.95, m	Ile-2,5,6	Ile-2,3,4b,5,6,NH
		1.30, m		Ile-3,4a,5,6
5	11.5, CH_3	0.76, t (7.8)	Ile-3,4a,4b	Ile-2,3,4a,4b,NH
6	16.1, CH_3	0.77, d (7.1)	Ile-2,3,4a,4b	Ile-2,3,4a,4b,NH
NH		8.18, d (9.7)		Lys-NH-ε,Ile-2,3,4a,5,6
*N*MeHty 1	169.4, qC		Ile-2NH NMeHty-2	
2	59.5, CH	4.60, dd (6.2, 8.3)	NMeHty-NMe,3a,3b, 4a,4b	Ile-NH, NMeHty-3a,3b,4b
3	30.8, CH_2	1.70, m	NMeHty-2,4a,4b	NMeHty-2,3b,4a,4b,NMe
		1.99, m		NMeHty-2,3a,4a,4b,NMe
4	31.4, CH_2	2.19, dt (4.9, 12.4)	NMeHty-3a,3b,6,6′	NMeHty-3a,3b,4b
		2.30, dt (5.1, 12.4)		NMeHty-3a,3b,4a
5	131.6, qC		NMeHty-4a,4b,7,7′	
6,6′	129.0, CH	6.95, d (8.3)	NMeHty-4a,4b,6,6′	NMeHty-7,7′
7,7′	115.4, CH	6.66, d (8.3)	NMeHty-6,6′,OH	NMeHty-6,6′
8	155.7, qC		NMeHty-6,6′,7,7′,OH	
*N*Me	28.7, CH_3	2.57, s	NMeHty-2,4a	NMeHty-3a,3b
OH		9.16, s		
Hph 1	172.3, qC		NMeTyr-NMe,Hph-2,3b,NH	
2	48.2, CH	4.70, m	Hph-4a,4b,3b,NH	Hph-3a,3b,4a,6,6′,NH
3	33.2, CH_2	1.75, m	Hph-2,4a,4b	Hph-2,3b,4b
		2.03, m		Hph-2,3a,4b
4	31.6, CH_2	2.70, m		Hph-4b
		2.81, m		Hph-3a,3b,4a
5	141.3, qC		Hph-3b,4a,4b,6,6′,7,7′,8	
6,6′	128.6, CH	7.25, m	Hph-4a,4b,7,7′	
7,7′	128.5, CH	7.23, m	Hph-6,6′,8	Hph-8
8	126.3, CH	7.19, m	Hph-7,7′	Hph-6,6′,7,7′
NH		8.98, d (4.3)		Val-2,Hph-2,3a,3b,4a,4b
Val 1	172.9, qC		Hph-NH,Val-2,3	
2	58.0, CH	3.97, t (6.8)	Val-3,4,5,NH	Val-3,4,5,NH,Hph-NH
3	30.1, CH	1.90, m	Val-2,4,5,NH	Val-2,4,5,NH
4	19.2, CH_3	0.88, d (6.9)	Val-2,3,5	Val-2,3,5,NH
5	18.7, CH_3	0.87, d (6.9)	Val-2,3,4	Val-2,3,4,NH
NH		6.81, d (6.1)	Hph-NH, Val-2,3	Val-2,3,4,5,Lys-2
Lys 1	170.7, qC		Val-NH,Lys-2	
2	58.1, CH	3.91, dt (10.7, 6.4)	Lys-3a,3b,NH	Lys-3a,3b,4a,4b,α-NH, Val-NH
3	36.0, CH_2	1.61, m	Lys-2,NH	Lys-2,4a,4b,α-NH
		1.66, m	Lys-2,NH	Lys-2,4a,4b,α-NH
4	24.2, CH_2	1.30, m	Lys-2,6b	Lys-2,3a,3b,5a,5b,6b,α-NH,ϵ-NH
		1.44, m		Lys-2,3a,3b,5a,5b,6b,α-NH,ϵ-NH
5	28.3, CH_2	1.40, m	Lys-4a,4b	Lys-4a,4b,6a,6b,α-NH,ϵ-NH
		1.43, m		Lys-4a,4b,6a,6b,α-NH,ϵ-NH
6	38.3, CH_2	2.81, m	Lys-5a,5b	Lys-5a,5b,6a,α-NH,ϵ-NH
		3.44, m		
α-NH		6.53, d (6.4		Lys-5a,5b,4a,4b,6b,α-NH
ϵ-NH		7.09, dd (6.4, 4.1)		Lys-2,3a,3b,4a,4b,5a,5b,6b
CO	157.5, qC		Arg-2,α-NH,Lys-2,α-NH	
Arg 1	174.3, qC		Arg-2,3a,3b,α-NH	
2	52.1, CH	4.09, dt (12.6, 7.7)	Arg-3a,3b,4,α-NH	Arg-3a,3b,4,α-NH
3	29.5, CH_2	1.53, m	Arg-2,4,5,α-NH	Arg-2,3b,4,α-NH
		1.72, m		Arg-2,3a,4,α-NH
4	25.6, CH_2	1.46, m	Arg-2,3a,3b,5	Arg-2,3a,3b,5
5	40.5, CH_2	3.11, t (6.0)	Arg-3a,3b,4,δ-NH	Arg-4,δ-NH
6	156.9, qC		Arg-5	-
α-NH		6.42, d (7.7)		Arg-2,3a,3b
δ-NH		7.57, t (5.3)		Arg-5
NH, NH_2		7.30, brm		

