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Bromine- and Chlorine-Containing Aeruginosins from *Microcystis aeruginosa* Bloom Material Collected in Kibbutz Geva, Israel

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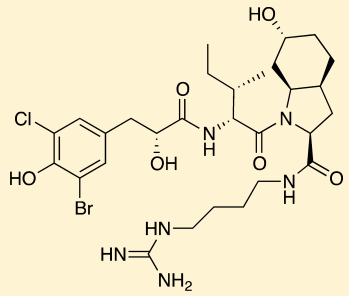
Supporting Information

ABSTRACT: Five new natural products, aeruginosins GE686 (**1**), GE766 (**2**), GE730 (**3**), GE810 (**4**), and GE642 (**5**), were isolated along with four known aeruginosins, 98C, 101, KY642, and DA688, from bloom material of the cyanobacterium *Microcystis aeruginosa* collected from a fish pond in Kibbutz Geva, Israel, in August 2007. Their structures were elucidated by a combination of various spectroscopic techniques, primarily NMR and MS, while the absolute configurations of the stereogenic centers were determined by Marfey's and chiral-phase HPLC methods. Two of the new aeruginosins, aeruginosins GE686 (**1**) and GE766 (**2**), contain the unprecedented D-*m*-Br-*m*′-Cl-*p*-hydroxyphenyllactic acid derivative. The structures and biological activities of the five new metabolites are described.

aeruginosin GE686 (**1**)



The aeruginosins are a group of linear modified peptides characterized by the presence of a hydroxyphenyllactic acid (Hpla) derivative at the *N*-terminus of the modified peptide, a variable lipophilic amino acid at the second position, a 2-carboxy-6-hydroxyoctahydroindole (Choi) derivative at the third position, and an arginine derivative (if any) at the fourth position.[1] They are produced mainly by aquatic bloom-forming cyanobacteria and possess serine-protease inhibitory activity.[2] To date, 27 variants of this group of protease inhibitors have been isolated and characterized from cyanobacteria extracts.[3] A wealth of additional aeruginosins have been identified by tandem mass spectrometry analyses of cyanobacteria bloom materials.[4] Three related metabolites, suomilide[5] and banyasides A and B,[6,7] were isolated from cyanobacteria bloom materials, while five other related metabolites, dysinosins A−D and chlorodysinosin A, were isolated from the marine sponge *Lamellodysidea chlorea*.[8] These linear modified peptides are biosynthesized in cyanobacteria from amino acids or amino acid precursors by a nonribosomal peptide synthetase (NRPS)-type enzyme assembly, which allows the incorporation of different precursors at the *N*-terminus and the second positions, but allows only stereochemical and substituent changes at the third and fourth positions.[9] As part of our continuing interest in the chemical ecology of cyanobacteria water blooms and the search for novel drugs for human diseases, we examined the extracts of a *Microcystis aeruginosa* bloom material collected in August 2007 from a fishpond in Kibbutz Geva, Israel. The 70% aqueous MeOH extract of the lyophilized bloom material yielded five new aeruginosins, aeruginosins GE686 (**1**), GE766 (**2**), GE730 (**3**), GE810 (**4**), and GE642 (**5**), along with four known ones, aeruginosins 98C (**6**),[10] 101 (**7**),[10] KY642 (**8**),[11] and DA688.[12] In this article we report the isolation, structure elucidation, and biological activity of the five new aeruginosins isolated from this bloom material.

The nine natural products were isolated from the 70% aqueous MeOH extract through separation on a reversed-phase open column, size exclusion chromatography on Sephadex LH-20, and repeated chromatography on various reversed-phase HPLC columns.

