Three Novel Protease Inhibitors from a Natural Bloom of the Cyanobacterium *Microcystis aeruginosa*

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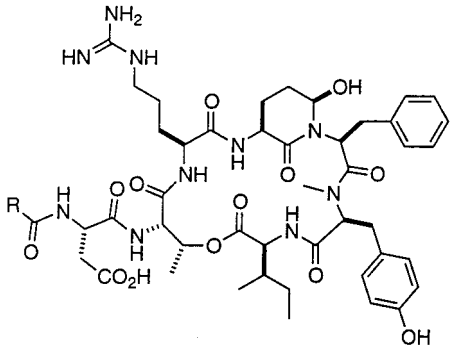
Two new trypsin inhibitors, micropeptins EI992 (**1**) and EI964 (**2**), and a modified linear peptide aeruginosin EI461 (**3**) were isolated from the hydrophilic extract of two samples of *Microcystis aeruginosa*, collected from the Einan Reservoir in Israel. Homonuclear and heteronuclear 2D-NMR techniques as well as HRFABMS determined the gross structures of **1**−**3**. The relative and absolute stereochemistry of **1**−**3** was deduced by a combination of spectral data and Marfey's method for HPLC. Aeruginosin EI461 (**3**) differs from the 14 known aeruginosins in the relative and absolute stereochemistry of the Choi-6-hydroxyl substituent.

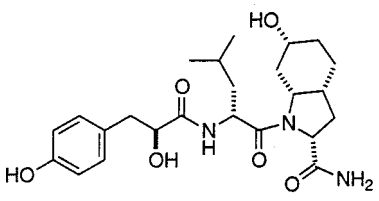
Toxic cyanobacteria of the genera *Microcystis*, *Anabaena*, *Planktothrix*, and *Nostoc* that produce toxic cyclic peptides, microcystins,[1] usually also produce considerable amounts of protease inhibitors.[2] Nontoxic strains of these genera of cyanobacteria occasionally produce the same classes of protease inhibitors. Five classes of protease inhibitors have been described from genera of cyanobacteria that produce toxic water blooms. All five classes of these protease inhibitors—micropeptins,[3] aeruginosins,[4] microginins,[5] anabaenopeptins,[6] and microviridins[7]—are peptides in nature. The micropeptins and aeruginosins each contain a transformed amino acid. In the micropeptins, the 3-amino-6-hydroxy-2-piperidone (Ahp) is produced by reduction of the δ-carboxyl of glutamic acid to the corresponding aldehyde, which, in turn, reacts with the amide nitrogen of a neighboring amino acid to produce the piperidone moiety. 2-Carboxy-6-hydroxyoctahydroindole (Choi), in the aeruginosins, is produced by the reduction of the aromatic ring of tyrosine and cyclization of its α-amine to form the new ring system. Both of these transformed amino acids interact with the catalytic site of the inhibited enzyme[8,9] and are important to the inhibition process. More than 45 micropeptins[10] and 14 aeruginosins[4] have, to date, been described from marine and freshwater cyanobacterial blooms. In this paper we describe the structure elucidation of two new micropeptins and a new aeruginosin, which differs from the known aeruginosins[4] in the stereochemistry of position 6 of Choi.

Results and Discussion

As part of our ongoing research on the chemistry of cyanobacterial blooms in Israeli bodies of water, nontoxic strains of the cyanobacterium *Microcystis aeruginosa* (strains IL-217 and IL-231) were collected, in the summer of 1998 and 1999, from the Einan Reservoir in the Hula Valley, Israel. The samples of the cyanobacterium were freeze-dried and extracted with 70% MeOH in H_2O. The extracts were found to inhibit trypsin and chymotrypsin. The active extract was flash-chromatographed on an ODS column. Six fractions eluted from the column, with 20−70% MeOH in H_2O, exhibited protease inhibitory activity and were further separated on a Sephadex LH-20 column and finally purified on a reversed-phase HPLC column. Three new protease inhibitors, micropeptin EI992 (**1**, 116.4 mg), micropeptin EI964 (**2**, 6.5 mg), and aeruginosin EI461 (**3**, 3.5 mg), were isolated. Examination of the ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** revealed that they are related in structure to micropeptin SD944[10] and other known micropeptins.[3]



Micropeptin EI992 (**1**) R = $CH_2CH_2CH_3$
Micropeptin EI964 (**2**) R = CH_3



Aeruginosin EI461 (**3**)

Micropeptin EI992 (**1**) was isolated as colorless oil. The molecular formula of **1**, $C_{48}H_{68}N_{10}O_{13}$, was deduced from high-resolution FABMS measurements of its protonated molecular cluster ion (*m*/*z* 993.5033). The ^{1}H NMR, in DMSO-d_6, revealed five doublet NH proton signals and three broad singlet (4H) signals between δ 6.6 and 9.9 ppm, pointing to seven amino acid residues (taking into account the four guanidine protons of the arginine moiety, the NMe-aromatic amino acid, and the Ahp residue that counts for two amino acids). The two two-proton doublets at 6.98 and 6.77 ppm and a two-proton doublet, a two-proton triplet, and a one-proton triplet at 6.83, 7.17, and 7.12 ppm, respectively, indicate that the aromatic amino acids of this peptolide are tyrosine and phenylalanine. Analysis of the

