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Protease Inhibitors from *Microcystis aeruginosa* Bloom Material Collected from the Dalton Reservoir, Israel

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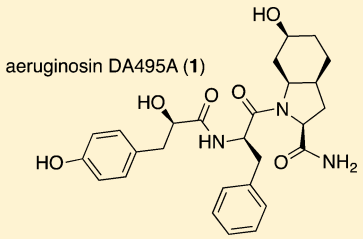
Supporting Information

ABSTRACT: Nine new metabolites, aeruginosins DA495A (**1**), DA511 (**2**), DA642A (**3**), DA642B (**4**), DA688 (**5**), DA722 (**6**), and DA495B (**7**), microguanidine DA368 (**8**), and anabaenopeptin DA850 (**9**), were isolated along with the known micropeptins MZ924, MZ939A, and MZ1019, cyanopeptolins S and SS, microcin SF608, and aeruginazoles DA1497, DA1304, and DA1274 from bloom material of the cyanobacterium *Microcystis aeruginosa* collected from the Dalton reservoir, Israel, in October 2007. Their structures were elucidated by a combination of various spectroscopic techniques, primarily NMR and MS, while the absolute configurations of the asymmetric centers were determined by Marfey's and chiral-phase HPLC methods. Two of the new aeruginosins, DA511 (**1**) and DA495A (**2**), contain a new Choi isomer, (2*S*,3a*S*,6*S*,7a*S*)-Choi. The structure elucidation and biological activities of the new metabolites are described.



During the past three decades, marine, freshwater, and terrestrial cyanobacteria have been documented as prolific producers of biologically active natural products.[1−3] Freshwater bloom-forming genera of cyanobacteria have been extensively studied, mainly due to their competence to biosynthesize toxins, which poses a serious threat to the health of humans and domesticated and wild animals.[4] The microcystins are the most abundant family of hepatotoxic metabolites found in freshwater bloom-forming cyanobacteria.[5] These toxins are usually accompanied by other groups of metabolites, mainly by the following five groups of protease inhibitors: micropeptins (139 isolated variants),[6] anabaenopeptins (38 isolated variants),[7] aeruginosins (38 isolated variants),[8] microginins (32 isolated variants),[9] and microviridins (15 isolated variants).[10] The aeruginosins are a group of linear modified peptides, which attracted the attention of synthetic chemists due to their modified peptidic nature and their capacity to inhibit important proteolytic processes.[11] They are characterized by the presence of the proline-mimicking 2-carboxy-6-hydroxyoctahydroindole (Choi) derivative at the third position, a hydroxyphenyl lactic acid (Hpla) derivative at the N-terminus of the modified peptide, a variable amino acid at the second position, and an arginine derivative (if present) at the fourth position. Analyses of the aeruginosin gene-cluster sequences revealed that these linear modified peptides are biosynthesized in cyanobacteria from amino acid precursors by nonribosmal peptide synthetase (NRPS)-type enzyme assembly.[12] To date, three natural Choi stereoisomers (out of 16 possible) have been characterized in the literature. The most frequent Choi isomer, 2*S*,3a*S*,6*R*,7a*S*-Choi (L-Choi), is contained in 33 known compounds (including those isolated in the present research),[8] 2*S*,3a*R*,6*R*,7a*R*-Choi (L-3a,7a-*diepi*-Choi) in one isolated compound,[13] and 2*R*,3a*R*,6*R*,7a*R*-Choi (D-3a,7a-*diepi*-Choi) in four published compounds.[14] The aeruginosins inhibit trypsin-type serine proteases (i.e., microcin SF608 inhibits trypsin with an IC_{50} value of 0.5 μg/mL[15]). It has been shown that extracts of *Microcystis aeruginosa*, and other toxic bloom-forming cyanobacteria, are more toxic than the pure microcystins themselves, and the enhanced toxicity is attributed to the presence of the different groups of protease inhibitors (i.e., aeruginosins, micropeptins, anabaenopeptins, and microviridins) in the extracts.[16,17] In the past decade attempts have been made to reveal the purpose(s) for the production of the microcystins and the accompanying protease inhibitors in cyanobacteria and to study the relationships between these groups of compounds. Inhibition of trypsin-type proteases and lowering the amounts of active glutathione S-transferase (GST)[18] in the water flea *Daphnia magna*, a cyanobacteria grazer, may imply that the areuginosins are produced in order to prevent the detoxification of the microcystins in the environment.[19] 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 *M. aeruginosa* bloom material collected in October 2007 from the Dalton reservoir in northern Israel.[20] The extract of this bloom material afforded 18 natural products: eight aeruginosins, aeruginosins DA495A (**1**), DA511 (**2**), DA642A (**3**), DA642B (**4**), DA688 (**5**), DA722 (**6**), and DA495B (**7**) and the known microcin SF608,[15] two of which, aeruginosins DA495A (**1**) and DA511 (**2**), contain a new Choi isomer, 2*S*,3a*S*,6*S*,7a*S*-Choi, microguanidine DA368 (**8**), anabaenopeptin DA850 (**9**), aeruginazoles DA1497, DA1304, and DA1274,[21] and the known micropeptins MZ924, MZ939A, and MZ1019[22] and cyanopeptolins S[23] and SS.[24] In this paper,

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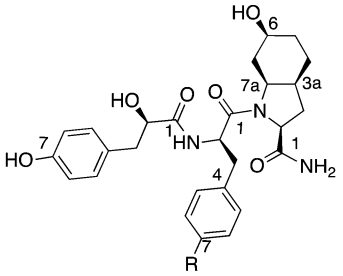
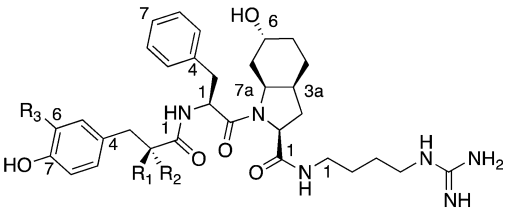
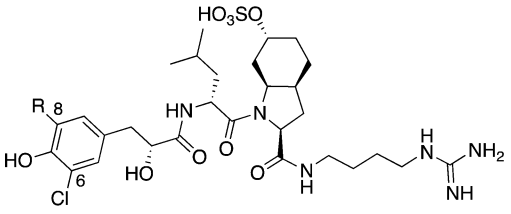
Chart 1



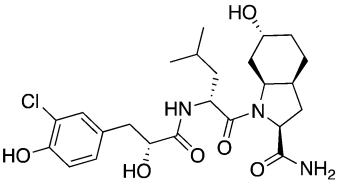
aeruginosin DA495A (**1**) R=H
aeruginosin DA511 (**2**) R=OH



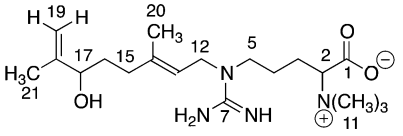
aeruginosin DA642A (**3**) R_1=OH, R_2=H, R_3=Cl
aeruginosin DA642B (**4**) R_1=H, R_2=OH, R_3=Cl
microcin SF608 R_1=OH, R_2=H, R_3=H



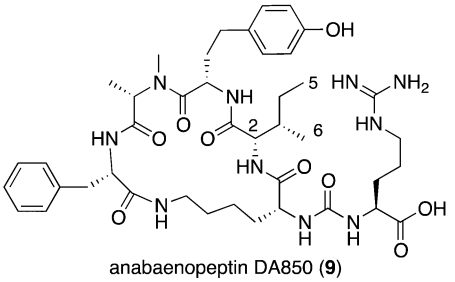
aeruginosin DA688 (**5**) R=H
aeruginosin DA722 (**6**) R=Cl



aeruginosin DA495B (**7**)



microguanidine DA368 (**8**)



anabaenopeptin DA850 (**9**)

we report the isolation, structure elucidation, and biological activities of the nine new metabolites isolated from this bloom material.