Aeruginosin GE686 (**1**) was isolated as a yellowish glassy material, which exhibited an HRESIMS complex molecular ion adduct at *m*/*z* 687.2285/689.2256/691.2254 (5:8:3 $[M + H]^+$), characteristic of a molecule that contains both bromine and chlorine atoms and corresponds to the molecular formula $C_{29}H_{44}BrClN_6O_6$. The ^1H and ^{13}C NMR spectra of **1** were characteristic of the aeruginosins and displayed two signals for each proton and carbon, as **1** appeared as a 13:1 mixture of the *trans* and *cis* rotamers.[3] The ^1H NMR spectrum (Table 1) of **1** presented a broad signal of a phenol, three pairs of amide protons (a pair of doublets and two pairs of broad triplets), two pairs of singlet signals in the aromatic region, two pairs of exchangeable doublet signals, four pairs of methine protons next to the electronegative atoms in the 5.5−3.8 ppm region, and two broad signals around 3.05 ppm corresponding to four protons. The aliphatic region was too complicated to be interpreted except for a pair of doublet methyl groups and a pair of distorted broad doublet methyl groups. The ^{13}C NMR spectrum (Table 1) revealed three pairs of amide/ester carbonyls, two pairs of sp^2 carbons characteristic of phenol and/or guanidine carbons, five signals of aromatic sp^2 carbons,

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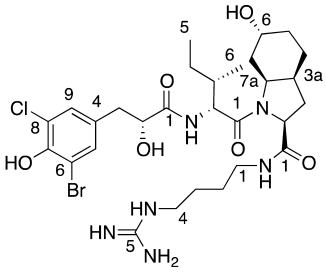
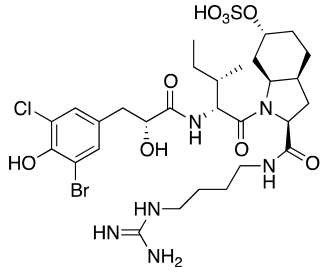
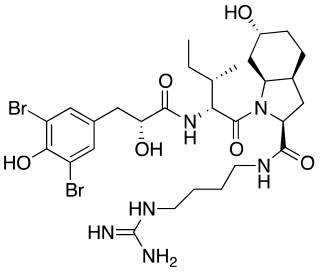
Chart 1



aeruginosin GE686 (**1**)



aeruginosin GE766 (**2**)



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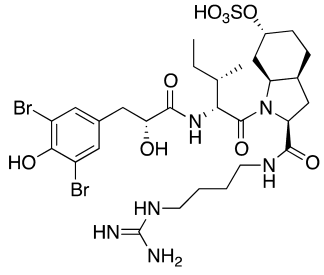
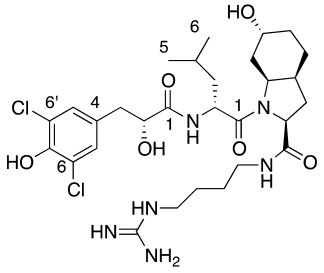
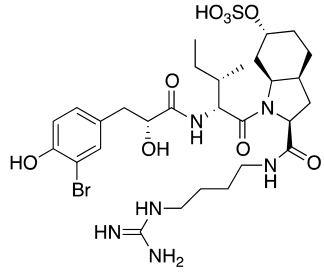


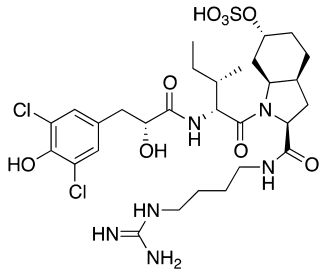
aeruginosin GE810 (**4**)



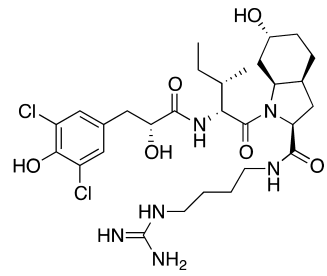
aeruginosin GE642 (**5**)



aeruginosin 98-C (**6**)



aeruginosin 101 (**7**)



aeruginosin KY642 (**8**)