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Table 1. NMR Data of Micropeptin EI992 (**1**)[a] in DMSO-d_6

position		δ_C, mult[b]	δ_H, mult, J (Hz)	LR H−C corr[c]	NOE corr[d]
Ile	1	172.4 s		Ile-2, Thr-3	
	2	55.3 d	4.71 dd 9.4,4.6	Ile-4,4′,6,NH	Ile-3,4,4′,6,NH, Tyr-NMe, Ahp-OH
	3	38.2 d	1.75 m	Ile-2,4,4′,5,6	Ile-2, Ahp-OH
	4	24.8 t	1.17 m; 1.10 m	Ile-6	Ile-2, Ahp-OH; Tyr-NMe
	5	11.7 q	0.81 t 6.3	Ile-4,4′	
	6	16.1 q	0.82 d 6.4	Ile-2,4,4′	Tyr-NMe
	NH		7.42 d 9.4		Ile-2,3,4,4′,6, Tyr-2,NMe, Ahp-OH
NMeTyr	1	169.0 s		Tyr-2, Ile-2,NH	
	2	60.9 d	4.89 brd 10.0	Tyr-3,3′,NMe	Ile-NH, Tyr-3,5,5′, Phe-2, Thr-2
	3	33.1 t	3.09 brd 12.9; 2.73 dd 12.9,10.0	Tyr-2,5,5′	Tyr-2,3′,5,5′; Tyr-3,5,5′
	4	127.7 s		Tyr-3,3′.6,6′	
	5,5′	130.6 d	6.98 d 8.2	Tyr-3,3′,5′	Tyr-2,3,3′,6,6′, Phe-2
	6,6′	115.5 d	6.77 d 8.2	Tyr-5,5′,6,6′	Tyr-5,5′,7, Phe-2
	7	157.5 s		Tyr-5,5′,6,6′	
	OH		9.35 brs		
	NMe	30.3 q	2.74 brs	Tyr-2	Ile-2,4′,6,NH, Ahp-OH
Phe	1	170.5 s		Phe-2, Tyr-2,NMe	
	2	50.5 d	4.74 brd 8.4	Phe-3,3′	Tyr-2,5,5,6,6, Phe-3′,5, Ahp-3,6
	3	35.4 t	2.86 m; 1.80 m	Phe-2,5,5′	Phe-3′,5,5′, Ahp-6; Phe-2,3,5,5′
	4	136.9 s		Phe-3,3′,6,6′	
	5,5′	129.6 d	6.83 d 7.2	Phe-3,3′,5,5′,6,6′,7	Phe-2,3,3′,6,6′ Ahp-3,5′,6
	6,6′	127.9 d	7.17 t 7.2	Phe-6′	Phe-5,5′
	7	126.3 d	7.12 t 7.2	Phe-5,5′	
Ahp	2	169.1 s		Ahp-3,4,6	
	3	48.6 d	3.65 m	Ahp-4,5,5′,NH	Ahp-NH, Phe-2,5,5′,6,6′
	4	21.7 t	2.40 m; 1.54 m	Ahp-3,6	Ahp-4′,5,NH,OH, Ile-NH, Thr-3; Ahp-3,4,6
	5	29.4 t	1.68 m; 1.52 m		Ahp-4,6
	6	73.9d	5.05 brs	Phe-2, Aph-4,4′	Ahp-5,5′,OH, Phe 3,5,5′,
	NH		6.97 d 9.2		Ahp-3,4,4′, Arg-2,NH, Thr-3
	OH		6.02 brs		Ahp-3,4,6, Ile-3,4,4′,NH, Tyr-NMe
Arg	1	170.0 s		Ahp-NH	
	2	50.7 d	4.17 m	Arg-NH	Arg-3,3′,5,NH, Ahp-NH
	3	26.2 t	1.85 m; 1.43 m		Arg-2,3′,5,5′; Arg-2,3,5,5′,NH
	4	24.4 t	1.44 m; 1.15 m		Arg-5,5′
	5	39.7 t	2.92 m (2H)		Arg-2,4,4′
	6(NH)		9.88 brs		
	7	156.4 s		Arg-5	
	NH,NH_2		6.63 m, 7.25 m		
	2-NH		8.54 d 8.1		Arg-2,3,4 Ahp-NH, Thr-2,3
Thr	1	169.7 s		Arg-NH, Thr-2	
	2	54.7 d	4.57 brd 8.8	Thr-4	Arg-NH, Thr-NH
	3	72.0 d	5.31 brq 6.2	Thr-4	Thr-2,4, Arg-NH, Ahp-NH,4, Ile-2
	4	17.6 q	1.12 d 6.2	Thr-2,3	Thr-2,3
	NH		7.35 brs		Asp-2,NH, Thr-2
Asp	1	173.0 s		Thr-2, Asp-2,3,3′	
	2	50.4 d	4.52 m	Asp-3,3′,NH	Asp-3,3′,NH, Thr-NH
	3	39.4 t	2.53 dd 16.7, 11.5; 2.13 brd 16.7	Asp-2	Asp-2,3′,NH Asp-2,3
	4	175.1 s		Asp-2,3,3′	
	NH		8.04 d 7.5		Thr-4, Asp-2,3,3′, But-3,3′
But	1	172.0 s		Asp-NH, But-2,3,3′	
	2	37.2 t	2.07 t 7.4	But-3,3′,4	Asp-NH, But-3,3′,4
	3	18.9 t	1.52 m; 1.50 m	But-2,4	But-2,2′,4
	4	13.6 q	0.85 t 7.2	But-2,3,3′	But-2,2′,3,3′