RESULTS AND DISCUSSION

Aeruginosins DA495A (**1**) and DA511 (**2**) were eluted closely one after the other from a reversed-phase HPLC column. They were isolated as colorless oils with comparable NMR spectra and molecular weights that differ by 16 mass units. Both appeared as ca. 55:45 mixtures, respectively, of two rotamers characteristic of the aeruginosins.[25] Full assignment of the ^{1}H and ^{13}C NMR data suggested that **1** contained a phenylalanine moiety at the second position of its short peptide backbone, while **2** contained a tyrosine moiety, and the rest of their structures were identical.

Aeruginosin DA495A (**1**) exhibited a positive HRESIMS molecular adduct ion at m/z 518.2266 ([M + Na]$^+$), in agreement with the molecular formula $C_{27}H_{33}N_3NaO_6$ and 13 degrees of unsaturation. The interpretation of the NMR data in DMSO-d_6 was rather complicated due to the high similarity of the chemical shifts of the proton and carbon signals of the above-mentioned rotamers. The aromatic region of the ^{1}H NMR spectrum of **1** (Table 1) presented pairs of phenol signals and three amide protons (two pairs of primary amide singlet signals and a pair of doublet secondary amides), overlapping signals indicating the presence of a phenyl moiety, and doublet signals of two pairs of *para*-substituted phenol moieties. In the mid-spectrum (5.5−3.8 ppm) two pairs of exchangeable doublet signals and four pairs of methine protons next to electronegative atoms were observed. The aliphatic region presented many overlapping signals that were too complex to be interpreted. The ^{13}C NMR spectrum (Table 1) revealed a similar doubling of signals corresponding to three pairs of amide/ester carbonyls, a pair of phenol sp^2 carbons, 13 signals of aromatic sp^2 carbons consistent with a pair of phenol and a pair of phenyl moieties, five pairs of methines in the midrange of the spectrum (73 to 50 ppm), and several additional methine and methylene signals in the aliphatic region. Both rotamers were fully characterized (see Table 1 for the NMR data of the *cis* rotamer and Tables S1a and S1b in the Supporting Information for the full NMR data of the *cis* rotamer and the *trans* rotamer, respectively), but for clarity only the structure elucidation of the major *cis* rotamer is discussed below. The nature of the *cis* and *trans* rotamers of the amide bond between Phe and Choi was distinguished through the NOEs of Phe-H-2 with Choi-H-2 in the *cis* rotamer and of Phe-H-2 with Choi-H-7a in the *trans* rotamer.[25] The assignment of the Phe and Hpla moieties was readily achieved through the interpretation of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (Tables 1, S1a, and S1b). The structure elucidation of the 2-carboxyamide-6-hydroxyoctahydroindole (Choi-amide) moiety was more challenging due to the overlapping signals in the aliphatic region. Analyses of the COSY and TOCSY correlations assembled the sequence of H-2 through

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Table 1. NMR Data of the Major *cis* Rotamer of Aeruginosins DA495A (**1**)[a] and DA511 (**2**)[b] in DMSO-d_6

aeruginosin DA495A (**1**)			aeruginosin DA511 (**2**)		
position	δ_C, mult.[c]	δ_H, mult., *J* (Hz)	position	δ_C, mult.[c]	δ_H, mult., *J* (Hz)
Hpla 1	173.4, C		Hpla 1	173.2, C	
2	72.5, CH	3.85, ddd (8.8, 6.1, 3.8)	2	72.4, CH	3.84, ddd (9.7, 6.0, 3.7)
3	39.9, CH_2	2.60, dd (14.0, 3.6)	3	39.9, CH_2	2.61, m
		2.29, dd (14.0, 8.5)			2.28, dd (14.0, 9.0)
4	128.7, C		4	128.7, C	
5,5′	130.3, CH	6.84, d (8.4)	5,5′	130.2, CH	6.86, d (8.4)
6,6′	115.0, d	6.59, d (8.4)	6,6′	114.9, d	6.61, m
7	155.8, C		7	155.8, C	
2-OH		5.29, d (6.3)	2-OH		5.29, d (6.0)
7-OH		9.07, s	7-OH		9.09, s
Phe 1	170.1, C		Tyr 1	170.1, C	
2	51.7, CH	4.28, dt (2.5, 8.4)	2	51.5, CH	4.19, m
3	36.7, CH_2	2.88, m	3	37.0, CH_2	2.79, m
		2.77, m			2.63, m
4	138.3, C		4	128.7, C	
5,5′	129.6, CH	7.19, m	5,5′	130.3, CH	6.99, m
6,6′	128.2, CH	7.22, m	6,6′	114.9, CH	6.62, m
7	126.4, CH	7.16, m	7	155.7, C	
NH		7.71, d (8.3)	NH		7.64, d (8.0)
			7-OH		9.06, s
Choi 1 amide	174.3, C		Choi 1 amide	174.2, C	
2	59.6, CH	4.78, d (9.0)	2	59.4, CH	4.76, d (8.8)
3	33.3, CH_2	2.32, m	3	33.2, CH_2	2.35, m
		1.77, dd (12.2, 5.7)			1.75, dd (11.6, 5.5)
3a	32.8, CH	2.23, m	3a	32.7, CH	2.25, m
4	22.8, CH_2	1.62, m	4	22.7, CH_2	1.58, m
		1.52, m			1.62, m
5	29.9, CH_2	1.60, m	5	29.8, CH_2	1.56, m
		1.21, m			1.20, m
6	67.1, CH	3.29, m	6	67.0, CH	3.28, m
7	36.3, CH_2	2.34, m	7	35.9, CH_2	2.37, m (eq)
		0.84, q (12.0)			0.83, q (11.8)
7a	56.7, CH	4.04, ddd (12.0, 6.3, 6.3)	7a	56.3, CH	4.01, ddd (11.8, 6.7, 6.1)
6-OH		4.49, d (4.3)	6-OH		4.51, d (4.1)
NH_2(a)		7.73, s	NH_2(a)		7.73, s
NH_2(b)		7.32, s	NH_2(b)		7.31, s

[a]500 MHz for ^{1}H, 125 MHz for ^{13}C. [b]400 MHz for ^{1}H, 100 MHz for ^{13}C. [c]Multiplicities and assignments from HSQC experiment.