[a] 500 MHz for ^{1}H, 125 MHz for ^{13}C. [b] Multiplicity and assignment from a HSQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH}$ = 8 Hz, recycle time 1 s, presented as a correlation of a proton with carbon in the row. [d] Selected NOEs from a ROESY experiment.

Micropeptin HU975 (**5**) was isolated as a glassy material with a molecular formula of $C_{40}H_{62}ClN_9O_{15}S$ based on the HR MALDI TOF quasi-molecular ion at *m/z* 998.3615/1000.3615 (3:1) corresponding to $[M + Na]^+$. These HRMS data suggested an additional methyl unit and one less sulfate unit when **5** is compared with **4**. Indeed, when the NMR data of **5** and **4** were compared, (i) a difference was noticed in the glyceric acid residue; H-2 in **5** is upfield shifted by 0.47 ppm relative to that of **4** and coupled with an acidic proton resonating at 6.09 ppm, while C-2 in **5** is upfield shifted by 4.1 ppm relative to that of **4**, in accordance with a 3-sulfated glyceric acid (in **5**), and (ii) in the aromatic residue an additional aromatic methoxy group (δ_H 3.77 s and δ_C 56.4 q) is found in the spectra of **5**. The analysis of the 1D (^{1}H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **5** (see Table 9, in the Supporting Information) allowed the full assignment of the following acid residues: valine, *N,O*-diMe-*o*-chlorotyrosine, *N,N*-disubstituted isoleucine, Ahp, arginine, threonine, and 3-sulfoglyceric acid. The amino acid sequence of **5** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent

Table 4. Protease Inhibition Data

compound	key structure components	trypsin IC_{50} μM	chymotrypsin IC_{50} μM
micropeptin HU1069 (**1**)	^{1}Ile, ^{2}*N*Me, *O*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}disulfo-GA	1.7	>100
micropeptin HU989 (**2**)	^{1}Ile, ^{2}*N*Me, *O*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}3-sulfo-GA	0.7	18.2
micropeptin HU1021 (**3**)	^{1}Ile, ^{2}*N*Me-Tyr, ^{4}Ahp, ^{7}disulfo-GA	2.2	>100
micropeptin HU1041 (**4**)	^{1}Val, ^{2}*N*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}disulfo-GA	1.2	>100
micropeptin HU975 (**5**)	^{1}Val, ^{2}*N*Me, *O*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}3-sulfo-GA	5.2	24.0
micropeptin HU895A (**6**)	^{1}Val, ^{2}*N*Me, *O*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}GA	3.5	19.6
micropeptin HU909 (**7**)	^{1}Val, ^{2}*N*Me, *O*Me-*o*-Cl-Tyr, ^{4}Amp, ^{7}GA	1.1	2.8
micropeptin HU895B (**8**)	^{1}Ile, ^{2}*N*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}GA	0.9	5.4
micropeptin 478-A (**10**)	^{1}Ile, ^{2}*N*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}3-sulfo-GA	0.7	5.2
micropeptin 478-B (**11**)	^{1}Ile, ^{2}*N*Me-*o*-Cl-Tyr, ^{4}Ahp, ^{7}disulfo-GA	2.4	72.0