five pairs of methine carbons between 73 and 50 ppm, and several additional methine, methylene, and methyl signals in the aliphatic region. The structure elucidation is based on the data of the major *trans* rotamer (Table 1) since the correlations of the *cis* rotamer were weak and resulted in only partial substructures. The signals of the aromatic residue in the NMR spectra of **1** in DMSO-d_6 presented two singlet protons (δ_H 7.20 and 7.32 s) and six carbons (δ_C 111.7, 121.4, 131.6, and 148.4 C's, 129.8 and 132.9 CH's), suggesting that the tetrasubstituted aromatic ring was not symmetric. The chemical shifts of the quaternary carbons suggested their substitution by bromine (δ_C 111.7), chlorine (δ_C 121.4), carbon (δ_C 131.6), and phenol (δ_C 148.4) groups (by comparison with the chemical shift of mixed halo-substituted *N*-acetyl-tyrosine derivatives).[13] HMBC correlations (Table S1 in the Supporting Information) established the aromatic ring as an *ortho*-bromo, *ortho*′-chloro, *para*-substituted phenol. The aromatic protons (δ_H 7.20 and 7.32 s) presented HMBC correlations with a methylene carbon (δ_C 38.5). Through COSY correlations the protons of this methylene (HSQC) were found to be part of an ABMX spin system of H-3,3′,2 and 2-OH of the novel disubstituted Hpla moiety, *m*-Br-*m*′-Cl-*p*-hydroxyphenyllactic acid. The carbonyl of this moiety was established through HMBC correlations of H-2,3,3′ with the carbonyl resonating at δ_C 171.7. The second residue that was established included the doublet methyl group (δ_H 0.57) and the distorted doublet methyl (δ_H 0.85, brd) that were correlated in the HSQC spectrum with two carbon signals (δ_C 13.8 and 12.0, respectively). These NMR signals were established as part of an isoleucine moiety. Methyl-5 of the Ile appeared as a distorted doublet due to the resonance of one of the methylene-4 protons at the same chemical shift (δ_H 0.85 m,

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Table 1. ^{1}H and ^{13}C NMR Data of Aeruginosin GE686 (1) and Aeruginosin GE766 (2) in DMSO-d_6[a]

	aeruginosin GE686 (1)		aeruginosin GE766 (2)	
position	δ_C, mult.[b]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)
bromo, chloro, Hpla 1	171.7, C		171.9, C	
2	71.5, CH	4.14, m	71.3, CH	4.12, m
3	38.5, CH_2	2.86, dd (14.0, 3.4)	39.5, CH_2	2.87, dd (14.0, 3.4)
3′		2.76, dd (14.0, 5.3)		2.71, dd (14.0, 5.3)
4	131.6, C		131.2, C	
5	129.8, CH	7.20, s	130.2, CH	7.22, s
6	121.4, C		121.3, C	
7	148.4, C		149.0, C	
8	111.7, C		111.5, C	
9	132.9, CH	7.32, s	132.6, CH	7.34, s
2-OH		6.04, d (5.3)		5.95, d (5.3)
7-OH		9.77, brs		
Ile	168.6, C		168.6, C	
2	51.6, CH	4.54, dd (8.3, 4.0)	52.5, CH	4.47, dd (6.6, 3.3)
3	38.1, CH	1.48, m	37.6, CH	1.51, m
4	26.3, CH_2	1.03, m	26.0, CH_2	1.10, m
4′		0.85, m		0.90, m
5	12.0, CH_3	0.85, brd (4.0)	11.9, CH_3	0.85, t (5.6)
6	13.8, CH_3	0.57, d (6.6)	13.7, CH_3	0.63, d (6.4)
NH		7.25, d (8.3)		7.35, d (10.0)
Choi 1	171.6, C		171.5, C	
2	60.1, CH	4.12, t (9.2)	59.9, CH	4.16, t (9.2)
3	30.8, CH_2	1.94, m	30.6, CH_2	2.02, m
3′		1.74, m		1.74, m
3a	36.2, CH	2.26, m	35.9, CH	2.28, m
4	19.1, CH_2	2.02, m	19.3, CH_2	1.98, m
4′		1.41, m		1.42, m
5	26.0, CH_2	1.42, m	23.3, CH_2	1.85, m
				1.32, m
6	64.1, CH	3.90, brs	70.8, CH	4.35, brs
7	33.7, CH_2	1.92, m	31.6, CH_2	2.24, m
7′		1.72, m		1.68, m
7a	54.0, CH	4.05, dt (10.8, 5.6)	54.0, CH	4.00, dt (10.0, 4.8)
6-OH		4.52, brd (2.0)		
Agm 1	38.1, CH_2	3.07, m	38.4, CH_2	3.03, m
1′		2.98, m		
2	26.2, CH_2	1.41, m	25.8, CH_2	1.42, m
3	26.4, CH_2	1.41, m	26.3, CH_2	1.44, m
4	40.6, CH_2	3.08, m	40.3, CH_2	3.08, m
1-NH		7.92, t (5.4)		7.85, t (5.4)
4-NH		7.55, t (5.0)		7.54, brt (5.0)
5	156.9, C		156.7, C	
5-NH,NH_2		6.90, brm		6.95, brm

