Micropeptins from an Israeli Fishpond Water Bloom of the Cyanobacterium *Microcystis* sp.†

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Seven new natural products, micropeptin MZ845 (**1**), micropeptin MZ859 (**2**), micropeptin MZ939A (**3**), micropeptin MZ925 (**4**), micropeptin MZ939B (**5**), micropeptin MZ1019 (**6**), and micropeptin MZ771 (**7**), as well as two known micropeptins, cyanopeptolin S (**8**) and cyanopeptolin SS (**9**), were isolated from the hydrophilic extract of the cyanobacterium *Microcystis* sp. that was collected from a fishpond in Kibbutz Ma'ayan Tzvi, Israel, in July 2006. The structures of the pure natural products were elucidated using spectroscopic methods, including UV, 1D and 2D NMR, and MS techniques. The absolute configuration of the chiral centers of the compounds was determined using Marfey's method for HPLC. The inhibitory activity of the compounds was determined for the serine proteases: trypsin, chymotrypsin, thrombin, and elastase. These micropeptins inhibited trypsin with IC_{50}'s that varied between 0.6 and 24.2 μM. The SAR of these micropeptins is discussed.

Cyanobacteria produce numerous natural products that are not used for primary metabolism, and their role in the producing organism is usually unknown. *Microcystis* spp. are responsible for water blooms that frequently produce potent hepatotoxins, and thus their environmental, toxicological, biological, and chemical properties have been extensively studied.[1] Extracts of toxic strains of *Microcystis* have been shown to be a rich source of unique modified peptides, such as microginins,[2] micropeptins,[3] aeruginosins,[4] anabaenopeptins,[5] and microviridines,[6] all of which are protease inhibitors. The micropeptins are the most abundant group of serine protease inhibitors from cyanobacteria, composed of more than 100 different members.[7] The selectivity of the micropeptins for the inhibition of different serine proteases is mainly influenced by the nature of the amino acid occupying the fifth position from the carboxy terminus. Basic amino acids (i.e., arginine and lysine) at this position select for inhibition of trypsin-like serine proteases, while aliphatic, aromatic, and other neutral amino acids select for inhibition of chymotrypsin-like serine proteases.[8] As part of our ongoing research on the chemistry and chemical ecology of cyanobacterial blooms in water bodies, a biomass (TAU strain IL-361) of the cyanobacterium *Microcystis* sp. was collected, in the summer of 2006, from a fishpond in Kibbutz Ma'ayan-Tzvi, Israel. The isolation and structure elucidation of micropeptins isolated from this cyanobacterial bloom biomass, as well as their biological activity and some SAR, is discussed below.

Results and Discussion

The cyanobacterium biomass was freeze-dried and extracted with 70% MeOH in H_2O. The extract that inhibited trypsin was flash-chromatographed on an ODS column. Two fractions eluted from the column with 40% and 50% MeOH in H_2O exhibited protease inhibitory activity and were further separated on a reversed-phase HPLC column. Seven new protease inhibitors, micropeptin MZ845 (**1**) (first identified by Martin Welker at al.[9] using an HPLC-MS method), micropeptin MZ859 (**2**), micropeptin MZ939A (**3**), micropeptin MZ925 (**4**), micropeptin MZ939B (**5**), micropeptin MZ1019 (**6**), and micropeptin MZ771 (**7**), along with two previously described natural products, cyanopeptolin S[10] (**8**) and cyanopeptolin SS[11] (**9**), were isolated from the cyanobacterial extract, their structures were elucidated, and their biological activities were studied.

Inspection of the NMR spectroscopic data of **1**−**7** reveals signals indicative of the micropeptin family. In the ^{1}H NMR spectrum a methyl singlet at ca. 2.70 ppm of an *N*Me moiety, a hydroxy of the amino hydroxy piperidone (Ahp) moiety at 6.10 ppm, a quartet signal at 5.40 ppm, and a doublet methyl at 1.21 ppm of the *O*-substituted Thr were all visible. Comparison of the ^{1}H NMR spectra of all of the nine members of this micropeptin family revealed their similarity: a doublet methyl (^{ll}Ile-6) at ca. −0.25 ppm, a triplet amide proton signal at ca. 7.50 ppm (Arg-δ-NH), a very broad signal of the guanidine moiety of Arg around 6.50−7.00 ppm, and overlapping signals of a monosubstituted phenyl moiety in the aromatic region (7.15−7.30 ppm) of the spectrum. The ^{13}C NMR spectrum revealed some other characteristic signals including a guanidine carbon at 156 ppm of the Arg moiety and three (two in the case of **7**) methine carbons adjacent to oxygen at around 73 ppm, in accordance with Ahp, Thr, and glyceric acid. The NMR data suggested a structure composed of the same seven acid units (except for **7**, which contains six acid units). The differences between the nine micropeptins were located at the amino hydroxy piperidone moiety and in the substitutions of the glyceric acid moiety. Five of the new micropeptins had an amino methoxy piperidone (Amp) moiety instead of an Ahp moiety, which was characterized by the appearance of a singlet methoxy group at 3.00 ppm, instead of the hydroxy group at 6.10 ppm. One of the micropeptins (**7**) was truncated and did not contain the glyceric acid moiety, while the remaining eight micropeptins differed in the substitution pattern of the glyceric acid (GA) by sulfate groups; two of the compounds possessed a sulfate substituent on position 2 of the glyceric acid. Such derivatives have not yet been described in the literature.

Micropeptin MZ845 (**1**) was isolated as an amorphous, white solid. The molecular formula of **1**, $C_{40}H_{63}N_9O_{11}$, was deduced from the high-resolution MALDI-TOF-MS measurements of its protonated molecular cluster ion at *m*/*z* 846.4641. Examination of the NMR spectra of **1** in DMSO-d_6 revealed its peptide nature, i.e., seven carboxylic carbons in the ^{13}C NMR spectrum (see Table 2) and four amide doublet protons in the ^{1}H NMR spectrum (see Table 1). Taking into account the *N*Me-aromatic amino acid and the *N,N*-disubstituted-amino acid of the micropeptins, this micropeptin was composed of six amino acids. The Ahp and glyceric acid structure was suggested on the basis of the COSY and HSQC spectra, which showed a singlet of a hydroxy group at 6.20 ppm of the Ahp moiety and two hydroxy signals at 4.76 ppm (t) and 5.80 ppm (brd) for GA. Analysis of the COSY, TOCSY, and HSQC 2D NMR spectra allowed the assignment of the side chains of two isoleucine units, a threonine, an arginine, a glyceric acid, and two