H-7a, while the correlations in the HSQC spectrum assigned the carbons directly attached to these protons (Tables 1 and S1a). H-2 was correlated to the adjacent protons resonating at δ_H 2.35 (a 9.0 Hz coupling constant) and 1.77 (<1 Hz) (COSY correlations) and presented NOEs (ROESY experiment) with both of them, suggesting dihedral angles of ca. 15° between H-2 and the *cis* proton resonating at δ_H 2.35 (H-3_{ax}) and of ca. 90° between H-2 and the *trans* proton resonating at δ_H 1.77 (H-3_{eq}). H-3a was coupled to H-3_{eq} with a coupling constant of 5.7 Hz and presented an NOE with H-2, suggesting that H-3a was *cis* oriented to H-2 in the pyrrolidine ring. H-3a exhibited an NOE and a 6.3 Hz coupling constant with H-7a, confirming their *cis* relationships. The *cis* relationships of H-2, H-3a, and H-7a in the pyrrolidine ring were thus confirmed by the mutual coupling constants of the latter and by NOEs.[26] In the cyclohexane ring, H-6 exhibited large coupling constants (12.0 Hz, based on the COSY correlations as H-6 co-resonated with the H_2O signal in DMSO-d_6) with H-5_{ax} and H-7_{ax}, suggesting that it occupied an axial position. In the *trans* rotamer, the NOE between H-7a and Tyr-H-2 suggested an *exo* orientation of Tyr and an envelope conformation of the pyrrolidine ring where the nitrogen was out of the ring plane, thus explaining the multiplicity of H-2 (doublet of 9.0 Hz). A comparison of the NMR data of Choi in **1** with those of microcin SF608 (containing L-Choi)[15] and aeruginosin KT608A (containing D-3a,7a-*diepi*-Choi)[14] revealed the similarity of the Choi moiety in **1** with those of the D-3a,7a-*diepi*-Choi moiety of aeruginosin KT608A. This suggested that the relative configuration of the Choi moiety of **1** was consistent with either 2*S*,3a*S*,6*S*,7a*S*-Choi or 2*R*,3a*R*,6*R*,7a*R*-Choi (L-6-*epi*-Choi or D-3a,7a-*diepi*-Choi, respectively). The sequence of the subunits that compose **1**, Hpla-Phe-Choi-amide, was based on the HMBC correlation of Hpla-CO with Phe-NH, the NOE correlation of Phe-H-2 and Choi-H-2, and the HMBC correlations of Choi-CO with the primary amide protons. These arguments established the relative configuration of the Choi moiety and the planar structure of **1**. The application of Marfey's method[27] (L-FDAA) and chiral-phase HPLC to determine the absolute configurations of the amino acids and the hydroxy acid, respectively, revealed the presence of D-Phe and D-Hpla moieties in **1**. The retention time of Choi-L-FDAA (35.1 min) from **1** was identical with that of the synthetic L-6-*epi*-Choi-L-FDAA (35.1

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Table 2. NMR Data of the Major *trans* Rotamer of Aeruginosins DA642A (3) and DA642B (4) in DMSO-d_6[a]

	aeruginosin DA642A (3)			aeruginosin DA642B (4)	
position	δ_C, mult.[b]	δ_H, mult., J (Hz)	HMBC correlations[c]	δ_C, mult.[b]	δ_H, mult., J (Hz)
Cl-Hpla 1	172.6, C			172.9, C	
2	71.9, CH	3.94, ddd (8.2, 6.0, 3.4)		71.8, CH	3.95, ddd (8.4, 6.3, 3.6)
3	39.0, CH_2	2.69, dd (14.1, 3.4)	Cl-Hpla-4	39.1, CH_2	2.67, dd (13.9, 3.6)
		2.45, dd (14.1, 8.2)	Cl-Hpla-2,4		2.41, dd (13.9, 8.4)
4	130.0, C			130.2, C	
5	130.7, CH	7.09, d (1.6)	Cl-Hpla-3,6,7,9	130.6, CH	7.08, d (1.8)
6	119.1, C			119.1, C	
7	151.5, C			151.4, C	
8	116.3 CH	6.81, d (7.9)	Cl-Hpla-4,6	116.2 CH	6.78, d (8.4)
9	129.2 CH	6.86, dd (7.9, 1.6)	Cl-Hpla-5,7	129.0 CH	6.83, dd (8.8, 1.8)
2-OH		5.54, d (6.0)	Cl-Hpla-1		5.32, d (6.3)
7-OH		9.88, s	Cl-Hpla-6,7		9.81, s
Phe 1	169.6, C			169.8, C	
2	50.6, CH	4.69, ddd (9.9, 8.2, 5.3)	Phe-1	51.1, CH	4.66, ddd (8.8, 7.7, 4.1)
3	38.4, CH_2	2.86, dd (14.5, 5.3)	Phe-1,2,4	38.4, CH_2	2.91, dd (14.0, 4.1)
		2.78, m	Phe-1,2,4		2.79, dd (14.0, 8.8)
4	137.0, C			137.4, C	
5,5′	129.6, CH	7.12, m	Phe-3,7	129.5, CH	7.22, m
6,6′	128.2, CH	7.23, m		128.3, CH	7.23, m
7	126.6, CH	7.19, m	Phe-5	126.6, CH	7.19, m
NH		7.58, d (8.2)	Cl-Hpla-1		7.79, d (7.7)
Choi 1	171.4, C			171.4, C	
2	59.9, CH	4.22, dd (9.2, 8.4)	Phe-1, Choi-1,3	59.9, CH	4.24, dd (9.4, 8.3)
3	30.5, CH_2	1.95, ddd		30.6, CH_2	1.95, m
		(12.4, 8.4, 6.8)	Choi-2,		1.83, m
		1.83, dt (9.2, 12.4)			
3a	36.6, CH	2.20, dtt (12.4, 6.8, 6.1)		36.6, CH	2.21, m
4	19.2, CH_2	2.01, m		19.2, CH_2	2.02, m
		1.39, m	Choi-3a,5,6,7a		1.41, m
5	26.2, CH_2	1.42, m (2H)		26.1, CH_2	1.36, m (2H)
6	64.1, CH	3.86, brs		64.1, CH	3.87, brs
7	34.3, CH_2	1.67, m (2H)	Choi-3a,5,7a	34.3, CH_2	1.68, m (2H)
7a	54.7, CH	4.40, dt (9.0, 6.8)	Choi-2,3,3a	54.6, CH	4.44, m
6-OH		4.54, d (2.5)	Choi-5,6		4.51, d (2.3)
Agm 1	38.1, CH_2	3.06, m	Choi-1,2	38.1, CH_2	3.07, m
2	25.9, CH_2	1.41, m		25.9, CH_2	1.41, m
3	26.5, CH_2	1.43, m	Agm-2	26.5, CH_2	1.43, m
4	40.5, CH_2	3.09, m	Agm-3,5	40.5, CH_2	3.09, m
5	156.8, C			156.8, C	
1-NH		7.84, t (5.7)	Choi-1, Agm-1		7.80, m
4-NH		7.48, t (5.5)			7.47, t (5.3)

[a]400 MHz for ^{1}H, 100 MHz for ^{13}C. [b]Multiplicities and assignments from HSQC experiment. [c]HMBC correlations, optimized for 8 Hz, are from the proton(s) stated to the indicated carbon.

min) and was different from the L-Choi-L-FDAA (36.6 min, synthetic) and the D-3a,7a-*diepi*-Choi-L-FDAA (31.0 min, obtained from hydrolysis of aeruginosin KT608B[14]) standards used for comparison. This procedure established this moiety as a new fourth natural Choi isomer, 2*S*,3a*S*,6*S*,7a*S*-Choi (L-6-*epi*-Choi) and the structure of aeruginosin DA495A (**1**) as a D-Hpla-D-Phe-L-6-*epi*-Choi-amide.

Aeruginosin DA511 (**2**) presented comparable ^{1}H and ^{13}C NMR data (Tables 1, S2a, and S2b) to those of **1** and exhibited a positive HRESIMS molecular adduct ion at m/z 534.2212 ([M + Na]$^+$) consistent with the molecular formula $C_{27}H_{33}N_3NaO_7$ and 13 degrees of unsaturation. Analyses of the 2D NMR data and the absolute configuration of the asymmetric centers, as discussed above for **1**, established the structure of **2** as D-Hpla-D-Tyr-L-6-*epi*-Choi-amide.