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment from HMQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s. [d] Selected correlations from ROESY experiment, spin lock delay of 200 ms.

1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data (see Table 1) revealed the seven amino acid units and the fatty acid that build compound **1**, namely, isoleucine, NMe-tyrosine, *N*,*N*-disubstituted phenylalanine, 3-amino-6-hydroxy-2-piperidone (Ahp), arginine, threonine, aspartic acid, and butyric acid. The ester linkage of **1** arises from the carbonyl of isoleucine, and the hydroxyl of threonine. All the proton and carbon signals of the latter residues, except the arginine carbonyl, were assigned by the COSY, TOCSY, HMQC, and HMBC data. No HMBC correlation was observed between the carbonyl of arginine and the arginine side chain protons. The assignment of the carbonyl at δ_C 170.0 ppm to arginine is based on an NOE correlation between the proton at position 2 of the arginine and Ahp-NH, as well as an HMBC correlation of the Ahp-NH proton with this carbonyl.

The amino acid sequence of micropeptin EI992 (**1**) was determined from HMBC correlations (see Table 1) of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Ile-NMe-Tyr, Ahp-Arg, Arg-Thr, and Asp-But), of the Tyr-NMe and H-2 with Phe-carbonyl, of the Phe-H-2 and C-6 of the Ahp residue, and of Thr H-2 with Asp-carbonyl. The ester bond was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of isoleucine. The amino acid sequence could also be assembled from the ROESY data (see Table 1). Acid hydrolysis of **1** and derivatization with Marfey's reagent,[11] followed by HPLC analysis, demonstrated the L-stereochemistry of the isoleucine, NMe-tyrosine, phenylalanine, arginine, threonine, and aspartic acid residues. Jones oxidation[12] of **1**, followed by a similar hydrolysis, derivatization, and HPLC analysis, demonstrated an L-stereo-

chemistry for the Ahp residue (the oxidation and subsequent hydrolysis liberated L-glutamic acid from Ahp). The stereochemistry of C-6 of the Ahp was determined as *R* on the basis of the *J*-values of H-6, <1, which points to an equatorial orientation of this proton and the chemical shift of the axial H-4, δ_H 2.40 brq, which is downfield shifted by the axial hydroxyl at position 6 and an NOE between the axial H-4 and 6-O*H*. We noticed some proton and carbon NMR chemical shift differences between two samples of **1** that were isolated with and without trifluoroacetic acid (TFA) in the mobile phase of the HPLC. The major chemical shift differences were observed for the guanidine residue of arginine and aspartic acid. In the carbon NMR spectrum, the guanidine carbon resonates at δ_C 157.8 ppm in the TFA adduct (δ_C of the quartet TFA carboxyl 158.1 ppm) and at 156.4 ppm in the TFA-free sample. The carboxyl (C-4) of the aspartic acid resonates at δ_C 171.7 ppm in the TFA adduct and at 175.1 ppm in the TFA-free sample. In the proton NMR spectrum the most dramatic differences are observed for methylene-3 of the aspartic acid (doublet of doublets at 2.75 and 2.49 ppm in the TFA adduct and 2.53 and 2.13 ppm in the TFA-free sample), for the amide proton of the aspartic acid (8.30 ppm in the TFA adduct and 8.04 in the TFA-free sample), and in the δ-NH of the arginine resonating at 7.58 ppm in the TFA adduct and 9.88 ppm in the TFA-free sample. These findings suggest that the TFA adduct is formed, as might be expected, with the guanidine unit of arginine, which is the strongest base in **1**, while in the TFA-free sample a bridging salt is formed between the carboxyl of the aspartic acid and the guanidine of arginine. This intramolecular salt forces some conformation changes that account for small chemical shift differences in other units of the cyclic peptide. The conformation changes are reflected in the HMBC correlations that appear for the Asp-carbonyl (C-1, δ_C 171.4 ppm). A correlation of Thr-NH (δ_H 7.58 d) with Asp C-1 appears in the HMBC map of the TFA adduct together with those of Thr H-2 (δ_H 4.56 d) and Asp H-2 (δ_H 4.62 d) that also appear in the HMBC map of the TFA-free sample, further supporting the connection of the Asp carbonyl to the amine of Thr and the assignment of Asp C-1.