[a]400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C. [b]Multiplicity and assignment from HSQC experiment.

based on the HSQC correlations). Interpretation of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments enabled the assignment of the Choi residue (Table S1 in the Supporting Information). An amide carbon resonating at 171.6 ppm showed an HMBC correlation with a methine proton resonating at 4.12 ppm, identified as the α-proton of an amino acid (δ_C 60.1 ppm). This methine proton showed COSY connectivity with methylene protons resonating at 1.74 and 1.94 ppm (δ_C 30.8 ppm). The latter two protons could be further correlated with a methine proton at 2.26 ppm (δ_C 36.2 ppm). This methine showed three additional COSY correlations with a methine proton resonating at 4.05 ppm (δ_C 54.0 ppm, characteristic of a methine adjacent to a nitrogen atom) and protons of a methylene resonating at 1.41 and 2.02 ppm (δ_C 19.1 ppm). The protons at 1.41 and 2.02 ppm show COSY and HMBC correlations to further methylene protons at 1.42 ppm (δ_C 26.0 ppm), which in turn showed a COSY correlation to a methine proton at 3.90 ppm (δ_C 64.1 ppm), assigned as a carbinol moiety, and reinforced by a COSY correlation and an HMBC correlation with an acidic proton at 4.52 ppm, the OH proton. The hydroxymethine proton at 3.90 ppm was connected (through COSY correlation) to protons of a methylene resonating at 1.72 and 1.92 ppm (δ_C 33.7 ppm), which were, in turn, connected to the methine at 4.05 ppm. These correlations closed a hexanol ring and identified the amino acid as Choi. The chemical shifts of the protons and carbons of the identified moiety were almost identical with those of the L-Choi moiety of aeruginosin KY642, which was isolated from the same bloom material and previously characterized by us from another bloom material.[11] The relative configurations of the asymmetric carbons of the Choi were deduced from the results of a ROESY experiment as follows: Choi-2 (δ_H 4.12 ppm) possessed a NOE correlation

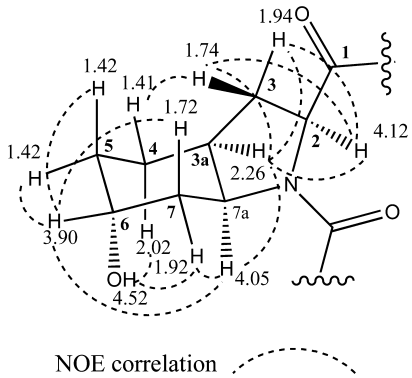

Figure 1. Relative configuration of L-Choi deduced from NOE correlations.

with a proton at 1.94 ppm (Choi-3). Choi-2 and -3 presented NOE correlations with Choi-3a, which in turn had an NOE correlation with Choi-7a. This set of NOEs was possible if all of these protons are pointing to the same face of the pyrrolidine residue. Proton 7a showed additional NOE correlation to a proton at 1.92 ppm (Choi-7), which in turn had a correlation with the hydroxy proton at 4.52 ppm. These correlations, together with the correlation of the hydroxy proton with the axial Choi-4 (δ_H 2.02 ppm), suggested that the hydroxy was axial in the cyclohexane ring. Choi-6 appeared as a broad singlet at 3.90 ppm, in accordance with its equatorial orientation. On the basis of these arguments the relative configuration of the Choi residue was suggested to be 2*S**,3a*S**,6*R**,7a*S**. Interpretation of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments enabled the assignment of the agmatine moiety (Table S1 in the Supporting Information). The four residues were connected through HMBC correlations (Hpla-CO with Ile-2, and NH and Choi-CO with Agm-1-NH)