Aeruginosins DA642A (**3**) and DA642B (**4**) were eluted from the reversed-phase HPLC column closely one after the other, presenting similar NMR properties (Table 2 and Figures S3a and S4a) and identical molecular weights. Their specific rotations had the same sign but differed in magnitude, −63 for **3** and −36 for **4**. The structures of **3** and **4** were closely related to that of microcin SF608, which was isolated along with those from the aqueous MeOH extract. They appeared as ca. 1:3 mixtures of two rotamers of the amide bond between Phe and Choi (29:71 ratio in **3** and 27:73 ratio in **4**).[25] The full assignment of the ^{1}H and ^{13}C NMR data suggested that they present opposite configurations of their Hpla moiety.[14]

Aeruginosin DA642A (**3**) exhibited a positive HRESIMS quasi-molecular ion at m/z 643.3016/645.3014 (3:1, [M + H]$^+$), which was consistent with the molecular formula

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Table 3. NMR Data of the Major Rotamer of Aeruginosins DA688 (5), DA722 (6), and DA495B (7) in DMSO-d_6[a]

	aeruginosin DA688 (5)		aeruginosin DA722 (6)		aeruginosin DA495B (7)	
position	δ_C, mult.[b]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)
Cl-Hpla 1	172.5, C		172.3, C		172.5, C	
2	71.6, CH	4.08, m	71.3, CH	4.14, m	71.8, CH	4.06, m
3	38.7, CH_2	2.83, dd (13.9, 3.7)	38.6, CH_2	2.85, dd (13.7, 2.7)	38.8, CH_2	2.80, dd (13.9, 3.8)
		2.67, dd (13.9, 7.4)		2.69, dd (13.7, 7.0)		2.67, dd (13.9, 6.6)
4	129.7, C		130.9, C		129.8, C	
5	130.7, CH	7.16, brd (1.4)	129.8, CH	7.17, s	130.9, CH	7.12, brs
6	118.9, C		121.7, C		118.9, C	
7	151.4, C		147.5, C		151.6, C	
8	116.1 CH	6.84, d (8.0)	121.7, C		116.2 CH	6.83, d (8.3)
9	129.2 CH	6.95, dd (8.0, 1.4)	129.8, CH	7.17, s	129.4 CH	6.93, brd (8.3)
2-OH		5.76, d (5.2)		5.83, d (5.5)		5.86, d (5.5)
7-OH		9.87, s		9.83, s		9.87, s
Leu 1	170.0, C		170.0, C		169.6, C	
2	48.5, CH	4.43, ddd (7.7, 7.2, 3.3)	48.6, CH	4.44, ddd (9.5, 8.0, 2.0)	47.9, CH	4.52, ddd (9.1, 8.7, 2.1)
3	41.3, CH_2	1.36, m	41.7, CH_2	1.35, m	42.4, CH_2	1.30, m
		1.24, m		1.22, m		1.20, m
4	24.0, CH	1.47, m	24.2, CH	1.43, m	23.8, CH	1.21, m
5	23.4, CH_3	0.81, d (6.0)	23.6, CH_3	0.79, d (5.5)	23.6, CH_3	0.78, d (5.8)
6	21.6, CH_3	0.85, d (5.6)	21.7 CH_3	0.83, d (5.5)	21.6, CH_3	0.85, d (5.8)
NH		7.51, d (7.2)		7.51, d (8.0)		7.37, d (8.7)
Choi 1 sulfate	171.5, C		171.7, C		173.5, C	
2	59.9, CH	4.17, dd (9.0, 8.4)	60.0, CH	4.16, t (9.1)	59.7, CH	4.12, dd (9.3, 8.2)
3	30.5, CH_2	2.02, m	30.6, CH_2	2.00, m	30.6, CH_2	1.97, m
		1.80, m		1.77, m		1.79, m
3a	35.9, CH	2.29, m	36.1, CH	2.30, m	36.3, CH	2.23, m
4	19.4, CH_2	1.95, m	19.5, CH_2	1.97, m	19.2, CH_2	2.02, m
		1.43, m		1.42, m		1.39, m
5	23.4, CH_2	1.84, m	23.6, CH_2	1.82, m	26.1, CH_2	1.40, m
		1.31, m		1.32, m		
6	70.7, CH	4.37, brs	70.9, CH	4.35, brs	64.0, CH	3.91, brs
7	31.6, CH_2	2.32, m	31.8, CH_2	2.26, m	33.7, CH_2	1.96, m
		1.61, brt (12.0)		1.60, brt (12.0)		1.65, brt (12.0)
7a	54.1, CH	3.98, ddd (11.1, 5.7, 5.4)	54.3, CH	3.96, ddd (11.0, 6.1, 5.8)	54.1, CH	4.00, ddd (11.2, 6.5, 5.9)
Agm 1	37.9, CH_2	3.04, m	38.0, CH_2	3.03, m		4.48, d (2.8)[c]
2	25.8, CH_2	1.40, m	26.0, CH_2	1.40, m		7.23, s[d]
3	25.9, CH_2	1.43, m	26.4, CH_2	1.41, m		6.83, s[d]
4	40.2, CH_2	3.09, m	40.5, CH_2	3.08, m		
5	156.6, C		156.8, C			
1-NH		7.71, t (5.2)		7.71, brt (5.5)		
4-NH		7.47, m		7.43, brt (5.5)		

[a]400 MHz for ^1H, 100 MHz for ^{13}C. [b]Multiplicities and assignments from HSQC experiment. [c]6-OH. [d]NH_2.

$C_{32}H_{45}{}^{35}ClN_6O_6$ and 14 degrees of unsaturation. The ^1H NMR spectrum (Table 2) of **3** in DMSO-d_6 was closely related to the spectrum of microcin SF608, which differed from **3** by 34 mass units. The main differences between the ^1H NMR spectra of the two compounds were located in the aromatic region of the spectra where the *para*-substituted phenol spin system in microcin SF608 was substituted by a nonsymmetric 1,2,4-trisubstituted phenyl spin system in **3**. The two rotamers of **3** were fully characterized (see Table 2 for the NMR data of the major *trans* rotamer and Tables S3a and S3b for the *cis* and *trans* rotamers). The trisubstituted phenyl moiety was established, based on the COSY correlations, as an *o,p*-disubstituted phenol, while the HMBC correlations (Table 2) suggested that an alkyl substituent was attached to the ring in the *para* position and a chlorine was attached in the *ortho* position. A comparison of the carbon chemical shifts of the latter moiety with that of the *o*-Cl-Hpla in aeruginosin KY608[28] confirmed this suggestion. HMBC correlations of C-4 with H-3 and H-3′, COSY correlations of H-2 with H-3, H-3′, and 2-OH, and an HMBC correlation of 2-OH with the carbonyl carbon resonating at δ_C 172.6 established this moiety as an *o*-Cl-Hpla. The application of Marfey's method[27] (L-FDAA) and the chiral-phase HPLC, as described above for **1**, revealed the presence of L-Choi, L-Phe, and L-*o*-Cl-Hpla moieties in **3**. On the basis of the evidence discussed above and the close similarity to microcin SF608 the structure of aeruginosin DA642A (**3**) was established as L-*o*-Cl-Hpla-L-Phe-L-Choi-agmatine.

Aeruginosin DA642B (**4**) exhibited analogous NMR data (Tables 2, S4a, and S4b) and the same molecular formula when compared with the data of **3**. Analyses of the data from the 2D NMR experiments revealed that **4** was assembled from the same four planar subunits, *o*-Cl-Hpla, Phe, Choi, and agmatine, in the same linear order as **3**. Some minor differences were observed in the chemical shifts of the Hpla-2-OH, Phe-C-2, and

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Phe-NH signals (Table 2 and Figures S3b and S4b) when the ^{1}H and ^{13}C NMR data of **3** and **4** were compared. These results were similar to those previously found for aeruginosins KT608A and KT608B,[14] suggesting that either Phe or Hpla differed in their absolute configurations in **3** and **4**. As discussed above for **1**, NMR analysis and applying Marfey's method[27] (L-FDAA) and chiral-phase HPLC revealed that the L-Phe and L-Choi were identical with those of **3**, while the *o*-Cl-Hpla moiety was of the D-series in **4**. This procedure established the structure of aeruginosin DA642B (**4**) as D-*o*-Cl-Hpla-L-Phe-L-Choi-agmatine.