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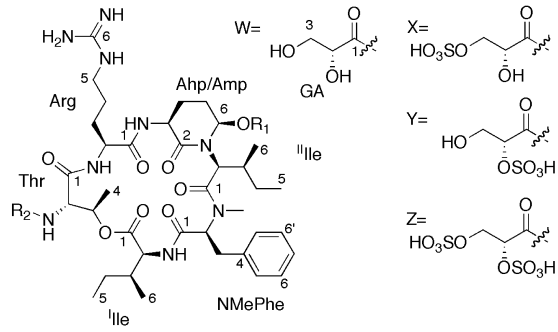
Chart 1



micropeptin MZ845 (**1**) R_1=H, R_2=W
micropeptin MZ859 (**2**) R_1=Me, R_2=W
micropeptin MZ939A (**3**) R_1=Me, R_2=X
micropeptin MZ925 (**4**) R_1=H, R_2=Y
micropeptin MZ939B (**5**) R_1=Me, R_2=Y
micropeptin MZ1019 (**6**) R_1=Me, R_2=Z
micropeptin MZ771 (**7**) R_1=Me, R_2=H
cyanopeptolin S (**8**) R_1=H, R_2=X
cyanopeptolin SS (**9**) R_1=H, R_2=Z

Table 1. Comparison of the ^{1}H NMR Data of Compounds **1**−**7** in DMSO-d_6[a]

position		**1**[c] δ_H, mult.	**2**[b] δ_H, mult.	**3**[c] δ_H, mult.	**4**[b] δ_H, mult.	**5**[c] δ_H, mult.	**6**[c] δ_H, mult.	**7**[c] δ_H, mult.
^{I}Ile	2	4.57, dd	4.58, dd	4.60, dd	4.72, dd	4.70, dd	4.70, m	4.85, dd
	3	1.74, m	1.72, m	1.73, m	1.76, m	1.78, m	1.78, m	1.77, m
	4a	1.27, m	1.34, m	1.33, m	1.26, m	1.34, m	1.33, m	1.30, m
	4b	1.06, m	1.13, m	1.11, m	1.00, m	1.11, m	1.06, m	1.10, m
	5	0.80, t	0.84, t	0.84, t	0.81, t	0.85, t	0.84, m	0.84, t
	6	0.83, d	0.87, d	0.86, d	0.84, d	0.86, d	0.86, d	0.87, d
	NH	7.77, d	7.00, d	6.95, d	7.61, d	6.89, d	6.88, d	6.89, d
*N*MePhe	2	5.12, dd	5.16, dd	5.15, dd	5.13, dd	5.17, dd	5.16, d	5.16, dd
	3a	3.29, m	3.30, m	3.32, m	3.29, m	3.30, m	3.30, m	3.29, m
	3b	2.79, dd	2.80, dd	2.79, dd	2.81, dd	2.81, dd	2.81, dd	2.82, dd
	5,5′	7.26, d	7.26, m	7.24, m	7.25, m	7.25, m	7.25, m	7.26, m
	6,6′	7.22, d	7.22, m	7.21, m	7.23, m	7.22, m	7.23, m	7.21, m
	7	7.19, m	7.19, m	7.17, m	7.20, m	7.19, m	7.20, m	7.20, m
	*N*Me	2.73, s	2.73, s	2.73, s	2.73, s	2.74, s	2.74, s	2.74, s
^{II}Ile	2	4.37, d	4.42, d	4.42, d	4.38, d	4.44, m	4.44, m	4.44, m
	3	1.74, m	1.80, m	1.76, m	1.74, m	1.79, m	1.80, m	1.81, m
	4a	1.07, m	1.05, m	1.05, m	1.05, m	1.07, m	1.06, m	1.05, m
	4b	0.60, m	0.59, m	0.58, m	0.60, m	0.60, m	0.60, m	0.59, brd
	5	0.60, brd	0.59, brd	0.58, brd	0.60, brd	0.60, brd	0.59, brd	0.59, brd
	6	−0.25, d	−0.28, d	−0.29, d	−0.26, d	−0.27, d	−0.29, d	−0.27, d
Ahp/Amp	3	4.44, m	4.47, m	4.46, m	4.44, m	4.44, m	4.47, m	4.45, m
	4pax	2.61, m	2.43, dd	2.42, dd	2.59, m	2.40, dd	2.40, m	2.33, m
	4peq	1.75, m	1.74, m	1.74, m	1.73, m	1.74, m	1.74, m	1.77, m
	5peq	1.75, m	2.10, m	2.06, m	1.73, m	2.04, m	2.07, m	2.10, m
	5pax	1.75, m	1.67, m	1.66, m	1.73, m	1.67, m	1.66, m	1.65, m
	6	4.91, brs	4.44, m	4.43, m	4.91, brs	4.44, m	4.43, m	4.45, m
	NH	7.32, d	7.19, m	7.15, d	7.30, d	7.17, d	7.17, d	7.24, m
	OH/OMe	6.20, brd	3.03, s	3.02, s	6.06, d	3.02, s	3.02, s	3.01, s
Arg	2	4.29, m	4.29, m	4.31, m	4.28, m	4.26, m	4.30, m	4.36, m
	3a	2.02, m	2.00, m	2.05, m	2.02, m	2.00, m	2.02, m	2.06, m
	3b	1.47, m	1.45, m	1.42, m	1.46, m	1.47, m	1.43, m	1.45, m
	4	1.47, m	1.45, m	1.41, m	1.46, m	1.44, m	1.43, m	1.46, m
	5	3.08, brq	3.08, m	3.07, m	3.06, m	3.06, m	3.07, m	3.10, m
	5-NH	7.53, t	7.45, t	7.44, t	7.46, t	7.47, t	7.44, t	7.49, m
	NH	8.60, d	8.63, d	8.58, d	8.53, brs	8.56, d	8.53, d	8.93, d
Thr	2	4.66, d	4.68, d	4.65, d	4.63, m	4.63, d	4.63, d	4.14, brs
	3	5.52, q	5.54, q	5.50, q	5.46, q	5.48, q	5.46, q	5.60, q
	4	1.21, d	1.21, d	1.20, d	1.22, d	1.22, d	1.21, d	1.40, d
	NH	7.64, d	7.66, d	7.64, d	7.83, d	7.83, d	7.75, d	8.40, brs
GA	2	4.07, q	4.07, m	4.24, dd	4.65, m	4.65, d	4.72, m	
	3a	3.60, m	3.60, m	3.97, dd	3.63, dd	3.63, m	3.99, m	
	3b	3.49, m	3.48, m	3.84, dd	3.63, dd	3.63, m	3.91, dd	
	2-OH	5.80, brd	5.80, d	6.09, brs				
	3-OH	4.76, t	4.76, t		4.77, t	4.73, dd		