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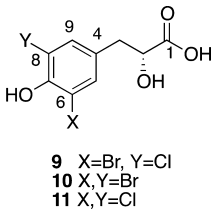
and ROESY correlations (Ile-2 with Choi-7a) to establish the planar structure of **1**. Applying Marfey's method[14] (L-FDAA) revealed the presence of L-Choi and D-Ile/*allo*Ile moieties in **1**. Chiral-phase HPLC chromatography and comparison of the Hpla derivative obtained from the hydrolysis of **1** with the corresponding synthetic Hpla derivatives synthesized according to the procedure developed for *N*-acetyl-tyrosine[13] established it as D-*m*-Br-*m*′-Cl-*p*-Hpla (**9**) in **1**. Despite the report in the literature,[15] we could not separate D-Ile from D-*allo*Ile, regardless of what column or mobile phase mixture we used. In the case of the micropeptins, our previous experience shows that the proton and carbon chemical shifts of Ile are highly sensitive to the conformation and configuration of its asymmetric centers.[16] The chemical shifts of the carbons of the isoleucine residue in **1** were similar to those of the D-*allo*Ile in aeruginosin 98-C[10] (its structure was established by X-ray diffraction, and it was isolated in this study as well). These arguments suggest that D-*allo*Ile and not D-Ile is incorporated into **1**. On the basis of the evidence discussed above, the structure of aeruginosin GE686 (**1**) was established as D-*m*-Br-*m*′-Cl-*p*-Hpla-D-*allo*Ile-L-Choi-agmatine.



9 X=Br, Y=Cl
10 X,Y=Br
11 X,Y=Cl

Figure 2. Synthetic Hpla derivatives.

Aeruginosin GE766 (**2**) was isolated as an amorphous, white material that presented an HRESIMS complex molecular ion adduct at *m*/*z* 789.1649/791.1637/793.1608 (5:8:3, $[M + Na]^+$) corresponding to the molecular formula $C_{29}H_{44}BrClN_6NaO_9S$. The 80 mass unit differences between **2** and **1** could be explained by a substitution of a hydroxy group in **1** with a sulfate group in **2**. The ^1H and ^{13}C NMR spectra of **2** (Table 1) were almost identical with those of **1** and appeared as a ca. 12:1 mixture of the *trans* and *cis* rotamers around the Ile-CO-Choi-N bond.[3] The major differences were the absence of the Choi-6-OH proton, the downfield shift of Choi-H-6 to δ_H 4.35 brs, and the downfield shift of Choi-C-6 to δ_C 70.8 in the NMR spectra of **3**. All of these differences were in accordance with a substitution of Choi-6-OH with a sulfate group similar to the Choi-6-sulfate of aeruginosin 98-C.[11] The interpretation of the COSY, HSQC, HMBC, and ROESY 2D NMR spectra (Table S2 in the Supporting Information) established the structure of *m*-Br-*m*′-Cl-*p*-Hpla, Ile, Choi-6-sulfate, and agmatine. Applying Marfey's method[14] (L-FDAA) and chiral-phase HPLC chromatography revealed the presence of L-Choi, D-Ile/*allo*Ile, and D-*m*-Br-*m*′-Cl-*p*-Hpla (**9**) moieties in **2**. The chemical shifts of the carbons of the isoleucine residue were almost identical with those of the D-*allo*Ile of aeruginosin 98-C,[11] suggesting that **2** contains D-*allo*Ile. ROESY and HMBC correlations, as described for **1**, established the structure of aeruginosin GE766 (**2**) as D-*m*-Br-*m*′-Cl-*p*-Hpla-D-*allo*Ile-L-Choi-6-sulfate-agmatine.