amino acid, Val-*N*,*O*-diMe-*o*-chloroTyr, Arg-Thr, and Thr-GA, the NMe of *N*,*O*-diMe-*o*-chloroTyr with Ile-carbonyl, and H-2 of Ile with C-6 of Ahp and by NOE correlation between α-NH of Ahp and α-NH of Arg. The ester linkage between the carbonyl of valine and the oxygen of threonine was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine. The configurations of the stereogenic centers of **5** were assigned as L-valine, L-isoleucine, L-*N*,*O*-diMe-*o*-chlorotyrosine, L-arginine, L-threonine, 3*S*,6*R*-Ahp, and D-glyceric acid by the same procedure described for **1**−**4**. On the basis of the above given arguments, structure **5** was assigned to micropeptin HU975.

Micropeptin HU895A (**6**), a glassy transparent material, exhibited a HR MALDI TOF quasi-molecular ion at *m*/*z* 934.3868/936.3869 (3:1) corresponding to $[M + K]^+$, for which the molecular formula $C_{40}H_{62}ClKN_9O_{12}$ was calculated with 14 degrees of unsaturation. It presented ^{1}H and ^{13}C NMR data similar to those of micropeptin HU975 (**5**). The only notable differences were located in the glyceric acid residue; H-2 in **6** is upfield shifted by 0.2 ppm relative to that of **5**, while H-3 and H-3′ in **6** are upfield shifted by ca. 0.4 ppm, relative to that of **5**, and coupled with an acidic proton resonating at 4.78 ppm. C-3 in **6** is upfield shifted by 4.6 ppm relative to that of **5**, and C-2 in **6** is downfield shifted by 2.1 ppm relative to that of **5**. These differences are in accordance with a nonsubstituted glyceric acid in **6**. Analysis of the 1D (^{1}H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **6** (see Table 10, in the Supporting Information) allowed the full assignment of the following acid residues: valine, *N,O*-diMe-*o*-chlorotyrosine, *N,N*-disubstituted isoleucine, Ahp, arginine, threonine, and glyceric acid. The amino acid sequence of **6** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Val-*N*,*O*-diMe-*o*-chloroTyr, Arg-Thr, and Thr-GA, the NMe of *N*,*O*-diMe-*o*-chloroTyr with Ile-carbonyl, and H-2 of Ile with C-6 of Ahp and by NOE correlation between α-NH of Ahp and α-NH of Arg. The ester linkage between the carbonyl of valine and the oxygen of threonine was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine. The configurations of the stereogenic centers of **6** were assigned as L-valine, L-isoleucine, L-*N*,*O*-diMe-*o*-chlorotyrosine, L-arginine, L-threonine, 3*S*,6*R*-Ahp, and D-glyceric acid by the same procedure described for **1**−**5**, to establish structure **6** for micropeptin HU895A.