[a] Complete NMR data are available in the Supporting Information (coupling constants, HMBC and ROESY correlations). [b] 500 MHz. [c] 400 MHz.

short fragments in accordance with a phenylalanine moiety and an amino hydroxy piperidone (Ahp). The structure of the side chains of the latter two amino acids and the assignment of the carboxyamide carbons to the side chains were achieved by analysis of the results of a ^{1}H−^{13}C HMBC experiment (see Table 4 in the Supporting Information). The sequence of the amino acids of the peptide, ^{I}Ile, *N*MePhe, ^{II}Ile, Ahp, Arg, Thr, and GA was assigned on the basis of HMBC correlations between the carboxyl of *N*MePhe and ^{I}Ile NH, the carboxyl of ^{II}Ile and the *N*Me of *N*MePhe, the C-6 methine of Ahp and H-2 of the ^{II}Ile, the carboxyl of Arg and NH of Ahp, the carboxyl of Thr and NH of Arg, the carboxyl of ^{I}Ile and H-3 of Thr (the lactone linkage), and the carboxyl of GA and the NH of Thr. The amino acid sequence could also be assembled from the ROESY data (see Table 4 in the Supporting Information). The relative configuration of Ahp-C-6 (*R**) is based on the *J*-values of H-6 (<1 Hz), which point to an equatorial orientation of this proton, the chemical shift of the pseudoaxial H-4 (δ_H 2.61), which is downfield shifted by the axial hydroxy group,

Table 2. Comparison of the ^{13}C NMR Data of Compounds **1**−**7** in DMSO-d_6

position		mult.[a]	**1**[b] δ_C	**2**[b] δ_C	**3**[b] δ_C	**4**[c] δ_C	**5**[b] δ_C	**6**[b] δ_C	**7**[b] δ_C
^{I}Ile	1	qC	173.0	172.7	172.7	−[d]	172.4	172.5	172.2
	2	CH	55.5	55.4	55.4	55.7	55.4	55.5	55.1
	3	CH	37.3	37.7	37.9	37.8	38.1	38.2	37.7
	4	CH_2	24.8	24.9	25.0	24.7	25.0	24.9	24.8
	5	CH_3	11.1	10.8	11.0	11.5	11.3	11.4	11.3
	6	CH_3	15.9	15.9	16.0	16.1	16.0	16.0	16.1
*N*MePhe	1	qC	169.3	169.2	169.1	169.0	169.0	169.2	169.2
	2	CH	60.6	60.8	60.8	60.7	60.8	60.8	60.9
	3	CH_2	34.3	34.2	34.2	34.4	34.4	34.3	34.3
	4	qC	137.8	137.7	137.8	137.9	137.8	137.9	137.6
	5,5′	CH	128.7	128.8	128.8	128.7	128.8	128.8	128.9
	6,6′	CH	129.7	129.7	129.7	129.8	129.7	129.8	129.7
	7	CH	126.8	126.9	126.8	126.8	126.8	126.9	126.9
	*N*Me	CH_3	30.3	30.3	30.3	30.3	30.2	30.3	30.2
^{II}Ile	1	qC	169.8	169.7	169.8	170.0	169.7	169.8	169.6
	2	CH	54.3	54.1	54.0	54.0	54.0	54.0	53.7
	3	CH_2	33.1	32.8	32.8	33.2	32.8	32.8	32.9
	4	CH_2	23.8	23.8	23.8	23.8	23.8	23.8	23.7
	5	CH_3	10.4	10.3	10.4	10.5	10.3	10.4	10.2
	6	CH_3	14.0	13.7	13.7	14.0	13.7	13.7	13.6
Ahp/Amp	2	qC	169.8	169.2	169.2	−[d]	169.3	169.0	169.3
	3	CH	49.2	49.4	49.3	49.1	49.3	49.2	49.1
	4	CH_2	21.6	21.6	21.6	21.8	21.7	21.8	21.9
	5	CH_2	29.9	23.8	23.8	29.8	23.8	23.7	23.7
	6	CH	74.2	83.3	83.2	73.9	83.1	83.1	83.2
	*O*Me	CH_3		55.4	55.4		55.4	55.5	55.5
Arg	1	qC	170.2	170.4	170.4	170.3	170.4	170.4	169.7
	2	CH	52.3	52.2	51.9	51.6	51.6	51.4	52.5
	3	CH_2	27.7	27.7	27.3	25.7	27.1	26.9	27.8
	4	CH_2	25.3	25.3	25.2	25.0	25.0	25.0	25.2
	5	CH_2	40.3	40.5	40.2	39.9	40.5	40.2	40.5
	6	qC	156.8	156.8	156.7	−[d]	156.7	156.7	156.9
Thr	1	qC	169.2	169.1	169.2	169.4	169.4	169.3	167.1
	2	CH	54.4	54.4	54.5	55.6	55.0	55.0	55.7
	3	CH	72.3	72.4	72.7	71.9	71.9	71.8	69.7
	4	CH_3	18.1	18.0	17.9	17.7	17.5	17.3	17.8
GA	1	qC	172.8	172.8	171.9	172.0	170.4	169.7	
	2	CH	72.7	72.7	70.6	77.0	77.0	74.7	
	3	CH_2	63.9	63.7	68.4	62.4	62.3	66.5	

[a] Multiplicity and assignment from an HSQC experiment. [b] 100 MHz. [c] 125 MHz. [d] Absent from spectrum due to limited amount of material that was isolated.

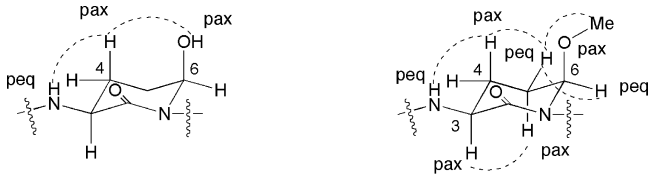


Figure 1. Relative configuration and NOEs of the Ahp and Amp moieties.

and the NOE correlation observed between H-4_{pax} and 6-OH (see Figure 1). On the basis of the results discussed above, the planar structure of micropeptin MZ845 was assigned as **1**.