Aeruginosin GE730 (**3**), a yellowish, amorphous solid, presented an HRESIMS complex molecular adduct ion at *m*/*z* 731.1768/733.1755/735.1782 (1:2:1, $[M + Na]^+$), which matched the molecular formula $C_{29}H_{45}Br_2N_6O_6$. The ^1H and ^{13}C NMR spectra of **3** (Table 2) were similar to those of **1** and appeared as a ca. 12:1 mixture of the *trans* and *cis* rotamers around the Ile-CO-Choi-N bond.[3] The differences observed in the NMR spectra of **3**, when compared with those of **1**, were the appearance of only one singlet proton signal (δ_H 7.35 s, 2H) in the aromatic region of the ^1H NMR spectrum and only four carbon signals (δ_C 111.5, 132.9, and 149.2 C's, 133.5 CH's) in the aromatic region of the ^{13}C NMR spectrum of **3**. This observation suggested that the Hpla moiety is symmetric and substituted in both *ortho* positions by two bromine atoms. The rest of the moieties that compose **3** were similar to that of **1**. An analysis of the 2D NMR spectra of **3** (Table S3 in the Supporting Information) established the structure of the *m*,*m*-di-Br-*p*-Hpla (**10**), Ile, Choi, and agmatine moieties. Applying the same methodology described above established the structure of aeruginosin GE730 (**3**) as D-*m*,*m*-di-Br-*p*-Hpla-D-*allo*Ile-L-Choi-agmatine.

Aeruginosin GE810 (**4**) was isolated as an amorphous solid presenting an HRESIMS complex molecular adduct ion at *m*/*z* 833.1169/835.1136/837.1133 (1:2:1, $[M + Na]^+$), corresponding to the molecular formula $C_{29}H_{45}Br_2N_6NaO_9S$. Its ^1H and ^{13}C NMR spectra (Table 2) resembled those of **3**, and as in the case of **1** and **2**, it seems that **4** is the Choi-6-sulfate derivative of **3**. An analysis of the COSY, HSQC, HMBC, and ROESY spectra of **4** (Table S4 in the Supporting Information) verified this suggestion, while Marfey's analysis[14] (L-FDAA) and chiral-phase HPLC chromatography revealed the presence of L-Choi, D-*allo*Ile, and D-*m*,*m*-di-Br-*p*-Hpla (**10**) moieties in **4**. On the basis of these results the structure of aeruginosin GE810 (**4**) was established as D-*m*,*m*-di-Br-Hpla-D-*allo*Ile-L-Choi-6-sulfate-agmatine.

Aeruginosin GE642 (**5**) was isolated as a glassy material, which exhibited an HRESIMS complex molecular ion adduct at *m*/*z* 643.2772/645.2777/647.2780 with (9:6:1, $[M + H]^+$), which is characteristic of a molecule that contains two chlorine atoms[11] and corresponds to the molecular formula $C_{29}H_{44}Cl_2N_6O_6$. The ^1H and ^{13}C NMR spectra of **5** were characteristic of the aeruginosins and displayed a 5:1 mixture of *trans* and *cis* rotamers.[3] The ^1H NMR spectrum (Table 2) of **5** presented three pairs of amide protons (a pair of doublets and two pairs of broad triplets), a pair of singlet signals in the aromatic region, two pairs of exchangeable doublet signals, four pairs of methine protons next to electronegative atoms in the 5.5−3.8 ppm region, and two broad signals around 3.05 ppm corresponding to four protons. The aliphatic region was too complicated to be interpreted except for two pairs of doublet methyl groups. The ^{13}C NMR spectrum (Table 2) revealed three pairs of amide/ester carbonyls, two pairs of sp^2 carbons characteristic of phenol and/or guanidine carbons, three signals of aromatic sp^2 carbons, five pairs of methine carbons between 73 and 50 ppm, and several additional methine, methylene, and methyl signals in the aliphatic region. The structure elucidation is based on the data of the major *trans* rotamer, as the correlations of the *cis* rotamer were weak and resulted in only partial substructures. The interpretation of the data from the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments enabled the assignment of the Leu and agmatine moieties (Table S5 in the Supporting Information). The structure of the *m*,*m*-dichloro-Hpla was elucidated as follows: COSY correlations established the connectivity of the ABMX spin system of H-3,3′,2 and 2-OH. The two-proton singlet aromatic signal at δ_H 7.11 (δ_C 129.8) suggested the existence of a symmetric