Aeruginosin DA688 (**5**) was obtained as a pale yellow, glassy material, which presented a positive HRESIMS molecular adduct ion at *m/z* 711.2540/713.2585 (3:1, $[M + Na]^+$), corresponding to the molecular formula $C_{29}H_{45}ClN_6NaO_9S$ and 12 degrees of unsaturation. The ^{1}H NMR spectrum (Table 3) in DMSO-d_6 suggested that **5** existed as a ca. 1:2 mixture of the *cis* and *trans* rotamers of the amide bond between the Choi moiety and the adjacent leucine moiety. The two rotamers were fully characterized (Table 3 for the NMR data of the major *trans* rotamer and Tables S5a and S5b for the full data of both rotamers). The structure elucidation of the major *trans* rotamer is discussed below. Analyses of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments assigned the structures of the agmatine, Leu, and *o*-Cl-Hpla (similar to those of **3** and **4**) moieties (Table S5a). The double doublet proton resonating at δ_H 4.17 and the broad singlet at δ_H 4.37 were characteristic of a Choi-6-sulfate moiety.[29] COSY correlations established all of the expected connectivities between the protons of the Choi moiety, except those of H_2-4 and H-3a. A comparison of the chemical shifts and multiplicities of the Choi moiety of **5** with those of aeruginosin 98-A[29] confirmed the suggested Choi-6-sulfate structure. The sequence of the subunits of **5** was determined as *o*-Cl-Hpla-Leu-Choi-6-sulfate-agmatine on the basis of the HMBC correlation of *o*-Cl-Hpla-CO with Leu-NH, the NOE correlation of Leu-H-2 and Choi-H-7a, and the HMBC correlation of Choi-CO with agmatine-1-NH. The application of Marfey's method[27] (L-FDAA) and chiral-phase HPLC revealed the presence of L-Choi, D-Leu, and D-*o*-Cl-Hpla moieties in **5**. On the basis of the evidence discussed above, the structure of aeruginosin DA688 (**5**) was established as D-*o*-Cl-Hpla-D-Leu-L-Choi-6-sulfate-agmatine.

Aeruginosin DA722 (**6**) eluted from the HPLC column right after **5** as a pale yellow, glassy material. It presented a negative HRESIMS molecular ion at *m/z* 721.2203/723.2231/725.2165 (9:6:1, $[M - H]^-$), which was characteristic of two chlorine atoms and corresponded to a molecular formula of $C_{29}H_{43}Cl_2N_6O_9S$. The ^{1}H NMR spectrum (Table 3) in DMSO-d_6 suggested that **6** existed as a ca. 1:2 mixture of the *cis* and *trans* rotamers and was similar to that of **5**. The major difference between the ^{1}H NMR spectra of **5** and **6** was noticed in the aromatic region, where the three pairs of protons of the *o*-Cl-Hpla in **5** were substituted by a pair of two-proton singlet signals in **6**, suggesting that the phenolic ring is symmetrically substituted by two chlorine atoms. The rest of the molecule was similar to that of **5**. The application of Marfey's method[27] (L-FDAA) and chiral-phase HPLC revealed the presence of L-Choi, D-Leu, and D-*o*,*o*-diCl-Hpla moieties in **6**. On the basis of the evidence discussed above, the structure of aeruginosin DA722 (**6**) was established as D-*o*,*o*-di-Cl-Hpla-D-Leu-L-Choi-6-sulfate-agmatine.

Aeruginosin DA495B (**7**) was isolated as a glassy material, which presented a positive HRESIMS molecular adduct ion at *m/z* 518.2031/520.2043 (3:1, $[M + Na]^+$), corresponding to the molecular formula $C_{24}H_{34}ClN_3NaO_6$ and nine degrees of unsaturation. The ^{1}H NMR spectrum (Table 3) in DMSO-d_6 revealed that **7** existed as a ca. 1:3 mixture of the *cis* and *trans* rotamers of the amide bond between the Choi moiety and the adjacent leucine moiety. The ^{13}C NMR spectrum (Table 3) of **7** was comparable to that of **5** except for the missing guanidine carbon, the shift of a methine carbon from 70.7 (in **5**) to 64.0 (in **7**) ppm, which indicated a Choi-6-OH moiety, and the abolition of four methylene carbons from the aliphatic region (Figure S7b). The two rotamers were fully characterized (Table 3 for the NMR data of the major *trans* rotamer and Tables S7a and S7b for the full data of both rotamers). The structure elucidation of the major, *trans*, rotamer is discussed below. Analyses of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments assigned the structures of *o*-Cl-Hpla (similar to that of **5**), Leu, and Choi-amide moieties (Table S7a). The sequence of the subunits was determined as *o*-Cl-Hpla-Leu-Choi-amide on the basis of NOE correlations of *o*-Cl-Hpla-H-2 with Leu-NH, of Leu-H-2 with Choi-H-7a, and of Choi-H-2 with the primary amide protons and the HMBC correlation *o*-Cl-Hpla-CO with the Leu-NH. Applying Marfey's method[27] (L-FDAA) and chiral-phase HPLC revealed the presence of L-Choi, D-Leu, and D-*o*-Cl-Hpla moieties in **7**. On the basis of the above evidence the structure of aeruginosin DA495B (**7**) was established as D-*o*-Cl-Hpla-D-Leu-L-Choi-amide.

Microguanidine DA368 (**8**) was isolated as a transparent, glassy material that exhibited an HRESIMS molecular cluster ion at *m/z* 369.2860 ($[M + H]^+$), which matched the molecular formula $C_{19}H_{37}N_4O_3$ and four degrees of unsaturation. The ^{1}H NMR spectrum of **8** in DMSO-d_6 (Table 4) was relatively broad, displaying a broad signal of three exchangeable protons (based on the HSQC spectrum) in the aromatic region, three vinyl protons and one exchangeable proton around 5 ppm, six protons adjacent to electronegative substituents [δ_H 3.96 brd (2H), 3.85 brs, 3.64 brd, 3.42 and 3.02 m], a nine-proton singlet (δ_H 3.10), and two broad singlet methyl groups (δ_H 1.71 and 1.65), reminiscent of the characteristic protons of microguanidine AL772, which was isolated and published by us several years ago.[30] The ^{13}C NMR spectrum (Table 4) revealed, among other signals, a carbonyl, guanidinium carbon, two carbons adjacent to electronegative substituents, two methylenes next to nitrogen, and a trimethylammonium residue (δ_C 50.9, CH_3). An interpretation of the COSY, TOCSY, HSQC, and HMBC data (Table 4) allowed for the full assignment of the structure of the oxidized geranyl moiety and of the *Nα*,*Nα*,*Nα*-trimethyl-*Nε*-substituted arginine, which were assembled to the complete structure through HMBC correlations of H-5 with C-12 and of H-5 and H_2-12 with C-7 (the guanidine carbon). This established the planar structure of amphiphilic microguanidine DA368 as **8**. The absolute configurations of C-2 and C-17 were not determined due to the small amount of material that was isolated.