Micropeptin MZ859 (**2**) was isolated as an amorphous, white solid. The molecular formula of **2**, $C_{41}H_{65}N_9O_{11}$, was deduced from the high-resolution MALDI-TOF-MS measurements of its protonated molecular ion cluster at *m*/*z* 860.4893. Comparison of the NMR data (see Tables 1 and 2) of micropeptin MZ859 (**2**) and micropeptin MZ845 (**1**) revealed that the difference between them was located in the piperidone moiety, where a methyl ether substituent in **2** replaced the hydroxy in **1**. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR data allowed the assignment of the side chains of the micropeptin as two isoleucines, a threonine, an arginine, a glyceric acid, and an *N*Me-phenylalanine (see Table 5 in the Supporting Information). The structure of the amino methoxy piperidone (Amp) was deduced as follows: COSY correlations established the connectivity of H-3 through H-6, and carbons 3 through 6 were assigned by correlations in the HSQC experiment. The amide carbonyl resonating at 169.2 ppm was assigned as C-2 of the Amp by its HMBC correlation with H-3, and the cyclic structure of Amp is suggested on the basis of the HMBC correlation of H-6 with the latter carbonyl. The HMBC correlation of the methoxy at δ_H 3.03 (s) with C-6, δ_C 83.3 ppm, established its attachment to C-6. The relative configuration of the Amp residue was the same as that of the Ahp residue, because 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 a substitution of a hydroxy group with a methoxy group. C-5 was upfield shifted by 6 ppm and C-6 was downfield shifted by 9 ppm in **2** relative to **1**. The pseudoequatorial H-5 in **2** was downfield shifted by ca. 0.4 ppm relative to **1**, while H-6 was upfield shifted by ca. 0.5 ppm. H-4_{pax} presented NOEs with H-5_{peq} and 3-NH and H-5_{peq} presented NOEs with H-4_{pax} and *O*Me, while H-3_{pax} presented NOEs with H-5_{pax} and H-4_{peq} and H-6_{peq} presented NOEs with H-5_{peq} (Figure 1). This established the relative configuration of the Amp to be the same as to that of Ahp, (3*R**,6*R**). The sequence of the amino acids of the peptide, ^{I}Ile-*N*MePhe-^{II}Ile-Amp-Arg-Thr-GA, was assigned on the basis of HMBC correlations between the carboxy of an amino acids and the amide proton of the adjacent acid in the pairs *N*MePhe-^{I}Ile, Arg-Amp, Thr-Arg, and GA-Thr; between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe; between C-6 of Amp and H-2 of ^{II}Ile; and between the carboxy of ^{I}Ile and H-3 of Thr

(the lactone linkage). The amino acid sequence could also be assembled from the ROESY data (see Table 5 in the Supporting Information). The planar structure **2** was thus assigned to micropeptin MZ859.

Micropeptin MZ939A (**3**) was isolated as an amorphous, white solid. The molecular formula of **3**, $C_{41}H_{65}N_9O_{14}S$, was deduced from the high-resolution MALDI-TOF-MS measurements of its sodiated molecular cluster ion at *m*/*z* 962.4260. The difference in the 1D NMR spectrum between micropeptin MZ939A (**3**) and micropeptin MZ859 (**2**) was located in the glyceric acid residue (see Tables 1 and 2). In **3**, H-3a and H-3b were downfield shifted by ca. 0.36 ppm, relative to those of **2**, and were not coupled to an alcoholic proton. In addition, C-3 in **3** was downfield shifted by 4.7 ppm relative to that of **2**. These differences were in accordance with a 3-sulfated glyceric acid on the basis of the comparison with the NMR chemical shifts of the 3-sulfated glyceric acid residue in cyanopeptolin S.[10] Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR spectra allowed the assignment of all the side chains that assemble this micropeptin as two isoleucine units, a threonine, an arginine, a 3-sulfated glyceric acid, an *N*Me-phenylalanine, and an amino methoxy piperidone (Amp) as described for **2** (see Table 6 in the Supporting Information). The sequence of the amino acids was determined by HMBC and ROESY 2D NMR experiments. HMBC correlations were observed between the carboxy of each amino acid and the amide proton of the adjacent amino acid in the pairs *N*MePhe-^{I}Ile, Arg-Amp, and Thr-Arg; between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe; between C-6 of Amp and H-2 of ^{II}Ile; between the carboxy of GA and NH of Thr; and between the carboxy of ^{I}Ile and Thr H-3 (the lactone linkage). The structure and relative configuration of the Amp moiety was established as for **2**. On the basis of the results discussed, the planar structure of micropeptin MZ939A was assigned as **3**.

Micropeptin MZ925 (**4**) was isolated as an amorphous, white solid. The molecular formula of **4**, $C_{40}H_{63}N_9O_{14}S$, was deduced from the high-resolution MALDI-TOF-MS measurements of its protonated fragment ion $[M + H - SO_3]^+$ at *m*/*z* 846.4741, which derives from a natural loss of sulfur trioxide, due to the low stability of the protonated sulfate moiety.[12] The difference between micropeptin MZ925 (**4**) and micropeptin MZ845 (**1**) was located in the glyceric acid residue (see Tables 1 and 2). In **4**, H-2 was downfield shifted by 0.58 ppm relative to that of **1** and not coupled to an alcoholic proton. In addition C-2 in **4** was downfield shifted by 4.3 ppm relative to that of **1**. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR data allowed the assignment of all the side chains that assemble this micropeptin as two isoleucine units, a threonine, an arginine, a 2-sulfated glyceric acid, an *N*Me-phenylalanine, and an amino hydroxy piperidone (Ahp) (see Table 7 in the Supporting Information). Due to the poor ^{13}C and HMBC NMR data, as a consequence of the limited amount of material that was isolated, the sequence of the amino acids was determined mainly on the basis of a ROESY experiment and the comparison of relevant chemical shifts with that of the related micropeptins **1** and **5**. HMBC correlations were observed between the carboxy of an amino acid and the amide proton of the adjacent amino acid of *N*MePhe-^{I}Ile and Arg-Ahp and between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe. ROESY correlations were observed between H-2 of an amino acid and the NH of the adjacent amino acid in Thr-Arg and GA-Thr, between H-2 of ^{I}Ile and NH of Thr (the lactone linkage), and between H-2 of ^{II}Ile and H-6 of Ahp. The relative configuration of C-6 of Ahp was established in the same way as for **1**. On the basis of the results discussed above, the planar structure of micropeptin MZ925 was assigned as **4**.