The proton NMR spectrum of micropeptin EI964 (**2**), the minor micropeptin in the extract of this cyanobacterial bloom, has shown minute chemical shift differences due to structural differences in the fatty acid substituent of the N-terminal amino acid, when compared with the spectrum of **1**. The molecular formula of **2**, $C_{46}H_{64}N_{10}O_{13}$, deduced from high-resolution FABMS measurements of its protonated molecular cluster ion (*m*/*z* 965.4740), suggested that **2** is shorter, by two methylenes, than **1**. This mass weight difference is in agreement with the NMR data that suggested the substitution of the butyrate in **1** with an acetate unit in **2**. The arguments used for the structure elucidation of **2** (see Table 2), are similar to those used for **1**. The small amount of material that was isolated resulted in a very diluted NMR sample, which gave a poorer HMBC map and fewer correlations between adjacent amino acid residues. The ROESY correlations were thus of great value in assembling the complete structure of **2**. Using Marfey's procedure,[11] as for **1**, to determine the absolute configuration of the amino acids in the peptide, revealed that all of the amino acids in **2** are of L-configuration. On the basis of these arguments, structure **2** was assigned to micropeptin EI964.

Aeruginosin EI461 (**3**) is the smallest of the protease inhibitors isolated from the extract of this *M. aeruginosa* water bloom. High-resolution FABMS measurements furnished a molecular formula of $C_{24}H_{35}N_3O_6$ for aeruginosin EI461 (**3**). This peptide is made up of two amino acids, leucine and 2-carboxy-6-hydroxyoctahydroindole amide (Choi amide), and *p*-hydroxy phenyl lactic acid (Hpla). A literature search revealed that the recently described aeruginosin 298-B[4] has a molecular weight, acid composition, and acid sequence similar to **3**. Comparison of the proton and carbon chemical shifts (see Supporting Information) revealed small chemical shift differences for the leucine and Hpla residues and considerable differences for the Choi amide moiety. Prior to the full assignment of the proton and carbon NMR data, the most striking chemical shift and multiplicity differences were observed for the carbinol at position 6 of Choi: δ_H 3.92 (brs), δ_C 63.7 (d) in the major component of aeruginosin 298-B[4] compared with δ_H 3.25 (brt, 11.5 Hz), δ_C 67.0 (d) in the major component of aeruginosin EI461 (**3**). These differences suggested that either the configuration of the hydroxyl at position 6 is reversed in both compounds or some conformation changes occur due to differences in another stereocenter or centers in the Choi moiety. In DMSO solution, compound **3** appears as a 3:2 mixture of *cis*- and *trans*-rotamers of the Choi-Leu peptidic bond, respectively. The structures of the acid residues were determined by 1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR data (see Table 3 for the NMR data of the major rotamer and Experimental Section and Supporting Information for the NMR data of the minor rotamer). The NMR data of the Hpla are in accordance with the data published for this residue in microcin SF608[13] and aeruginosin 298-B.[4]

The relative stereochemistry and conformation of the Choi residue, as well as the nature of its two rotamers, were elucidated by analysis of the protons' *J*-values and the NOE correlations from a ROESY experiment (see Figure 1 and Supporting Information). H-6 was determined as axial, based on its two *trans* diaxial coupling constants (with H-5*ax* and H-7*ax*) and its NOEs with the axial protons at positions 4 and 7a. These coupling constants and NOEs also confirmed the chair conformation of the cyclohexanol moiety. The medium size coupling constant and the NOE between H-3a and H-7a determined the *cis* fusion of the perhydroindolol moiety. The NOEs between H-2, H-3a, and H-7a determine that these three protons are oriented to the same face of perhydroindole as H-6*ax* and H-4*ax*. Finally, the NMR data of **3** were compared with the NMR data of the synthetic 6α- and 6*β*-isomers of Boc-L-Choi-OMe[14] (kindly provided by Prof. J. Bonjoch of the University of Barcelona, Spain) (see Supporting Information). The comparison of **3** with the two synthetic Choi isomers revealed good correlation between **3** and Boc-L-6*β*-OH-Choi-OMe, further supporting the suggested structure of **3**. Due to the modest size of aeruginosin EI461, the two sets of proton and carbon NMR signals of the two rotamers were well resolved, and hence the sequence of residues could be determined by HMBC and ROESY experiments. The Choi-Leu connectivity was determined by the ROESY correlation between Choi H-2 and Leu H-2 and Choi H-7a and Leu H-3′ for the major isomer, and between Leu H-2 and Choi H-2 and H-3a and H-7a for the minor isomer. The Leu-Hpla sequence was determined by HMBC correlation between Leu NH and Hpla carbonyl. Using Marfey's procedure,[11] as for **1** and **2**, revealed that the Leu in **3** is of D-configuration. The retention time of the Choi-Marfey's reagent derivative from **3** (57.1 min) is longer than that obtained for L-6*β*-OH-Choi derivative (53.8 min), suggesting that it is of the D-configuration, and thus the