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Table 2. ^{1}H and ^{13}C NMR Data of Aeruginosins GE730 (3), GE810 (4), and GE642 (5) in DMSO-d_6

	aeruginosin GE730 (3)[a]		aeruginosin GE810 (4)[b]		aeruginosin GE642 (5)[a]	
position	δ_C, mult.[c]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)	δ_C, mult.[b]	δ_H, mult., J (Hz)
di-Br/Cl- Hpla 1	171.7, C		171.9, C		171.7, C	
2	71.5, CH	4.14, m	71.3, CH	4.13, m	71.3, CH	4.10, m
3	38.4, CH_2	2.86, dd (14.0, 3.6)	37.8, CH_2	2.86, m	38.6, CH_2	2.83, dd (14.4, 4.0)
3′		2.76, dd (14.0, 6.0)		2.70, m		2.77, dd (14.4, 6.4)
4	132.9, C		132.3, C		130.8, C	
5,5′	133.5, CH	7.35, s	133.2, CH	7.36, s	129.8, CH	7.11, s
6,6′	111.5, C		111.4, C		121.7, C	
7	149.2, C		149.0, C		147.5, C	
2-OH		6.03, d (6.0)		5.92, d (5.0)		5.96, d (4.0)
7-OH		9.60, brs				9.70, brs
Ile/Leu	168.6, C		168.9, C		169.6, C	
2	51.6, CH	4.54, m	52.4, CH	4.45, dd (8.5, 4.0)	47.9, CH	4.52, m
3	38.1, CH	1.48, m	37.5, CH	1.52, m	42.4, CH_2	1.32, m
						1.20, m
4	26.2, CH_2	1.02, m	25.8, CH_2	1.10, m	24.1, CH	1.20, m
4′		0.85, m		0.91, m		
5	12.1, CH_3	0.85, brd (4.0)	11.9, CH_3	0.85, t (6.5)	23.5, CH_3	0.76, d (6.5)
6	13.8, CH_3	0.57, d (6.6)	13.7, CH_3	0.63, d (6.5)	21.5, CH_3	0.85, d (6.0)
NH		7.25, d (9.2)		7.35, d (8.0)		7.35, d (8.4)
Choi 1	171.6, C		171.5, C		171.5, C	
2	60.1, CH	4.12, t (9.2)	59.9, CH	4.16, dd (9.5, 8.5)	60.1, CH	4.12, m
3	30.7, CH_2	1.94, m	30.6, CH_2	2.00, m	30.8, CH_2	1.98, m
3′		1.74, dt (16.8, 12.0)		1.76, dt (11.5, 10.5)		1.76, m
3a	36.2, CH	2.26, m	35.8, CH	2.27, m	36.2, CH	2.27, m
4	19.1, CH_2	2.02, m	19.3, CH_2	1.98, m	19.1, CH_2	2.02, m
4′		1.41, m		1.42, m		1.41, m
5	26.0, CH_2	1.42, m	23.3, CH_2	1.85, m	26.0, CH_2	1.42, m
				1.32, m		
6	64.1, CH	3.90, brs	70.7, CH	4.35, brs	64.0, CH	3.92, brs
6-OH		4.52, brd (1.2)				4.49, brs
7	33.7, CH_2	1.92, m,	31.6, CH_2	2.18, m	33.7, CH_2	1.98, m
7′		1.72, m		1.68, m		1.72, m
7a	54.0, CH	4.05, dt (10.8, 5.6)	53.9, CH	4.01, dt (10.5, 6.0)	54.0, CH	4.06, dt (11.6, 6.0)
Agm 1	38.1, CH_2	3.07, m	38.2, CH_2	3.08, m	38.1, CH_2	3.03, m
1′		2.98, m		3.02, m		
2	26.3, CH_2	1.41, m	26.2, CH_2	1.42, m	26.0, CH_2	1.42, m
3	26.4, CH_2	1.41, m	26.5, CH_2	1.41, m	26.4, CH_2	1.41, m
4	40.6, CH_2	3.08, m	39.8, CH_2	3.08, m	40.5, CH_2	3.07, m
1-NH		7.92, t (5.0)		7.82, brt (5.0)		7.83, brt (5.2)
4-NH		7.51, brt (4.5)		7.56, brm		7.67, brt (4.9)
5	156.9, C		156.7, C		156.8, C	
5-NH,NH_2		6.95, brm		6.95, brm		6.95, brm