Micropeptin MZ939B (**5**) was isolated as an amorphous, white solid. The molecular formula of **5**, $C_{41}H_{65}N_9O_{14}S$, was deduced from the high-resolution ESI-MS measurements of its protonated fragment ion $[M + H]^+$ at *m*/*z* 940.4472. The only difference between micropeptin MZ939B (**5**) and micropeptin MZ925 (**4**) was located in the piperidone moiety, where an Amp moiety appears in **5**, instead of an Ahp moiety in **4** (see Tables 1 and 2). Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR spectra allowed the assignment of the side chains of the amino acids of this micropeptin, as two isoleucines, a threonine, an arginine, a 2-sulfated glyceric acid, an Amp, and an *N*Me-phenylalanine (see Table 8 in the Supporting Information). The sequence of the amino acids was determined by HMBC and ROESY 2D NMR experiments. The HMBC correlations were observed between the carboxy of an amino acid and the amide proton of the adjacent amino acid for *N*MePhe-^{I}Ile, Arg-Amp, Thr-Arg, and GA-Thr; between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe; between C-6 of Amp and H-2 of ^{II}Ile; and between the carboxy of Ile and Thr H-3 (the lactone linkage). The relative configuration of C-6 of Amp was established in the same way as for **2**. On the basis of the results discussed here, the planar structure of micropeptin MZ939B was assigned as **5**.

Micropeptin MZ1019 (**6**) was isolated as an amorphous, white solid. High-resolution ESIMS measurements in the positive mode gave a sodiated cluster ion $[M + Na - SO_3]^+$ at *m*/*z* 962.4274, derived from a natural loss of sulfur trioxide, due to a low stability of the protonated sulfate moiety.[12] The molecular formula of **6**, $C_{41}H_{65}N_9O_{17}S_2$, was deduced from the negative mode HR-ESIMS measurment, which presented a molecular ion $[M - H]^-$ at *m*/*z* 1018.3912. Micropeptin MZ1019 (**6**) differs from micropeptin MZ939B (**5**) in the glyceric acid residue (see Tables 1 and 2). In **6**, H-3a and 3b were downfield shifted by 0.30−0.36 ppm relative to that of **5** and not coupled to an alcoholic proton. In addition, C-3 in **6** was downfield shifted by 4.2 ppm relative to that of **5**. These differences are in accordance with 2,3-disulfated glyceric acid, on the basis of a comparison with the NMR chemical shifts of the 2,3-disulfated glyceric acid residue in cyanopeptolin SS (**9**).[11] Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR spectra allowed the assignment of the side chains of the amino acids that are contained in **6**: two isoleucines, a threonine, an arginine, a disulfated glyceric acid, an Amp, and an *N*Me-phenylalanine (see Table 9 in the Supporting Information). The sequence of the amino acids was determined by HMBC and ROESY 2D NMR experiments. The HMBC correlations were observed between the carboxy of the amino acid and an amide proton of the adjacent amino acid in Arg-Ahp and GA-Thr; between C-6 of Amp and H-2 of ^{II}Ile; and between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe. The ROESY correlations were observed between H-2 of *N*MePhe and ^{I}Ile-NH; between Thr-2 and Arg-NH; and between Thr-NH and ^{I}Ile-2 (the lactone linkage). The relative configuration of C-6 of Amp was established in the same way as for **2**. On the basis of the results discussed, the planar structure of micropeptin MZ1019 was assigned as **6**.

Micropeptin MZ771 (**7**) was isolated as an amorphous, white solid. The molecular formula of **6**, $C_{38}H_{61}N_9O_8$, was deduced from the high-resolution MALDI-TOF-MS measurements of its protonated molecular cluster ion at *m*/*z* 772.4764. Examination of the NMR spectrum of **7** in DMSO-d_6 revealed that the Thr moiety in **7** was different from all of the known micropeptins. In the ^{1}H NMR spectrum, H-2 was upfield shifted by 0.5 ppm, H-3 and H_3-4 were downfield shifted by 0.14 and 0.19 ppm, respectively, and the amide proton was substituted by a two-proton signal at 8.40 ppm. In the ^{13}C NMR spectrum C-2 was downfield shifted by 0.7 ppm, while C-1, -3, and -4 were upfield shifted by 2.2, 2.1, and 0.5 ppm, respectively. The signals for a glyceric acid were missing from both of the ^{1}H and ^{13}C NMR spectra of **7** (see Tables 1 and 2). These findings were in accordance with a nonsubstituted threonine moiety. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR data allowed the assignment of the side chains of the amino acids in this micropeptin: two isoleucines, a threonine, an arginine, an Amp, and an *N*Me-phenylalanine (see Table 10 in the Supporting Information). The sequence of the amino acids was determined by

Table 3. Comparison of the Relative Trypsin Inhibition Potency of **1**−**9** as a Function of the Glyceric Acid and Piperidone Substitution

	Ahp	Amp
GA-substitution	compound (IC_{50} μM)	compound (IC_{50} μM)
diol	**1** (2.6)	**2** (0.6)
3-sulfate	**8** (4.1)	**3** (1.5)
2-sulfate	**4** (24.2)	**5** (7.4)
disulfate	**9** (2.9)	**6** (1.6)

analysis of the HMBC and ROESY 2D NMR experiments. HMBC correlations were observed between the carboxy of an amino acid and an amide proton of the adjacent amino acid of *N*MePhe-^{I}Ile, Arg-Amp, and Thr-Arg; between the carboxy of ^{II}Ile and the *N*Me of *N*MePhe, between C-6 of Amp and H-2 of ^{II}Ile, and between the carboxy of ^{I}Ile and Thr H-3 (the lactone linkage). The relative configuration of C-6 of Amp was established in the same way as for **2**. On the basis of the results discussed, the planar structure of micropeptin MZ771 was assigned as **7**.