Table 2. NMR Data of Micropeptin EI964 (**2**)[a] in DMSO-d_6

position		δ_C, mult[b]	δ_H, mult, J (Hz)	LR H−C corr[c]	NOE corr[d]
Ile	1	172.4 s		Thr-3, Ile-2	
	2	55.4 d	4.68 dd 9.4,4.6	Ile-6	Ile-3,6,NH
	3	37.8 d	1.75 m	Ile-5,6	Ile-2
	4	24.8 t	1.00 m; 1.23 m		
	5	11.6 q	0.80 t 6.3	Ile-4′	
	6	16.2 q	0.83 d 6.4	Ile-4′	Ile-2
	NH		7.45 d 9.4		Tyr-2,NMe
NMeTyr	1	169.2 s		Tyr-2, Ile-NH	
	2	61.0 d	4.87 brd 10.0	Tyr-3,3′,NMe	Ile-NH, Tyr-3,5,5′, Phe-2
	3	33.0 t	3.10 brd 12.9; 2.71 dd 12.9,10.0	Tyr-5,5′	Tyr-2,3′,5,5′; Tyr-3,5,5′
	4	127.7 s		Tyr-3,0.6,6′	
	5,5′	130.6 d	6.98 d 8.2	Tyr-3,5,5′	Tyr-2,3,3′,6,6′, Phe-2
	6,6′	115.5 d	6.77 d 8.2		Tyr-5,5′,OH, Phe-2
	7	156.4 s		Tyr-5,5′,6,6′	
	OH		9.36 s		Tyr-6,6′
	NMe	30.4 q	2.73 brs	Tyr-2	Ile-NH
Phe	1	170.5 s		Tyr-NMe	
	2	50.5 d	4.73 brd 8.4	Phe-3′	Tyr-2,5,5′, Phe-3′,5,5′
	3	35.5 t	2.85 dd 12.0,8.4; 1.80 m	Phe-2,5,5′	Phe-3′,5,5′, Ahp-6; Phe-2,3,5,5′
	4	136.9 s		Phe-3,3′,6,6′	
	5,5′	129.6 d	6.82 d 7.2	Phe-3,5,5′,6,6′,7	Phe-2,3, Ahp-5′,6
	6,6′	127.9 d	7.15 t 7.2	Phe-6′	Phe-5,5′
	7	126.4 d	7.10 t 7.2	Phe-5,5′	
Ahp	2	169.0 s		Ahp-6	
	3	48.8 d	3.58 m		Ahp-4,5,NH
	4	21.7 t	2.37 m; 1.57 m		Phe-2; Ahp-4′
	5	29.4 t	1.70 m; 1.55 m		Ahp-6,OH; Ahp-3,6,OH
	6	73.9d	5.04 brs		Ahp-3,5,OH, Phe 2,3,4
	NH		7.06 d 9.2		Ahp-3,4, Arg-2,NH
	OH		6.04 brs		Ahp-5,6, Ile-NH
Arg	1	169.9 s		Ahp-NH	
	2	51.4 d	4.15 m		Arg-3,3′,NH, Ahp-NH
	3	26.9 t	1.85 m; 1.42 m		Arg-2,3′,5,5′; Arg-2,3,5,5′,NH
	4	24.6 t	1.45 m; 1.30 m		Arg-5,5′
	5	40.2 t	3.00 m (2H)		Arg-2,4,4′
	6(NH)		6.95 m		
	7	155.9 s		Arg-5,NH_2,NH	
	NH_2,NH		6.61 m		
	NH		8.54 d 8.1		Ahp-NH, Thr-2,3
Thr	1	169.4 s		Arg-NH, Thr-2	
	2	54.8 d	4.55 brd 8.2	Thr-4	Arg-NH, Thr-3,4
	3	72.2 d	5.35 brq 6.4	Thr-4	Thr-2,4, Arg-NH,
	4	17.9 q	1.15 d 6.4	Thr-2,3	Thr-2,3
	NH		7.57 d 8.2		Asp-2,NH, Thr-4
Asp	1	172.2 s		Asp-2	
	2	50.0 d	4.57 m	Asp-3	Asp-3,3′,NH, Thr-NH
	3	39.4 t	2.63 dd 16.7,11.5; 2.37 brd 16.7		Asp-2,3′,NH; Asp-2,3
	4	173.2 s		Asp-3	
	NH		8.23 d 7.5		Thr-NH, Ac-2
Ac	1	169.4 s		Ac-2	
	2	22.6 q	1.84 s		Asp-NH

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment from HMQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s. [d] Selected correlations from ROESY experiment, spin lock delay of 200 ms.

absolute configuration of the chiral centers of the Choi moiety in **3** is suggested to be 2*R*, 3a*R*, 6*R*, and 7a*R*. The (−)-menthol method[15] was used to determine the absolute configuration of the Hpla moiety as *S*. On the basis of this evidence, we suggest structure **3** for aeruginosin EI461.