Anabaenopeptin DA850 (**9**) presented an HRESIMS molecular cluster ion at *m/z* 851.4774 ($[M + H]^+$) and NMR spectra in DMSO-d_6 similar to that of anabaenopeptin F.[31,32] A complete structure elucidation of **9**, based on the interpretation of the COSY, TOCSY, HSQC, and HMBC data (Table S8), revealed that indeed its planar structure is identical with that of anabaenopeptin F, *cyclo*-(Phe-*N*MeAla-Hty-Ile-Lys-

Table 4. NMR Data of Microguanidine DA369 (8) in DMSO-d_6[a]

position	δ_C, mult.[b]	δ_H, mult., J (Hz)	HMBC correlations[c]	NOE correlations[d]
1	168.6, C			
2	76.3, CH	3.64, d (11.2)	1,3,11,11′,11″	4,4′11,11′,11″
3	22.4, CH_2	1.81, m		
		1.66, m	5	
4	23.8, CH_2	1.77, m		2,4′,11,11′,11″
4′		1.52, m		2,4
5	46.6, CH_2	3.42, t (10.6)		5′
5′		3.08, m	7	5,12
7	156.0, C			
8,9		7.49, brs		12
11−11″	50.9, CH_3	3.10, s	2,11′,11″	2,4
12	45.6, CH_2	3.91, d (5.3)	5,7,13,14	5′,8,9,13,15,20
13	119.2, CH	5.07, brt (5.3)	12,15,20	12,20
14	140.9, C			
15	27.8, CH_2	2.06, t (7.4)	13,14,16,17,20	12,16,16′,17
16	33.4, CH_2	1.52, m	15,17,18	15,17
		1.45, m	14,15,17,18	15
17	73.5, CH	3.85, brs		15,16,17-OH,19,19′
18	148.5, C			
19	110.1, CH	4.89, s	17,21	17,17-OH,19′,21
19′	$_2$	4.75, s	17,21	17,17-OH,19,21
20	23.3, CH_3	1.71, s	13,14,15	12,13
21	18.1, CH_3	1.65, s	17,18,19	19,19′
17-OH		4.98, brs	16,17	17,19,19′

[a]500 MHz for ^{1}H, 125 MHz for ^{13}C. [b]Multiplicities and assignments from HSQC experiment. [c]HMBC correlations, optimized for 8 Hz, are from the proton(s) stated to the indicated carbon. [d]Selected NOEs from ROESY experiment.

Nε)-*Nα*-CO-Arg-CO_2H. The ^{1}H and ^{13}C NMR spectra of **9** and anabaenopeptin F could be superimposed on one another, except for the signals of their Ile moieties (Tables 5 and S8). Such chemical shift differences were noticed by us in the micropeptins where Ile or *allo*-Ile are situated at the C-terminus of the peptide[33] or at the fourth position.[14] In anabaenopeptin F, the latter amino acid was determined as L-*allo*-Ile.[32] Applying Marfey's method[27] (L-FDAA) to the hydrolysate of **9** established the absolute configuration of the amino acids that build **9** as L-Phe, L-*N*MeAla, L-Hty, L-Ile, D-Lys, and L-Arg, confirming that the difference between the isoleucine moieties in both compounds is in the *β*-position. On the basis of these arguments the structure of anabaenopeptine DA850 (**9**) was established as *cyclo*-(L-Phe-L-*N*MeAla-L-Hty-L-**Ile**-D-Lys-*Nε*)-*Nα*-CO-L-Arg-CO_2H.

Table 5. Comparison of the NMR Data of the Ile Moieties of Anabaenopeptins DA850 (9) and F in DMSO-d_6[a]

	anabaenopeptin DA850 (**9**)		anabaenopeptin F	
position	δ_C, mult.[b]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)
Ile 1	172.6, C		172.7, C	
2	56.3, CH	4.08, dd (7.5, 7.0)	56.6, CH	3.97, dd (8.5, 7.3)
3	36.3, CH	1.77, m	35.7, CH	1.78, m
4	25.0, CH_2	1.16, m	24.7, CH_2	1.14, m
		1.61, m		
5	11.5, CH_3	0.89, t (7.5)	10.3, CH_3	0.82, t (7.3)
6	14.9, CH_3	0.88, d (6.5)	14.9, CH_3	0.88, d (6.4)
NH		6.91, d (7.0)		6.97, d (7.3)

[a]500 MHz for ^{1}H, 125 MHz for ^{13}C. [b]Multiplicities and assignments from HSQC experiment.

Biological Activities. The extracts of strain IL-374 exhibited significant inhibition of the serine protease trypsin at a concentration of 1 mg/mL. The activity-guided purification of the protease-inhibiting components of the extract revealed that the known micropeptins MZ924, MZ939A, MZ1019,[22] cyanopeptolin S,[23] and cyanopeptolin SS[24] and the new aeruginosins DA642A (**3**), DA642B (**4**), DA688 (**5**), and DA722 (**6**) were responsible for the inhibition of trypsin.

The inhibitory activities of **1**−**9** were determined for the serine proteases trypsin and thrombin. Aeruginosin DA642A (**3**) inhibited trypsin with an IC_{50} of 30.8 *μ*M and thrombin with an IC_{50} of 15.5 *μ*M. Aeruginosin DA642B (**4**) inhibited trypsin with an IC_{50} of 19.0 *μ*M, but not thrombin at a concentration of 45.5 *μ*M. Aeruginosins DA688 (**5**) and DA722 (**6**) inhibited trypsin with IC_{50} values of 9.5 and 7.3 *μ*M, respectively, but not thrombin at a concentration of 45.5 *μ*M. Aeruginosins DA495A (**1**) and DA511 (**2**), microguanidine DA368 (**8**), and anabaenopeptin DA850 (**9**) did not inhibit trypsin and thrombin at a concentration of 45.5 *μ*M.

CONCLUDING REMARKS

Aeruginosins **1** and **2** compose a new subgroup of aeruginosins that contain a new stereoisomer of the Choi core. This isomer, 2*S*,3a*S*,6*S*,7a*S*-Choi (L-6-*epi*-Choi), adds to the three known isomers (2*S*,3a*S*,6*R*,7a*S*)-Choi (L-Choi),[8] (2*S*,3a*R*,6*R*,7a*R*)-Choi (L-3a,7a-*diepi*-Choi),[13] and 2*R*,3a*R*,6*R*,7a*R*-Choi (D-3a,7a-*diepi*Choi).[14] A proposed biogenesis (Scheme S1) is based on the one suggested by Ishida et al. for L-Choi biosynthesis in aeruginoside 126.[12] The variety of enzymatic processes demonstrates the versatility of the biosynthetic machinery utilized by cyanobacteria for the synthesis of secondary metabolites. This strategy allows cyanobacteria to produce peptide-mimicking building blocks that interfere with the degradation of these short peptides.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1010 polarimeter. UV spectra were recorded on an Agilent 8453 spectrophotometer. IR spectra were recorded on a Bruker Vector 22 spectrometer. NMR spectra were recorded on a Bruker DMX-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, and gHMBC spectra were recorded using standard Bruker pulse sequences. Mass spectra were recorded on a Waters MaldiSynapt instrument. 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 multi-wavelength detector), on a Merck Hitachi HPLC system (model L-6200A pump and model L-4200 UV−vis detector), and on a Merck Hitachi HPLC system (L-7000A intelligent pump and model L-6200 UV−vis detector). An Elisa EL_x808 reader (Bio-Tek Instruments, Inc.) was used for protease inhibition assays.

Biological Material. *Microcystis aeruginosa*, TAU strain IL-374, was collected in October 2007, from the Dalton water reservoir, Gush Halav, Israel. Samples of the cyanobacteria are deposited at the culture collection of Tel Aviv University.