Micropeptin HU909 (**7**) presented ^{1}H NMR data similar to those of micropeptin HU895A (**6**). The only notable difference was found around 3 ppm, where a singlet methyl resonating at δ_H 3.03 ppm appeared in the spectrum of **7**. This additional methyl group was also reflected in the molecular formula of **7**, $C_{41}H_{64}ClN_9NaO_{12}$, which was determined from its high-resolution MALDI TOF quasi-molecular ion, $[M + Na]^+$, at *m*/*z* 932.4220/934.4220 (3:1). The NMR data of **7** (see Table 11, in the Supporting Information) allowed the full assignment of the following acid residues: valine, *N,O*-diMe-*o*-chlorotyrosine, *N,N*-disubstituted isoleucine, amino-methoxy-piperidone (Amp), arginine, threonine, and glyceric acid. The relative configuration of the Amp residue seemed to be similar to that of the Ahp residue, in the rest of the micropeptins, since the proton and carbon chemical shifts of positions 2, 3, and 4 of the Ahp and Amp were almost identical. The chemical shifts of carbons 5 and 6 were changed as expected from the substitution of a hydroxy group with a methoxy group. C-5 is upfield shifted by 6 ppm and C-6 is downfield shifted by 9 ppm in **7** relative to **6**. The pseudoeqatorial H-5 in **7** is downfield shifted by ca. 0.4 ppm, relative to **6**, while H-6 is upfield shifted by ca. 0.5 ppm. An attempt to determine the relative configuration of the Amp from *J* values and NOE correlations from the NMR data of **7** in DMSO-d_6 failed due to overlapping of H-3peq and H-4pax and the absence of NOE correlation between H-3pax and 6-OMe. This obstacle was overcome by dissolving **7** in pyridine-d_5 and running ^{1}H, COSY, and ROESY experiments for it, which established the *J* values and through-space conectivities in the Amp residue. The protons of this spin system were assigned as follows: H-3pax (4.94 ddd, 13.6,8.8,4.0), 3-NH (7.80 d 8.8), H-4pax (2.70 brq 13.6), H-4peq (1.86 m), H-5peq (1.84 m), H-5pax (1.49 brt 13.6), H-6peq (4.45 brs), and 6-OMe (3.01 s). The trans diaxial relationships, between H-3pax, H-4pax, and H-5pax, were established by the COSY correlations and *J* values (13.6 Hz). H-5pax presented NOEs with H-3pax, H-5peq, and H-6peq, while H-4pax presented NOEs with 3-NH, H-4peq, and H-5peq, thus establishing the relative configuration of the Amp, which is similar to that of the Ahp, 3*S**,6*R**. The amino acid sequence of **7** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Val-*N*,*O*-diMe-*o*-chloroTyr, Arg-Thr, and Thr-GA, the NMe of *N*,*O*-diMe-*o*-chloroTyr with Ile-carbonyl, and H-2 of Ile with C-6 of Amp and by a NOE correlation between α-NH of Amp and H-2 of Arg. The ester linkage between the carbonyl of valine and the oxygen of threonine was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine. The configurations of the chiral centers of **7** were assigned as L-valine, L-isoleucine, L-*N*,*O*-diMe-*o*-chlorotyrosine, L-arginine, L-threonine, 3*S*,6*R*-Amp, and D-glyceric acid by the same procedure described for **1**−**6**. On the basis of the above given arguments, structure **7** was assigned to micropeptin HU909.

Micropeptin HU895B (**8**) was isolated as a glassy material with a molecular formula of $C_{40}H_{63}ClN_9O_{12}$ based on the HR MALDI TOF quasi-molecular ion at *m*/*z* 896.5133/898.5133 (3:1) corresponding to $[M + H]^+$. Its molecular formula was found to be identical with that of micropeptin 895A (**6**) and similar to its NMR spectra. A singlet methoxy signal in the proton NMR spectrum of **6**, δ_H 3.76 ppm, was missing from the spectrum of **8**, and instead a phenolic singlet proton appeared at δ_H 9.94 ppm. In the aliphatic region of the proton NMR spectrum the doublet methyl signal of **6**, δ_H 0.85 ppm, is substituted by a triplet methyl signal, δ_H 0.88 ppm, in the spectrum of **8**. These differences are in accordance with a structure of **8** that is similar to that of **6**, where **8** is a des-*O*-methyl analogue, with isoleucine replacing valine. Analysis of the 1D (^{1}H, ^{13}C, and DEPT) (see Tables 1 and 2) and 2D (COSY, TOCSY, ROESY, HMQC, and

HMBC) NMR data of **8** (see Table 12, in the Supporting Information) allowed the full assignment of the following acid residues: isoleucine^{I}, *N*-Me-*o*-chlorotyrosine, *N,N*-disubstituted isoleucine^{II}, Ahp, arginine, threonine, and glyceric acid. The amino acid sequence of **8** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Ile^{I}-*N*-Me-*o*-chloroTyr, Ahp-Arg, Arg-Thr, and Thr-GA, the NMe of *N*-Me-*o*-chloroTyr with Ile^{II}-carbonyl, and H-2 of Ile^{II} with C-6 of Ahp. The ester linkage between the carbonyl of isoleucine and the oxygen of threonine was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of isoleucine. The configurations of the stereogenic centers of **8** were assigned as L-isoleucine (×2), L-*N*-Me-*o*-chlorotyrosine, L-arginine, L-threonine, 3*S*,6*R*-Ahp, and D-glyceric acid by the same procedure described for **1**−**7**. On the basis of the above given arguments, structure **8** was assigned to micropeptin HU895B.