To determine the absolute configuration of the chiral centers of micropeptins **1**−**7**, Marfey's method for HPLC was used. This involved acid hydrolysis to liberate the free amino acids, followed by derivatization with Marfey's reagent[13] and HPLC analysis. In order to liberate glutamic acid from the Ahp/Amp residues, a small amount of **1**−**7** was oxidized with Jones' reagent,[14] following the same procedure. These two procedures demonstrated the L-configuration of all amino acids. The observed *J*-value (0−1 Hz) between H-2 and H-3 of the substituted threonine in **1**−**7** suggested that, as in the case of all known micropeptins, it should be threonine and not *allo*-threonine.[15] The possibility that D- or L-*allo*-Ile is present in these cyclic depsipeptides was ruled out by the comparison of the ^{13}C chemical shifts of C-5 and C-6 of the isoleucine residues in known micropeptins. For the Ile at the carboxy terminus of the peptide the measured chemical shifts in L-*allo*-Ile (established for oscillamide J by chiral-GC-MS[16]) are 11.4 (C-5) and 14.3 (C-6) ppm, while for L-Ile (established for nostopeptins A and B by chiral-GC-MS[17]) they are 11.3 (C-5) and 16.1 (C-6) ppm, in accordance with the values measured for **1**−**7**. In the case of the *N,N*-disubstituted-Ile, only values for L-Ile are available, 10.3 (C-5) and 13.9 (C-6) ppm (established for nostopeptins A and B by chiral-GC-MS[17]), which are similar to those measured for compounds **1**−**7**. Ether extracts of the acid hydrolysates followed by chiral HPLC analysis demonstrated the D-configuration for the glyceric acid moieties of micropeptins **1**−**6**. This established the absolute stereostructures, **1**−**7**, for the new micropeptins.

To rule out the possibility that compounds **1**−**7** are isolation artifacts of cyanopeptolins SS (**9**), either derived from methoxylation of the Ahp moiety or hydrolysis of the sulfate esters, we dissolved **9** in a 1:1 methanol/0.1% aqueous TFA solution and allowed it to stand at RT for a week. Comparison of the proton NMR and negative ESIMS spectra of the sample, before and after the treatment with the aqueous acidic methanol solution, verified our previous findings that the micropeptins are stable under these conditions; namely, the sulfate esters do not hydrolyze and the Ahp does not convert to the Amp residue.

Micropeptins **1**−**9** were isolated through serine proteases (trypsin and chymotrypsin) inhibition assay-guided separation. The inhibitory activities of pure **1**−**9** were determined for the serine proteases trypsin (see Table 3), thrombin, chymotrypsin, and elastase. Thrombin was mildly inhibited by micropeptin MZ859 (**2**), with an IC_{50} of 52.9 μM, and cynopeptolin SS (**9**) with an IC_{50} of 45.2 μM, but not by the rest of the compounds. Micropeptin MZ771 (**7**) was inactive in these inhibition assays. Micropeptins **1**−**9** were inactive in the chymotrypsin and elastase inhibition assays. Compounds **1**−**9** exhibit potent to moderate inhibitory activity against trypsin (IC_{50} 0.6−24.2 μM), as expected for micropeptins that contain arginine at position 5, but almost no activity against thrombin. Comparison of the potency of trypsin inhibition by **1**−**9** (see Table 3) reveals that the Amp derivatives are more potent than the Ahp derivatives, and GA at position 7 is the most potent of the GA derivatives. Recently we described a related family of micropeptins, which differed from **1**−**9** in the aromatic amino acid. It presents a substituted *N*MeTyr moiety instead of *N*MePhe in **1**−**9**. This related family of compounds exhibit both trypsin and chymotrypsin inhibition, most probably due to the chlorine substitution on the tyrosine aromatic ring.[18]

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO P-1010 polarimeter. UV spectra were recorded on an Agilent 8453 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, gHMBC, and gHMBC spectra were recorded using standard Bruker pulse sequences. High-resolution MS were recorded on an Applied Biosystems Voyager System 4312 instrument and Waters MALDI Synapt System. HPLC separations were performed on a JASCO HPLC system (model PU-2080 Plus pump, model LG-2080-04 Quaternary Gradient unit, and model PU-2010 Plus multiwavelength detector) and on a Merck HPLC system (model L-6200A pump and model L-4200 UV−vis detector). ELISA was used for the protease inhibition assay, EL_x808, BIO-TEK Instruments, Inc.

Biological Material. *Microcystis* sp., TAU strain IL-361, was collected in July 2006, from a fishpond at Kibbutz Ma'ayan-Tzvi, Israel. The cell mass was frozen after it was collected and lyophilized. A sample of the cyanobacterium is deposited at the culture collection of Tel-Aviv University.

Isolation Procedure. Lyophilization of the first batch of IL-361 cells produced 0.54 kg of dry cells. Extraction with 7:3 MeOH/H_2O (3 × 2 L) and evaporation yielded 20 g of extract. The extract was chromatographed, in 5 g portions, on a reversed-phase (ODS) flash column (YMC-GEL, 120A, 4.4 × 6.4 cm) eluted with increasing percentage of MeOH in H_2O. Fraction 5 (2:3 MeOH/H_2O; 190 mg) was further separated on a Sephadex LH-20 column with 7:3 MeOH/H_2O to obtain 10 fractions (fractions 5a−j). Fraction 6 from the initial RP column (1:1 MeOH/H_2O; 220 mg) was separated on a Sephadex LH-20 column eluted with 7:3 MeOH/H_2O to obtain 11 fractions (fractions 6a−k). Fractions 5c−g and 6d−f were combined (a, 188 mg) and separated again on a Sephadex LH-20 column eluted with 1:1 MeOH/H_2O to obtain 10 fractions (a1−a10). Fractions a5−a8 were combined, and the combined fraction (b, 106 mg) was separated on a reversed-phase HPLC column (YMC-Pack C-8 250 mm × 20.0 mm, DAD at maximum absorbance, flow rate 5.0 mL/min) eluted with 4:1 0.1% TFA in H_2O/acetonitrile (ACN) to obtain four fractions (b1−b4). Fraction b3 was further separated on a RP HPLC column eluted with 2:3 ACN/0.1% TFA in H_2O to obtain 10 fractions (b3a−j), seven of which were found to be pure compounds. Compound **9** (b3c, 1.9 mg) was eluted from the column with a retention time of 23.7 min (it was isolated twice more from other fractions, adding to a total weight of 6.4 mg, 0.0015% yield based on the dry weight of the cyanobacterium). Compound **4** (b3d, 1.4 mg) was eluted from the column with a retention time of 30.2 min (it was reisolated from another fraction, adding to a total weight of 1.1 mg, 0.0005% yield). Compound **8** (b3e, 3.4 mg) was eluted from the column with a retention time of 32.6 min (it was reisolated five more times from other fractions, adding to a total weight of 8.9 mg, 0.002% yield). Compound **6** (b3f, 1.4 mg, 0.0003% yield) was eluted from the column with a retention time of 35.6 min. Compound **1** (b3g, 1.7 mg) was eluted from the column with a retention time of 37.5 min (it was reisolated four more times from other fractions, adding to a total weight of 7.7 mg, 0.0017% yield). Compound **5** (b3h, 0.9 mg) was eluted from the column with a retention time of 49.3 min (it was reisolated one more time, adding to a total of 2.1 mg, 0.0006% yield). Compound **2** (b3j, 2.6 mg) was eluted from the column with a retention time of 64.4 min (it was reisolated three more times from other fractions, adding to a total weight of 24.1 mg; the total yield is 0.0045%). Fraction b3j was further separated on the same RP HPLC column with 4.7:5.3 ACN/0.1% TFA in H_2O to obtain six fractions (b3j1−6). Compound **3** (b3j−5, 1.2 mg, 0.0002% yield) was eluted from the column with a retention time of 19.6 min. A second batch of lyophilized cells (1.1 kg) was extracted with 7:3 MeOH/H_2O (3 × 1.5