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Isolation Procedure. The freeze-dried cells (IL-374, 1600 g) were extracted with 7:3 MeOH/H_2O (3 × 3 L). The extract was evaporated to dryness (98.5 g) and separated on an ODS (YMC-GEL, 120A, 4.4 × 6.4 cm, 10 portions of 10 g each) flash column, eluting with increasing amounts of MeOH in H_2O. Fraction 6 (1:1 MeOH/H_2O, 2.645 g) was subjected to a Sephadex LH-20 column eluting with 7:3 MeOH/H_2O to obtain 14 fractions. Fractions 5−7 from the Sephadex LH-20 column were again subjected to a Sephadex LH-20 column eluting with 1:1 MeOH/H_2O to obtain 14 fractions. Fraction 7 from the second Sephadex LH-20 column (30.5 mg) was separated on a reversed-phase HPLC column (Hibar, 5 μm, 250 mm × 25.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) eluting with 77:23 0.1% aqueous TFA/CH_3CN to obtain pure compounds **5** (1.1 mg, retention time 32.0 min, 0.0001% yield based on the dry weight of the bacteria) and **6** (0.9 mg, retention time 40.8 min, 0.0001% yield). Fraction 9 from the second Sephadex LH-20 column (28.4 mg) was separated on a reversed-phase HPLC column (Hibar, 5 μm, 250 mm × 25.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) eluting with 72:28 0.1% aqueous TFA/CH_3CN to obtain pure compounds **1** (1.7 mg, retention time 20.8 min, 0.0001% yield) and **2** (2.0 mg, retention time 22.6 min, 0.0001% yield). Combined fractions 7 and 8 from the initial reversed-phase column (6:4 and 7:3 MeOH/H_2O, 2.929 g) was loaded on a Sephadex LH-20 column eluting with 1:1 $CHCl_3$/MeOH to obtain 14 fractions. Combined fractions 3 and 4 from this Sephadex LH-20 column were subjected to an additional Sephadex LH-20 column eluting with 7:3 MeOH/H_2O to obtain 14 fractions. Fractions 8−11 from the second Sephadex LH-20 column (95.4 mg) were separated on a reversed-phase HPLC column (YMC-Pack ODS-A, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 1:1 0.1% aqueous TFA/MeOH to obtain pure compound **3** (4.0 mg, retention time 17.2 min, 0.0003% yield), compound **4** (3.0 mg, retention time 14.9 min, 0.0002% yield), compound **7** (2.4 mg, retention time 16.5 min, 0.0002% yield), and microcin SF608 (4.5 mg, retention time 21.3 min, 0.0003% yield). Fractions 4−7 from the second Sephadex LH-20 column (379 mg) were separated on a reversed-phase HPLC column (YMC-Pack C-8, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 1:1 0.1% aqueous TFA/MeOH to obtain pure anabaenopeptin DA850 (**9**) (2.1 mg, retention time 23.9 min, 0.0001% yield). Fraction 9 from the initial reversed-phase column (4:1 MeOH/H_2O, 1.842 g) was subjected to a Sephadex LH-20 column eluting with 1:1 $CHCl_3$/MeOH to obtain 13 fractions. Fractions 4−8 from the Sephadex LH-20 column were subjected to an additional Sephadex LH-20 column eluting with 7:3 MeOH/H_2O to obtain 15 fractions. Fractions 3−5 from the second Sephadex LH-20 column (184.8 mg) were separated on a reversed-phase HPLC column (YMC-Pack ODS-A, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 1:1 H_2O/CH_3CN to obtain five fractions, some of which contain aeruginazoles DA1497, DA1304, and DA1274.[21] Fraction 2 from the reversed-phase HPLC was separated again by a reversed-phase HPLC column (YMC-Pack C-8, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 55:45 0.1% aqueous TFA/MeOH to obtain pure compound **8** (2.6 mg, retention time 29.0 min, 0.0002% yield). Fraction 5 from the initial reversed-phase column was subjected to a Sephadex LH-20 column eluting with 1:1 $CHCl_3$/MeOH to obtain 10 fractions. Fractions 4−7 from the second Sephadex LH-20 column (211.5 mg) were separated on a reversed-phase HPLC column (YMC-Pack C-8, 5 μm, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 55:45 0.1% aqueous TFA/MeOH to obtain micropeptin MZ1019 (2.2 mg, retention time 20.7 min, 0.0001% yield), cyanopeptolin SS (1.4 mg, retention time 30.0 min, 0.000 06% yield), micropeptin MZ924 (0.7 mg, retention time 39.7 min, 0.000 03% yield), micropeptin MZ939A (1.8 mg, retention time 42.1 min, 0.000 11% yield), and cyanopeptolin S (1.3 mg, retention time 51.2 min, 0.000 05% yield).

Aeruginosin DA495A (1): colorless oil; $[\alpha]^{25}_D$ −48 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.14), 225 (3.69), 277 (2.94) nm; IR (KBr) ν_{max} 3450, 1680, 1206, 1138 cm^{-1}; NMR data, see Tables 1, S1a and S1b in Supporting Information; HRESIMS *m/z* 518.2266 $[M + Na]^+$ (calcd for $C_{27}H_{33}N_3NaO_6$, 518.2267). Retention time of hydroxy acid on chiral-phase column: D-Hpla 4.24 min (L-Hpla 3.95, D-Hpla 4.24 min). Retention times of AA Marfey's derivatives: D-phenylalanine 46.9 min (L-Phe 44.9, D-Phe 46.9 min), L-*6-epi*-Choi 34.9 min (L-Choi 36.5, L-*6-epi*-Choi 34.9 min, D-3a,7a-*diepi*-Choi 31.0 min).

Aeruginosin DA511 (2): colorless oil; $[\alpha]^{25}_D$ −134 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.06), 225 (3.95), 277 (3.41) nm; IR (KBr) ν_{max} 3460, 1684, 1205, 1141 cm^{-1}; NMR data, see Tables 1, S2a, and S2b; HRESIMS *m/z* 534.2212 $[M + Na]^+$ (calcd for $C_{27}H_{33}N_3NaO_7$, 534.2216). Retention time of hydroxy acid on chiral-phase column: D-Hpla 4.28 min (L-Hpla 3.99, D-Hpla 4.28 min). Retention times of AA Marfey's derivatives: D-tyrosine 52.1 min (L-Tyr 50.4, D-Tyr 52.1 min), L-*6-epi*-Choi 35.1 min (L-Choi 36.6, L-*6-epi*-Choi 35.1 min, D-3a,7a-*diepi*-Choi 31.0 min).

Aeruginosin DA642A (3): colorless oil; $[\alpha]^{25}_D$ −63 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.42), 229 (3.72), 281 (3.18) nm; IR (KBr) ν_{max} 3450, 2935, 1678, 1204, 1138 cm^{-1}; NMR data, see Tables 2, S3a, and S3b; HRESIMS *m/z* 643.3016/645.3014 (3:1), $[M + H]^+$ (calcd for $C_{32}H_{45}{}^{35}ClN_6O_6$, 643.3011). Retention time of hydroxy acid on chiral-phase column: L-Cl-Hpla 3.93 min (L-Cl-Hpla 3.93, D-Cl-Hpla 4.29 min). Retention times of AA Marfey's derivatives: L-phenylalanine 44.6 min (L-Phe 44.6, D-Phe 46.5 min), L-Choi 36.5 min (L-Choi 36.5, L-*6-epi*-Choi 34.9 min).

Aeruginosin DA642B (4): colorless oil; $[\alpha]^{25}_D$ −36 (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.32), 228 (3.63), 281 (3.01) nm; IR (KBr) ν_{max} 3450, 2934, 1678, 1205, 1138 cm^{-1}; NMR data, see Tables 2, S4a, and S4b; HRESIMS *m/z* 643.3014/645.3006 (3:1), $[M + H]^+$ (calcd for $C_{32}H_{45}{}^{35}ClN_6O_6$, 643.3011). Retention time of hydroxy acid on chiral-phase column: D-Cl-Hpla 4.27 min (L-Cl-Hpla 3.93, D-Cl-Hpla 4.27 min). Retention times of AA Marfey's derivatives: L-phenylalanine 44.6 min (L-Phe 44.6, D-Phe 46.5 min), L-Choi 36.5 min (L-Choi 36.5, L-*6-epi*-Choi 35.2 min).