The new anabaenopeptin HU892 (**9**), isolated from this *M. aeruginosa* bloom material, belongs to a relatively rare subgroup of the anabaenopeptins, presenting an aliphatic amino acid at the carboxylic end of the peptide and an *N*-methyl-homoaromatic amino acid at the second position. In a recent article, we described three new anabaenopeptins and summarized the different variation in the structure of the anabaenopeptins.[9] Since then, two additional, closely related (to **9**) anabaenopeptins, anabaenopeptins 908 and 915, were described in the literature,[10] and the configuration of the lysine residue of the braunsvicamides[11] was established by total synthesis to be D, in accordance with all of the other cyanobacterial-derived anabaenopeptins.[12] Anabaenopeptin HU892 presented a proton NMR spectrum characteristic of the anabaenopeptins where the two amide protons of the urea bridge resonate around δ_H 6.5 ppm. It presented a HR MALDI TOF quasi-molecular ion at *m/z* 893.5284, $[M + H]^+$, in accordance with a molecular formula of $C_{45}H_{68}N_{10}O_9$. Analysis of the 1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data of **9** (see Table 3) revealed the six amino acid units, namely, isoleucine, *N*-Me-homotyrosine (*N*-Me-Hty), homophenylalanine (Hph), valine, and lysine, of anabaenopeptin HU892 (**9**). The amino acid sequence of **9** was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid, Ile-*N*-Me-Hty, Hph-Val, and Val-Lys and the NMe of *N*-Me-Hty with Hph-carbonyl. The closure of the peptide ring was established through an HMBC correlation between Lys-ϵ-NH and the carbonyl of Ile, while the occurrence of the urea bridge is suggested on the basis of the HMBC correlation between the urea carbonyl (δ_C 157.5 ppm) and the H-2 and α-NH of the lysine and arginine residues.[13] Acid hydrolysis of **9** and derivatization with Marfey's reagent,[7] followed by HPLC analysis, demonstrated the L-configuration of the isoleucine, valine, homophenylalanine, and arginine residues and D-configuration for the lysine residue. The configuration of the *N*-Me-homotyrosine was not determined. On the basis of this data, structure **9** was established for anabaenopeptin HU892.

Compounds **1**−**8**, **10**, and **11** exhibit potent inhibitory activity against trypsin (IC_{50} 0.7−5.2 μM), as expected for micropeptins that contain arginine at position 5. Nevertheless, some of the compounds (**2**, **5**−**8**, **10**, **11**) also exhibit moderate (to potent) inhibition of chymotrypsin. Comparison of the potency of trypsin inhibition by the various compounds reveals that isoleucine at position 1 correlates to lower IC_{50}'s than valine (see compounds **2** and **10** versus **5** and **4**, in Table 4, respectively); 3-sulfo-GA at position 7 is more potent than GA, which in turn is more potent than disulfo-GA (see compounds **10**, **8**, and **11**, in Table 4, respectively), and *N,O*-diMe-*o*-chlorotyrosine is more potent than *N*Me-tyrosine, which is more potent than *N*Me-*o*-chlorotyrosine (see compounds **1**, **3**, and **11**, in Table 4, respectively). The lowest inhibition concentrations of chymotrypsin were measured for glyceric acid at position 7, while 3-sulfoglyceric acid derivatives were less potent and disulfoglyceric acid derivatives exerted much higher IC_{50}'s (see compounds **2**, **5**−**8**, **10**, and **11**, in Table 4, respectively). It is important to mention that a related group of compounds containing a *N*Me-phenylalanyl moiety at the second position do not inhibit chymotrypsin at similar concentrations, thus suggesting that the *o*-chlorophenol moiety is responsible for this unusual behavior.[14] The reason for the biosynthesis of such an array of compounds by toxic bloom forming cyanobacteria remains as yet unsolved, although some evidence points toward protection of the microcystins from proteolytic enzymes.[15]

Experimental Section

Instrumentation. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). UV spectra were obtained on a Varian Cary 5000 UV−vis−NIR spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C and a Bruker Avance 400 spectrometer at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. DEPT, COSY-45, gTOCSY, gROESY, gHSQC, gHMQC, gHMBC, gNHHSQC, and gNHHMBC spectra were recorded using standard Bruker pulse sequences. High-resolution MS were recorded on an Applied Biosystems Voyager System 4312 instrument. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem diode-array detector and Merck-Hitachi HPLC system (model L-4200 UV−vis detector and model L-6200A Intelligent pump).