L) and then evaporated *in vacuo* to produce 42 g of extract. The extract was separated on an ODS Flash column with an increasing amount of MeOH in H_2O. Fraction 5′ (2:3 MeOH/H_2O; 540 mg) was further separated on a Sephadex LH-20 column with 7:3 MeOH/H_2O to obtain 11 fractions (5′k−u). Fraction 6 from the RP column (1:1 MeOH/H_2O; 720 mg) was separated on a Sephadex LH-20 column with 7:3 MeOH/H_2O to obtain 11 fractions (6′l−w). Fractions 5′k−n and 6′l−o were combined (c, 300 mg) and separated again on a Sephadex LH-20 column eluted with 1:1 MeOH/H_2O to obtain 10 fractions (c1−c10). Fractions c5−c10 were combined with fractions 5′m and 6′p. The combined fraction (d, 503 mg) was separated on a Sephadex LH-20 column eluted with 1:1 MeOH/H_2O to obtain 11 fractions (d1−d11). Fractions d5−d7 were combined (e, 188 mg) and separated on a RP HPLC column YMC-Pack C-8 eluted with 5:15 ACN/0.1% TFA in H_2O to obtain six fractions (e1−e6), fraction e4 was found to be a pure compound, and compound **7** (2.6 mg, 0.0006% yield) was eluted from the column with a retention time of 43.4 min.

Micropeptin MZ845 (1): amorphous, white solid; $[\alpha]^{23}_D$ −160 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.20), 250 (2.88) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS *m/z* 846.4641 $[M + H]^+$ (calcd for $C_{40}H_{63}N_9O_{11}$, 846.4720). Retention time of amino acids (AA) Marfey's derivatives: L-Arg 18.2 min (D-Arg 18.6 min), L-Thr 23.3 min (D-Thr 26.9 min), L-Glu 25.4 min (D-Glu 27.0 min), L-Ile 39.8 min (D-Ile 44.1 min), and L-*N*Me-Phe 41.8 min. Retention time of D-glyceric acid on the chiral column 3.8 min (L-glyceric acid 3.4 min).

Micropeptin MZ859 (2): amorphous, white solid; $[\alpha]^{22}_D$ −38 (*c* 0.53, MeOH); UV (MeOH) λ_{max} (log ε) 203 (5.04), 254 (2.71) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS *m/z* 860.4893 $[M + H]^+$ (calcd for $C_{41}H_{66}N_9O_{11}$, 860.4876). Retention time of AA Marfey's derivatives: L-Arg 18.3 min (D-Arg 18.4 min), L-Thr 21.1 min (D-Thr 24.6 min), L-Glu 23.1 min (D-Glu 24.9 min), L-Ile 40.5 min (D-Ile 44.3 min), and L-*N*Me-Phe 42.3 min. Retention time of D-glyceric acid on the chiral column 3.7 min (L-glyceric acid 3.4 min).

Micropeptin MZ939A (3): amorphous, white solid; $[\alpha]^{23}_D$ −119 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.22), 250 (2.93) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS *m/z* 962.4260 $[M + Na]^+$ (calcd for $C_{41}H_{65}N_9NaO_{14}S$, 962.4260). Retention time of AA Marfey's derivatives: L-Arg 18.3 min (D-Arg 18.5 min), L-Thr 25.1 min (D-Thr 28.5 min), L-Glu 25.9 min (D-Glu 27.4 min), L-Ile 41.2 min (D-Ile 44.9 min), and L-*N*Me-Phe 42.9 min. Retention time of D-glyceric acid on the chiral column 3.7 min (L-glyceric acid 3.3 min).

Micropeptin MZ925 (4): amorphous, white solid; $[\alpha]^{22}_D$ −89 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 203 (5.04), 254 (3.00) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS *m/z* 846.4741 $[M + H - SO_3]^+$ (calcd for $C_{40}H_{64}N_9O_{11}$, 846.4720). Retention time of AA Marfey's derivatives: L-Arg 17.2 min (D-Arg 17.6 min), L-Thr 22.9 min (D-Thr 26.4 min), L-Glu 24.7 min (D-Glu 26.5 min), L-Ile 39.4 min (D-Ile 43.5 min), and L-*N*Me-Phe 41.3 min. Retention time of D-glyceric acid on the chiral column 3.8 min (L-glyceric acid 3.4 min).

Micropeptin MZ939B (5): amorphous, white solid; $[\alpha]^{23}_D$ −80 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.07), 252 (2.83) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR-ESIMS *m/z* 940.4472 $[M + H]^+$ (calcd for $C_{41}H_{66}N_9O_{14}S$, 940.4450). Retention time of AA Marfey's derivatives: L-Arg 18.5 min (D-Arg 18.6 min), L-Thr 23.4 min (D-Thr 26.8 min), L-Glu 26.2 min (D-Glu 27.7 min), L-Ile 40.6 min (D-Ile 46.0 min), and L-*N*Me-Phe 41.8 min. Retention time of D-glyceric acid on the chiral column 3.7 min (L-glyceric acid 3.3 min).