Aeruginosin DA688 (5): pale yellow, glassy material; $[\alpha]^{25}_D$ −160 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.19), 229 (3.78), 279 (3.34) nm; IR (KBr) ν_{max} 2924, 1682, 1205, 1140 cm^{-1}; NMR data, see Tables 3, S5a, and S5b; HRESIMS *m/z* 711.2540/713.2585 (3:1), $[M + Na]^+$ (calcd for $C_{29}H_{45}{}^{35}ClN_6NaO_9S$, 711.2555). Retention time of hydroxy acid on chiral-phase column: D-Cl-Hpla 4.28 min (L-Cl-Hpla 3.81, D-Cl-Hpla 4.28 min). Retention times of AA Marfey's derivatives: D-leucine 47.1 min (L-Leu 44.4, D-Leu 47.1 min), L-Choi 36.6 min (L-Choi 36.6, L-*6-epi*-Choi 35.3 min).

Aeruginosin DA722 (6): pale yellow, glassy material; $[\alpha]^{25}_D$ −135 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.23), 226 (3.68), 285 (3.04) nm; IR (KBr) ν_{max} 2928, 1666, 1205 cm^{-1}; NMR data, see Tables 3 and S6a; HRESIMS *m/z* 721.2203/723.2231/725.2165 (9:6:1), $[M - H]^-$ (calcd for $C_{29}H_{43}{}^{35}Cl_2N_6O_9S$, 721.2189). Retention time of hydroxy acid on chiral-phase column: D-di-Cl-Hpla 4.31 min (L-di-Cl-Hpla 3.81, D-di-Cl-Hpla 4.31 min). Retention times of AA Marfey's derivatives: D-leucine 46.6 min (L-Leu 43.9, D-Leu 46.6 min), L-Choi 36.4 min (L-Choi 36.4, L-*6-epi*-Choi 35.0 min).

Aeruginosin DA495B (7): colorless, glassy material; $[\alpha]^{25}_D$ −44 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.88), 227 (3.20), 280 (2.69) nm; IR (KBr) ν_{max} 1685, 1206, 1140 cm^{-1}; NMR data, see Tables 3, S7a, and S7b; HRESIMS *m/z* 518.2031/520.2043 (3:1), $[M + Na]^+$ (calcd for $C_{24}H_{34}{}^{35}ClN_3NaO_6$, 518.2034). Retention time of hydroxy acid on chiral-phase column: D-Cl-Hpla 4.20 min (L-Cl-Hpla 3.88, D-Cl-Hpla 4.20 min). Retention times of AA Marfey's derivatives: D-leucine 47.1 min (L-Leu 44.4, D-Leu 47.1 min), L-Choi 36.8 min (L-Choi 36.8, L-*6-epi*-Choi 35.6 min).

Microguanidine DA368 (8): transparent, glassy material; $[\alpha]^{25}_D$ −78 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.81), 223 (3.74), 278 (3.05); IR (KBr) ν_{max} 3445, 1647, 1206, 1139 cm^{-1}; NMR data, see Table S8; HRESIMS *m/z* 369.2860 ($[M + H]^+$ (calcd for $C_{19}H_{37}N_4O_3$ *m/z* 369.2871).

Anabaenopeptin DA850 (9): white, glassy material; $[\alpha]^{25}_D$ −145 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.21), 273 (2.63); IR (KBr) ν_{max} 3448, 1664, 1206, 1139 cm^{-1}; NMR data, see Table S9; HRESIMS *m/z* 851.4774 $[M + H]^+$ (calcd for $C_{42}H_{63}N_{10}O_9$, 851.4779). Retention times of AA Marfey's derivatives: L-Phe 45.4 min (L-Phe 45.4, D-Phe 57.5 min), L-***N*Me**-alanine 35.5 min (L-***N*Me**Ala 35.5, D-***N*Me**Ala 36.2 min), L-homotyrosine 40.8 min, L-isoleucine 44.2

min (L-Ile 44.2, D-Ile 47.2 min), D-lysine 46.1 min (L-Lys 45.7, D-Lys 46.1 min), and D-arginine 30.8 min (L-Arg 29.7, D-Arg 30.8 min).

Determination of the Absolute Configurations of the Amino Acids. Compounds **1**−**7** and **9** (0.3 mg portions) 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 the removal of HCl, by repeated evaporation *in vacuo*, the hydrolysate was resuspended in H_2O (250 μL). A solution of (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA) in acetone (1.2 equiv) and 1 N $NaHCO_3$ (120 μL) was added to each reaction vessel, and the reaction mixture was stirred at 70 °C for 3 h. A 2 N HCl solution (60 μL) was added to each reaction vessel, and the solution was evaporated *in vacuo*. The *N*-[(dinitrophenyl)-5-L-alanine amide]-amino acid derivatives, from hydrolysates, were compared with similarly derivatized standard amino acids by HPLC analysis: Knauer GmbH Eurospher 100 C_{18}, 10 μm, 4.6 × 300 mm, flow rate 1 mL/min, UV detection at 340 nm, linear gradient elution from 100%, 0.1% TFA in H_2O to 1:1, 0.1% TFA in H_2O/CH_3CN, within 60 min. The absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids.

Determination of the Absolute Configurations of the Hydroxy Acids. Compounds **1**−**7** (0.25 mg portions) were dissolved in 6 N HCl (1 mL), and the reaction mixture was then placed in a sealed glass bomb at 110 °C for 20 h. The ethereal extract of the acid hydrolysate of **1**−**7** was removed *in vacuo*, and the residue was dissolved in MeOH (1 mL). The MeOH solution was analyzed on an Astec, Chirobiotic, LC stationary phases, 250 × 4.6 mm flow rate 1 mL/min, UV detection at 210 nm, linear elution 1:9 1% triethylamine, 1% acetic acid aqueous buffer, pH 4/MeOH. The Hpla residues from the aeruginosins were compared with standard L,D-Hpla.

Protease Inhibition Assays. Trypsin and thrombin were purchased from Sigma Chemical Co. Trypsin (1 mg/mL) was 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, the trypsin substrate, was dissolved in a solution of 1:9 DMSO/Tris-buffer (0.85 g/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 micro titer plate well and preincubated at 37 °C for 5 min. Then, 100 μL of substrate solution was added, and the kinetics of the reaction were measured at 405 nm, 37 °C for 30 min. Thrombin (0.5 mg/mL) was dissolved in 0.2 M Tris-HCl, pH 8 buffer solution. z-Gly-Pro-Arg-4M*β*NA-acetate salt of the substrate for thrombin was dissolved in water (0.5 mg/mL). The test samples were dissolved in DMSO (1 mg/mL). For thrombin, 170 μL of buffer solution, 10 μL of enzyme solution, and 10 μL of sample solution were added to each micro titer plate well and preincubated at 25 °C for 20 min. Then, 30 μL of substrate solution was added, and the kinetics of the reaction were measured at 405 nm, 25 °C for 20 min. Microcin SF608 (IC_{50} of 0.5 μg/mL)[15] served as a positive control. The percent inhibition of the reaction was calculated from a control reaction containing only the compound vehicle (DMSO). IC_{50} curves were generated by fitting percentage inhibition values to a four-parameter logistic model using a data analysis computer program.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full tabulated NMR data, 1D and 2D NMR spectra, and HRMS data of compounds **1**−**9**, and a biogenetic scheme. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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