Biological Material. *Microcystis* sp., TAU strain IL-342, was collected on October 25, 2004, from a water reservoir next to Kibbutz Hulda, Israel. A sample of the cyanobacterium is deposited at the culture collection of Tel Aviv University.

Isolation Procedure. The freeze-dried cells (135 g) were extracted with 7:3 MeOH/H_2O. The crude extract (18.0 g) was evaporated to dryness and separated on an ODS (YMC-GEL, 120A, 4.4 × 6.4 cm) flash column with increasing amounts of MeOH in water. Fraction 2 (1:9 MeOH/H_2O, 1.24 g) was subjected to a Sephadex LH-20 column in 1:1 MeOH/H_2O to obtain 12 fractions. The combined fractions 9−11 (380.3 mg) were separated again on a Sephadex LH-20 column in 1:1 $CHCl_3$/MeOH to give 12 fractions. The combined fractions 3−6 were subjected to a reversed-phase HPLC (YMC-Pack C-8 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 7:3 0.1% TFA in H_2O/CH_3CN to obtain five fractions. Fraction 5 (9.6 mg) was subjected to a reversed-phase HPLC (YMC-Pack C-8, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 65:35 0.1% TFA in H_2O/CH_3CN to obtain six fractions. Micropeptin HU1069 (**1**) (1.8 mg, 0.0013% yield based on the dry weight of the cyanobacteria) was eluted from the column with a retention time of 21.3 min, and micropeptin HU989 (**2**) (1.0 mg, 0.0007% yield based on the dry weight of the cyanobacteria) was eluted from the column with a retention time of 39.3 min. Fraction 5 (2:3 MeOH/H_2O, 314.4 mg) from the initial flash column was subjected to a Sephadex LH-20 column in 1:1 MeOH/H_2O to obtain 14 fractions. The combined fractions 5−8 (142.8 mg) were subjected to a reversed-phase HPLC (YMC-Pack C-8, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 8:2 0.1% TFA in H_2O/CH_3CN to obtain five fractions. Fraction 3 from this HPLC separation (4.9 mg) was separated on a C-18 reversed-phase HPLC column (YMC-Pack C-18, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) with 8:2 0.1% TFA in H_2O/CH_3CN to obtain pure micropeptin HU1021 (**3**) (1.3 mg, 0.001% yield) with a retention time of 24.8 min. Fraction 4 (6.0 mg) was purified on the same column with the same conditions to afford pure micropeptin HU1041 (**4**) (1.5 mg, 0.0011% yield) with a retention time of 28.6 min. Fraction 5 (10.4 mg) was separated on the C-8 HPLC column with the same conditions to afford micropeptin 478-A (**10**) (1.0 mg, 0.0007% yield, t_R 28.9 min) and micropeptin 478-B (**11**) (1.0 mg, 0.0007% yield, t_R 52.8 min). Fraction 6 (1:1 MeOH/H_2O, 293.1 mg) from the initial flash column was subjected to a C-18 reversed-phase HPLC column (YMC-Pack C-18, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 3:1 0.1% TFA in H_2O/CH_3CN

to obtain 10 fractions. Fraction 4 contained micropeptin 478-B (**11**) (4.0 mg, 0.003% yield, t_R 34.9 min). Fraction 8 from this column (19.5 mg) was separated on a reversed-phase HPLC (YMC-Pack C-8, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) with 55:45 0.1% TFA in H_2O/CH_3CN to obtain pure micropeptin HU975 (**5**) (2.0 mg, 0.0015% yield, t_R 34.9 min), micropeptin HU895A (**6**) (2.5 mg, 0.0019% yield, t_R 41.4 min), and micropeptin HU909 (**7**) (4.9 mg, 0.0036% yield, t_R 54.5 min). Fraction 7 (3:2 MeOH/H_2O, 372.0 mg) from the initial flash column was subjected to a Sephadex LH-20 column in 1:1 MeOH/H_2O to obtain 10 fractions. The combined fractions 5 and 6 (117.7 mg) were subjected to reversed-phase HPLC (YMC-Pack C-8, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 8:2 0.1% TFA in H_2O/CH_3CN to yield seven fractions. Fraction 7 (18.6 mg) from this separation was purified on the same column with 7:3 0.1% TFA in H_2O/CH_3CN to obtain micropeptin HU895B (**8**) (1.8 mg, 0.0013% yield, t_R 34.8 min). Fraction 8 (7:3 MeOH/H_2O, 354.2 mg) from the initial chromatography was subjected to a Sephadex LH-20 column in 1:1 $CHCl_3$/MeOH to obtain 10 fractions. The combined fractions 3−5 (154.4 mg) were separated again on a reversed-phase HPLC column (YMC-Pack C-8, 250 mm × 20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 28:72 0.1% TFA in H_2O/CH_3CN to yield nine fractions. Anabaenopeptin HU892 (**9**) (6.6 mg, 0.0045% yield) was obtained from this separation with a retention time of 56.2 min.