The inhibitory activity of **1**, **2**, and **3** was determined against the serine proteases trypsin and chymotrypsin. Micropeptin EI992 (**1**) and micropeptin EI964 (**2**) inhibited trypsin with IC_{50} values of 3.8 and 4.2 μg/mL, respectively, but not chymotrypsin at a concentration 45.5 μg/mL. Aeruginosin EI461 (**3**) inhibited only 15% of the activity of trypsin at a concentration of 45.5 μg/mL.

Experimental Section

Instrumentation. IR spectra were recorded on a Nicolet FTIR in $CHCl_3$ or neat. Low- and high-resolution MS were recorded on a Fisons VG AutoSpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. Optical rotations were measured on a Jasco P-1010 polarimeter. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 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. ^{1}H, ^{13}C, DEPT, gCOSY, gTOCSY, gROESY, gHMQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.

Biological Material. *Microcystis aeruginosa* TAU strains IL-217 and IL-231 were collected from the Einan Reservoir in the Hula Valley, Israel, in June 1998 and June 1999, respectively. The two strains are currently maintained in the culture collection at Tel Aviv University. The cells were freeze-dried and kept at −20 °C until extracted.

Isolation Procedure. The freeze-dried cells (99 g) were extracted with 7:3 MeOH−H_2O. The crude extract (16.8 g) was evaporarated and separated on an ODS (YMC-GEL, 120A, 4.4

Table 3. NMR Data of the Major Rotamer of Aeruginosin EI461 (**3**)[a] in DMSO-d_6

position		δ_C, mult[b]	δ_H, mult, J (Hz)	LR H−C corr[c]	NOE Corr[d]
Choi amide	1	173.9 s		Choi-NH(7.60)	
	2	59.3 d	4.71 d 9.3	Choi-NH(7.10)	Choi-3′,7a,NH, Leu-2
	3	33.3 t	2.28 m; 1.69 dd 12.8,5.7	Choi-2	Choi-3′; Choi-2,3,3a
	3a	32.6 d	2.18 m		Choi-3′,7a,NH
	4	22.7 t	1.63 m (2H)	Choi-3,3a	Choi-6,7a
	5	29.9 t	1.54 m (*eq*[e]); 1.21 brdq 4.2,12.8		Choi-6; Choi-7*ax*
	6	67.0 d	3.25 brt 11.2	Choi-5*ax*,7*ax*[e]	Choi-4*ax*,5*eq*,6-OH, 7*eq*,7a
	7	36.2 t	2.31 brdd 11.7,6.7; 0.80 q 11.7		Choi-6,7*ax*,7a; Choi-5*ax*,7*eq*
	7a	56.5 d	3.93 ddd 11.4,6.5, 5.7	Choi-3′,7*ax*	Choi-2,3a,4*ax*,6,7*e*, Leu-3′
	6-OH		4.51m		Choi-6
	NH_2		7.60 brs 7.10 brs		Choi-2,3′,3a
Leu	1	170.5 s			
	2	48.0 d	4.14 ddd 11.7,8.5, 3.0		Choi-2, Leu-3,3′,5,6
	3	39.9 t	1.45 m; 1.22 m	Leu-5,6	Leu-2; Choi-7a, Leu-2
	4	24.1 d	1.27 m		Leu-NH
	5	23.7 q	0.76 d 6.4	Leu-6	Leu-2
	6	21.3 q	0.70 d 6.2	Leu-5	Leu-2
	NH		7.36 d 8.4		Leu-2,3,3′,4, Hpla-2,3, 3′,2-OH
Hpla	1	172.9 s		Leu-NH,	
	2	72.1 d	4.02 m	Hpla-3′	Hpla-3,3′,5,5′,2-OH
	3	39.5 t	2.80 ddd 13.9,4.9, 3.8	Hpla-5,5′	Hpla-2,5,5′,2-OH
			2.60 ddd 13.9,7.4, 6.0		Hpla-2,5,5′,2-OH
	4	128.2 s		Hpla-2,3,3′,6,6′	
	5,5′	130.5 d	6.95 d 8.4	Hpla-3,3′,5′,6,6′	Hpla-2,3,3′,6,6′
	6,6′	114.8 d	6.61 d 8.4	Hpla-5,5′,6′	Hpla-5,5′,7-OH
	7	155.9 s		Hpla-5,5′,6,6′	
	2-OH		5.49 brd 5.8		Hpla-2,3,3′
	7-OH		9.09 brs		Hpla-6,6′

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment from HMQC and HSQC experiments. [c] Determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s. [d] Selected correlations from ROESY experiment, spin lock delay of 200 ms. [e] Axial proton, *ax*; equatorial proton, *eq*.