Micropeptin MZ1019 (6): amorphous, white solid; $[\alpha]^{23}_D$ −44 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.13), 227 (3.66), 277 (3.03) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR-ESIMS *m/z* 962.4274 $[M + Na - SO_3]^+$ (calcd for $C_{41}H_{65}N_9NaO_{14}S$, 962.4269), *m/z* 1018.3912 $[M - H]^-$ (calcd for $C_{41}H_{64}N_9O_{17}S_2$, 1018.3862). Retention time of AA Marfey's derivatives: L-Arg 18.0 min (D-Arg 18.2 min), L-Thr 22.8 min (D-Thr 26.2 min), L-Glu 24.7 min (D-Glu 26.5 min), L-Ile 39.4 min (D-Ile 43.3 min), and L-*N*Me-Phe 41.4 min. Retention time of D-glyceric acid on the chiral column 3.7 min (L-glyceric acid 3.4 min).

Micropeptin MZ771 (7): amorphous, white solid; $[\alpha]^{23}_D$ −176 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.58), 248 (2.14) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS *m/z* 772.4764 $[M + H]^+$ (calcd for $C_{38}H_{62}N_9O_8$, 772.4716). Retention time of AA Marfey's derivatives: L-Arg 18.5 min (D-Arg 18.7 min), L-Thr 23.4 min (D-Thr 27.1 min), L-Glu 27.9 min (D-Glu 29.5 min), L-Ile 40.0 min (D-Ile 44.3 min), and L-*N*Me-Phe 42.0 min.

Cyanopeptolin S (8): amorphous, white solid; HR MALDI-TOF-MS *m/z* 948.4117 $[M + Na]^+$ (calcd for $C_{40}H_{63}N_9NaO_{14}S$, 948.4107). Identical in all respects with the published data.[10]

Cyanopeptolin SS (9): amorphous, white solid; HR-ESIMS *m/z* 1004.3729 $[M - H]^-$ (calcd for $C_{40}H_{62}N_9O_{17}S_2$, 1004.3705). Identical in all respects with the published data.[11]

Determination of the Absolute Configuration of the Amino Acids. Compounds **1**−**7** (0.3 mg each) were hydrolyzed in 6 M HCl (1 mL). The reaction mixture was maintained in a sealed glass bomb at 110 °C for 16 h. The acid was removed *in vacuo*, and the residue was resuspended in 250 μL of H_2O. FDAA solution [(1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide] in acetone (115 μL, 0.03 M) and $NaHCO_3$ (120 μL, 1 M) were added to each reaction vessel. The reaction mixture was stirred at 40 °C for 2 h. Then HCl (2 M, 60 μL) was added to each reaction vessel, and the solution was evaporated *in vacuo*. The FDAA-amino acid derivatives from the hydrolysate were dissolved in 1 mL of ACN and compared with standard FDAA-amino acids by HPLC analysis: LiChrospher 60, RP-select B (5 μm), flow rate 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer, pH 3/ACN, within 60 min. The absolute configuration of each amino acid was determined by spiking the derivatized hydrolysates with a D,L-mixture of the standard derivatized amino acids.

Determination of the Absolute Configuration of Ahp and Amp Derivatives. Compounds **1**−**7** (0.1 mg each) were oxidized with Jones' reagent (1 drop from a solution of 1.34 g of K_2CrO_7, 1 mL of H_2SO_4 in 8 mL of H_2O) in 0.5 mL of acetone at 0 °C for 10 min. The mixture was allowed to warm to room temperature, and a few drops of MeOH were added. The bluish residue that developed was filtered, and the solvent was evaporated *in vacuo*. The resultant products were treated as described above.

Determination of the Absolute Configuration of Glyceric Acid. Extraction of the acid hydrolysates of compounds **1**−**6** with ethyl ether separated the glyceric acid from the amino acid salts. The ether was removed *in vacuo*, and the residue was dissolved in MeOH (1 mL). The MeOH solution was analyzed on an Astec, Chirobiotic, LC stationary phase, 250 × 4.6 mm flow rate 1 mL/min, UV detection at 210 nm, linear elution 1:49 1% triethylamine, 1% acetic acid (TEAA) buffer, pH 4/MeOH. The glyceric acid from the micropeptins was compared with standard D,L-glyceric acids.

Protease Inhibition Assays. Trypsin, thrombin, chymotrypsin, and elastase were purchased from Sigma Chemical Co. Trypsin (1 mg/mL) and chymotrypsin (10 mg/mL) were dissolved in 0.05 M Tris-HCl/100 mM NaCl/1 mM $CaCl_2$, pH 7.5 buffer solution. Benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA), the trypsin substrate, was dissolved in a solution of 1:9 DMSO/Tris-buffer (0.85 g/mL). Suc-Gly-Gly-*p*-nitroanilide (SGGPNA), the substrate for chymotrypsin, was dissolved in Tris buffer (1 mg/mL). Test samples were dissolved in DMSO (1 mg/mL). A 100 μL buffer solution, 10 μL of enzyme solution, and 10 μL of sample solution were added to each microtiter plate well and preincubated at 37 °C for 10 min. Substrate solution (10 μL) was added, and the kinetics of the reaction were measured at 405 nm, 37 °C, for 30 min. Elastase (75 mg/mL) and thrombin (0.5 g/mL) were dissolved in 0.2 M Tris-HCl, pH 8 buffer solution. Z-Gly-Pro-Arg-4MβNA-acetate salt, the thrombin substrate, was dissolved in Tris buffer (0.5 mg/mL). *N*-Suc-Ala-Ala-Ala-*p*-nitroanilide, the elastase substrate, was dissolved in Tris buffer (1 mg/mL). The test samples were dissolved in DMSO (1 mg/mL). For elastase, 150 μL of buffer solution, 10 μL of enzyme solution, and 10 μL of sample solution were added to each microtiter plate well and preincubated at 30 °C for 20 min. Substrate solution (30 μL) was added, and the kinetics of the reaction were measured at 405 nm, 37 °C, for 20 min. For thrombin, 170 μL of buffer solution, 10 μL of enzyme solution, and 10 μL of sample solution were added to each microtiter plate well and preincubated at 25 °C for 5 min. Substrate solution (10 μL) was added, and the kinetics of the reaction were measured at 405 nm, 37 °C, for 20 min.

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Supporting Information Available: Tables 4−10, summarizing the ^{1}H, ^{13}C, HMQC or HSQC, HMBC, and ROESY data of **1**−**7**, as well as the ^{1}H and ^{13}C NMR spectra of compounds **1**−**9**, are available free of charge via the Internet at http://pubs.acs.org.

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