Micropeptin HU1069 (1): transparent oil; $[\alpha]^{25}_D$ −43.1 (*c* 1.7, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.38), 280 (3.36) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); MALDI TOF MS *m/z* 1012.29/1014.29 (3:1) $[MNa - SO_3]^+$, HR MALDI TOF MS *m/z* 910.4486/912.4485 (3:1) $[MH - 2SO_3]^+$ (calcd for $C_{41}H_{65}{}^{35}ClN_9O_{12}$, 910.4436). Retention times of AA Marfey derivatives: L-arginine 24.5 min, L-glutamic acid 29.7 min, L-threonine 28.9 min, L-isoleucine 48.6 min, and L-*N,O*-diMe-*o*-chlorotyrosine 53.2 min. Retention time of D-glyceric acid on a chiral column: 4.8 min.

Micropeptin HU989 (2): transparent oil; $[\alpha]^{25}_D$ −77 (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.17), 280 (3.30) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 1012.3863/1014.3863 (3:1) $[M + Na]^+$ (calcd for $C_{41}H_{64}{}^{35}ClN_9NaO_{15}S$, 1012.3823). Retention times of AA Marfey derivatives: L-arginine 24.7 min, L-glutamic acid 27.6 min, L-threonine 29.4 min, L-isoleucine 48.4 min, and L-*N,O*-diMe-*o*-chlorotyrosine 54.8 min. Retention time of D-glyceric acid on a chiral column: 4.0 min.

Micropeptin HU1021 (3): transparent oil; $[\alpha]^{25}_D$ −150 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 229 (4.23), 277 (3.63) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); MALDI TOF MS *m/z* 1012.29/1014.29 (3:1) $[M + Na - SO_3]^+$; HR MALDI TOF MS *m/z* 964.4083 $[M + Na - SO_3]^+$ (calcd for $C_{40}H_{63}N_9NaO_{15}S$, 964.4057). Retention times of AA Marfey derivatives: L-arginine 17.9 min, L-glutamic acid 19.5 min, L-threonine 21.4 min, L-isoleucine 42.3 min, and L-*N*Me-tyrosine 48.4 min. Retention time of D-glyceric acid on a chiral column: 4.1 min.

Micropeptin HU1041 (4): transparent oil; $[\alpha]^{25}_D$ −57 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.07), 282 (3.53) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 984.3421/986.3420 (3:1) $[M + Na - SO_3]^+$ (calcd for $C_{39}H_{60}{}^{35}ClN_9NaO_{15}S$, 984.3510). Retention times of AA Marfey derivatives: L-arginine 20.4 min, L-glutamic acid 24.8 min, L-threonine 25.9 min, L-valine 39.9 min, L-isoleucine 44.5 min, and L-*N*Me-*o*-chlorotyrosine 59.9 min. Retention time of D-glyceric acid on a chiral column: 4.0 min.

Micropeptin HU975 (5): transparent oil; $[\alpha]^{25}_D$ −26 (*c* 2.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.09), 280 (3.31) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 998.3615/1000.3615 (3:1) $[M + Na]^+$ (calcd for $C_{40}H_{62}{}^{35}ClN_9NaO_{15}S$, 998.3667). Retention times of AA Marfey derivatives: L-arginine 20.5 min, L-glutamic acid 25.4 min, L-threonine 25.3 min, L-valine 39.4 min, L-isoleucine 45.4 min, and L-*N,O*-diMe-*o*-chlorotyrosine 50.3 min. Retention time of D-glyceric acid on a chiral column: 4.2 min.