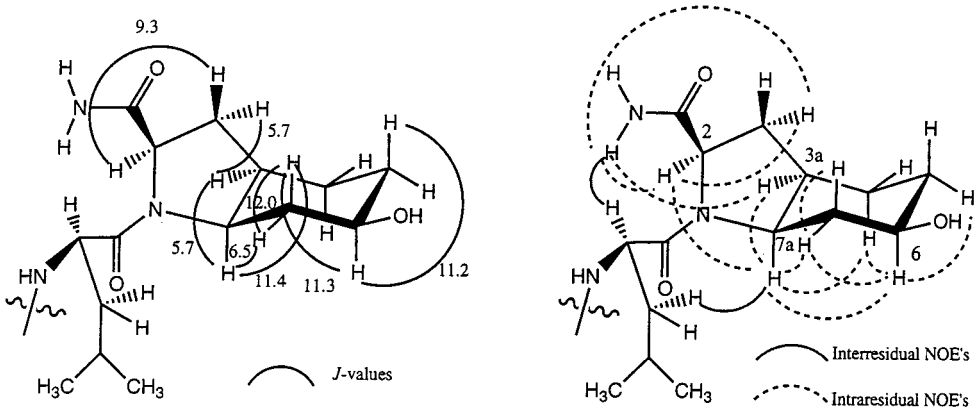

Figure 1. Selected *J*-values and NOE correlations of the major, *cis*, rotamer of aeruginosin EI461 (**3**).

× 6.4 cm) flash column with increasing amounts of MeOH in water. Fractions 3−8 (2:8, 3:7, 4:6, 1:1, 6:4, and 7:3 MeOH−H_2O) were combined, and the combined fraction was separated on a Sephadex LH-20 gel-filtration column (3.6 × 30 cm) eluted with 1:1 $CHCl_3$−MeOH (20 mL each fraction). The combined fractions (2−7), from the Sephadex LH-20 column, were subjected to a reversed-phase HPLC (YMC ODS-A, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 6:4 MeOH−water to obtain semipure **2** in fraction 4 (retention time of 33.1 min) and pure **1** (100 mg, 0.1% yield based on the dry weight of the bacteria) in fraction 5 (retention time of 36.0 min). In another experiment, compound **1** was subjected to a reversed-phase HPLC (YMC ODS-A, 5 μm, DAD at 238 nm, 1:1 acetonitrile−0.1% TFA in water, flow rate 5.0 mL/min). Compound **1** (16.4 mg) was isolated from the column as TFA salt (retention time 26.0 min). Fraction 4 was subjected to reversed-phase HPLC (YMC ODS-A, 5 μm, DAD at 238 nm, 3:7 acetonitrile−water, flow rate 5.0 mL/min). Compound **2** (6.5 mg), 0.0067% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 34.0 min. Fraction 8 from the Sephadex LH-20 column was subjected to reversed-phase HPLC (YMC ODS-A, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 6:4 MeOH−water to obtain in fraction 9 (retention time 29 min) a compound that seemed pure in the chromatogram but a mixture of two related compounds in the NMR spectrum. Further purification of the later fraction on a semipreparative HPLC column (YMC ODS-A, 5 μm, 250 mm × 10.0 mm, DAD at 238 nm, flow rate 3.0 mL/min) afforded two pure peaks (retention times 7.0 and 10.5 min) that presented proton NMR spectra identical to the NMR spectrum of the parent fraction. The compound was proven to be a mixture of two rotamers by temperature-dependent proton NMR measurements. The two fractions were combined to give compound **3** (3.5 mg, 0.0035% yield based on the dry cell weight).

Micropeptin EI992 (1): colorless oil; $[\alpha]^{25}_D$ −12° (*c* 0.05, MeOH); UV (MeOH) λ_{max} (ϵ) 224 (16500), 280 (5000) nm; for ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 993.5033 $[MH]^+$ (calcd for $C_{48}H_{69}N_{10}O_{13}$, 993.5045).

Micropeptin EI964 (2): colorless oil; $[\alpha]^{25}_D$ −13° (*c* 0.003, MeOH); UV (MeOH) λ_{max} (ϵ) 224 (17200), 278 (5000) nm; for ^{1}H and ^{13}C NMR data, see Table 2; HRFABMS *m/z* 965.4740 $[MH]^+$ (calcd for $C_{46}H_{65}N_{10}O_{13}$, 965.4732).

Aeruginosin EI461 (3): colorless oil; $[\alpha]^{25}_D$ +5° (*c* 0.006, MeOH); UV (MeOH) λ_{max} (ϵ) 220 (10200), 278 (4200) nm; for ^{1}H and ^{13}C NMR data of the major rotamer, see Table 3; minor

isomer: ^{1}H NMR (500 MHz, DMSO-d_6) δ Choi: 6.97 and 6.95 (each 1H, brs NH_2), 4.10 (1H, d, J = 9.3 Hz, 2-H), 2.17 (2H m, 3-H_2), 2.41 (1H, brdt, J = 11.8, 6.0 Hz, 3a-H), 1.63 (2H, m, 4-H_2), 1.62 and 1.24 (each 1H, m, 5-H_2), 3.34 (1H, brt, J = 12.0, 13.2 Hz, 6-H), 2.00 and 1.18 (each 1H, m, 7- H_2), 4.20 (1H, ddd, J = 12.0, 6.3, 6.0 Hz, 7a-H), Leu: 4.51 (1H, ddd, J = 11.7, 8.7. 3.8 Hz, 2-H), 1.56 and 1.25 (each 1H, m, 3-H_2), 1.43 (1H, m, 4-H), 0.81 (3H, d, J = 6.4 Hz, 5-H_3), 0.85 (3H, d, J = 6.2 Hz, 6-H_3), 7.53 (1H, d, J = 8.4 Hz, 2-NH), Hpla: 4.02 (1H, m, 2-H), 5.74 (1H, brs, 2-OH), 2.60 (1H, dd, J = 13.9, 7.4 Hz, 3-H), 2.80 (1H, dd, J = 13.9, 4.9 Hz, 3-H′), 6.98 (2H, d, J = 8.4 Hz, 5,5′-H), 6.62 (2H, d, J = 8.4 Hz, 6,6′-H); ^{13}C NMR (125 MHz, DMSO-d_6) δ Choi: 173.7 (s, C-1), 59.3 (d, C-2), 33.8 (t, C-3), 34.7 (d, C-3a), 22.7 (t, C-4), 29.9 (t, C-5), 67.0 (d, C-6), 39.1 (t, C-7), 56.5 (d, C-7a), Leu: 170.5 (s, C-1), 48.0 (d, C-2), 41.1 (t, C-3), 23.6 (d, C-4), 23.6 (d, C-5), 21.6 (d, C-6), Hpla: 173.5 (s, C-1), 72.1 (d, C-2), 39.5 (t, C-3), 128.2 (s, C-4), 130.6 (d × 2, C-5,5′), 114.8 (d × 2, C-6,6′), 155.9 (s, C-7); HRFABMS m/z 484.2432 $[MNa]^+$ (calcd for $C_{24}H_{35}N_3O_6Na$, 484.2423).

Determination of the Absolute Configuration of the Amino Acids. Portions of compounds **1**−**3** (0.5 mg) were dissolved in 6 N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 104 °C for 20 h. In another experiment, 0.25 mg portions of compounds **1** and **2** were first oxidized with Jones reagent (1 drop) in acetone (1 mL) at 0 °C for 10 min. Following the usual workup, the residue was dissolved in 6 N HCl (1 mL) and placed in a sealed glass bomb at 104 °C for 18 h. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (40 mL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA). The *N*-[(-dinitrophenyl)-5-L-alanine amide]-amino acid (AA) derivatives, from hydrolysates, were compared with similarly derivatized standard amino acids by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10 μm, 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)−acetonitrile to 1:1 TEAP−acetonitrile within 60 min. A 90 min linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)−acetonitrile to 1:1 TEAP−acetonitrile was used to differentiate between the Choi-isomers. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids. Retention times of the derivatized amino acids were L-Arg, 29.6 min; D-Arg, 30.3 min; L-Asp, 35.5 min; D-Asp, 38.2 min; L-Glu, 34.5 min; D-Glu, 40.1 min; L-Ile, 56.1 min; D-Ile, 63.8 min; L-Leu, 52.6 min; D-Leu, 58.9 min; L-Phe, 57.0 min; D-Phe, 62.3 min; l-Thr, 34.3 min; D-Thr, 40.1 min; L-NMe-Tyr, 69.7 min; D-NMe-Tyr, 70.5 min. The *N*-[(dinitrophenyl)-5-L-alanine amide]-6α and -6β isomers of L-Choi eluted from the column with retention times of 55.6 and 53.8 min, respectively. The *N*-[(dinitrophenyl)-5-L-alanine amide]-Choi from **3** eluted from the column with a retention time of 57.1 min. HPLC analysis of derivatized hydrolysates of **1** established L-Arg, L-Asp, L-Ile, L-Phe, L-Thr, L-NMe-Tyr; that of **2** established L-Arg, L-Asp, L-Ile, L-Phe, L-Thr, L-NMe-Tyr; that of **3** established D-Leu. HPLC analysis of the FDAA derivatives of oxidized **1** and **2** hydrolysates established L-Glu for both compounds and thus confirmed the L configuration of the Ahp units in these compounds.

Determination of Absolute Configuration of the Hydroxy Acids. A 0.25 mg portion of **3** was dissolved in 6 N HCl (1 mL), and the reaction mixture was then placed in a sealed glass bomb at 104 °C for 20 h. The ethereal extract of the acid hydrolysate was dried, and the residue was treated with (−)-menthol in toluene with *p*-toluenesulfonic acid under reflux for 36 h. The reaction mixture was washed with $NaHCO_3$ solution and water, dried, and analyzed on a Knauer GmbH Eurospher 100 C18, 10 μm, 4.6 × 300 mm HPLC column using 1:1 water−acetonitrile as eluent at a flow rate of 1 mL/min. The D-isomer showed a retention time of 13.5 min and the L-isomer a retention time of 16.4 min. The Hpla residue, from **3**, was determined as the L-form.

Protease Inhibition Assays. 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 enzyme solution, and 10 mL of 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 compounds **1**−**3**, NMR data of the minor rotamer of aeruginosin EI461 (**3**), comparison of NMR data of aeruginosin EI461 (**3**) and aeruginosin 298-B, and comparison of NMR data of major rotamers of 6α- and 6β-isomers of Choi methyl esters.

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