Micropeptin HU895A (6): transparent oil; $[\alpha]^{25}_D$ −65 (*c* 1.7, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.11), 280 (3.21) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 934.3868/936.3869 (3:1) $[M + K]^+$ (calcd for $C_{40}H_{62}{}^{35}ClKN_9O_{12}$, 934.3838). Retention times of AA Marfey derivatives: L-arginine 25.7 min, L-glutamic acid 29.8 min, L-threonine 30.6 min, L-valine 44.4 min, L-isoleucine 50.0 min, and L-*N,O*-diMe-tyrosine 54.1 min. Retention time of D-glyceric acid on a chiral column: 3.5 min.

Micropeptin HU909 (7): transparent oil; $[\alpha]^{25}_D$ −53 (*c* 2.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.15), 280 (3.46) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 932.4220/934.4220 (3:1) $[M + Na]^+$ (calcd for $C_{41}H_{64}{}^{35}ClN_9NaO_{12}$, 932.4255). Retention times of AA Marfey derivatives: L-arginine 23.1 min, L-glutamic acid 26.4 min, L-threonine 27.6 min, L-valine 40.6 min, L-isoleucine 47.2 min, and L-*N,O*-diMe-*o*-chlorotyrosine 51.9 min. Retention time of D-glyceric acid on a chiral column: 4.9 min.

Micropeptin HU895B (8): transparent oil; $[\alpha]^{25}_D$ −108 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.21), 280 (3.35) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI TOF MS *m/z* 896.4235/898.4234 (3:1) $[M + H]^+$ (calcd for $C_{40}H_{63}{}^{35}ClN_9O_{12}$, 896.4279). Retention times of AA Marfey derivatives: L-arginine 22.6 min, L-glutamic acid 25.8 min, L-threonine 26.9 min, L-isoleucine 45.9 min, and L-*N*Me-*o*-chlorotyrosine 59.8 min. Retention time of D-glyceric acid on a chiral column: 4.0 min.

Anabaenopeptin HU892 (9): transparent oil; $[\alpha]^{25}_D$ −36 (*c* 4.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 225 (4.28), 281 (3.24) nm; ^{1}H and ^{13}C NMR (see Table 3); HR MALDI TOF MS *m/z* 893.5284 $[M + H]^+$ (calcd for $C_{45}H_{68}N_{10}O_9$, 893.5243). Retention times of AA Marfey derivatives: L-arginine 26.2 min, D-lysine 42.8 min, L-valine min, L-isoleucine 51.5 min, and L-homophenylalanine 56.8 min.

Determination of the Absolute Configuration of the Amino Acids. Portions of compounds (0.5 mg) **1**−**9** were dissolved in 6 N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110 °C for 20 h. After removal of HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (40 mL). A solution of (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA) (4.2 mmol) in acetone (150 mL) and 1 N $NaHCO_3$ (20 mL) were added to each reaction vessel, and the reaction mixture was stirred at 40 °C for 2 h. A 2 N HCl solution (10 mL) was added to each reaction vessel, and the solution was evaporated in vacuo. The *N*-[(2,4-dinitrophenyl)-5-L-alanine amide]-amino acid derivatives, from hydrolysates, were compared with similary derivatized standard amino acids by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10 m, 4.6 × 300 mm, flow rate 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/CH_3CN to 1:1 TEAP/CH_3CN within 60 min. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids.

Determination of the Absolute Configuration of Glyceric Acid. The acid hydrolysates (1 mL) of compounds **1**−**8** were extracted with ethyl ether (1 mL × 3) to separate the glyceric acid from the amino acids mixture. The ether was removed and the residue dissolved in MeOH (1 mL). The MeOH solution was analyzed on an Astec Chirobiotic HPLC column, 250 × 4.6 mm flow rate 1 mL/min, UV detection at 210 nm, linear elution with 1:49 1% triethylammonium acetate (TEAA) buffer (pH 4)/MeOH. The authentic samples were spiked with a standard mixture of L- and D-glyceric acid.

Protease Inhibition Assay. Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$ to prepare a 1 mg/mL solution. Chymotrypsin was dissolved in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$/1 mM HCl to prepare a 1 mg/mL solution. A 2 mM solution of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (for trypsin) and Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) in the appropriate buffer solution was used as a substrate solution. The test sample was dissolved in ethanol and diluted with the same buffer solution that was used for the enzyme and substrate. A 100 μL buffer solution, 10 μL of enzyme solution, and 10 μL of the test solution were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then, 100 μL of substrate solution was added to begin the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37 °C for 30 min.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of all new compounds as well as full NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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