[a]400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C. [b]500.13 MHz for ^{1}H, 125.76 MHz for ^{13}C. [c]Multiplicity and assignment from HSQC experiment.

tetrasubstituted aromatic ring. This was supported by the appearance of only four pairs of aromatic carbons in the region of 148 to 121 ppm. The aromatic proton presented HMBC correlations with the carbon signals resonating at δ_C 147.5, 121.7, which were thus assigned as C-7 and C-6,6′, and with C-3 (δ_C 38.6). On the basis of a comparison with the chemical shifts of the same residue in aeruginosin KY642[11] (that contains Ile instead of Leu) the remaining aromatic carbon (δ_C 130.8) was assigned as C-4 of this residue, *m,m*-di-Cl-*p*-Hpla. The structure of the Choi moiety was established mainly based on the COSY correlations. The four characteristic protons of this moiety, H-2 (δ_H 4.12 m), H-3a (δ_H 2.27, m), equatorial H-6 (δ_H 3.92, brs), and H-7a (δ_H 4.06, m), could be sequentially extended to the entire Choi spin system through the COSY correlations. HSQC correlations assigned the carbons of the Choi moiety, which were identical with the chemical shifts of the protons and carbons of the same moiety in aeruginosin KY642.[11] The four subunits were assembled through ROESY correlations of Hpla-H-2 and Leu-NH and of Leu-H-2 and Choi-H-7a and HMBC correlation of Choi-CO and Agm-1-NH. Applying Marfey's method[14] (L-FDAA) and chiral-phase HPLC chromatography revealed the presence of L-Choi, D-Leu, and D-*m,m*-di-Cl-*p*-Hpla (**11**) moieties in **5**. On the basis of the evidence discussed above the structure of aeruginosin GE642 (**5**) was established as D-*m,m*-di-Cl-*p*-Hpla-D-Leu-L-Choi-agmatine.

BIOLOGICAL ACTIVITY

The extracts of strain IL-377 exhibited significant inhibition of the serine protease trypsin at a concentration of 1 mg/mL. The

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activity-guided purification of the trypsin-inhibiting components of the extract revealed that the known as well as the new aeruginosins were responsible for the inhibition of trypsin. The inhibitory activities of **1**−**5** were determined for the serine proteases trypsin and thrombin (Table 3). These results indicate that compounds containing 6-OH-Choi inhibit trypsin and thrombin more potently than their 6-sulfated counterparts.

Table 3. Summary of the IC_{50}'s of Compounds 1−5 against Trypsin and Thrombin

compound	trypsin	thrombin
aeruginosin GE686 (**1**)	3.2 μM	12.8 μM
aeruginosin GE766 (**2**)	12.2 μM	>45.5 μM
aeruginosin GE730 (**3**)	2.3 μM	12.9 μM
aeruginosin GE810 (**4**)	18.2 μM	>45.5 μM
aeruginosin GE642 (**5**)	8.5 μM	>45.5 μM

CONCLUDING REMARKS

Cyanobacteria present high biosynthetic versatility, which is reflected in the synthesis of modified peptides such as the aeruginosins. In the aeruginosins the biosynthetic versatility is demonstrated in the ability to synthesize at least three different isomers of the core modified amino acid Choi, (2*S*,3a*S*,6*R*,7a*S*)-Choi (L-Choi),[10] (2*S*,3a*R*,6*R*,7a*R*)-Choi (L-3a,7a-*diepi*Choi),[16] and 2*R*,3a*R*,6*R*,7a*R*-Choi (D-3a,7a-*diepi*Choi),[3] as well as the ability to synthesize different derivatives of arginine at the *C*-termius and to modify the configuration of C-2 and the substituents of the aromatic ring of the Hpla residue in the *N*-termius of these linear peptides.[10] Alternation of the configuration of Hpla-C-2 occurs even in isomers from the same organisms (i.e., aeruginosins KT608A and B[3]), and mono- and dihalogenation of the phenolic ring of Hpla with chlorine and bromine increase the diversity of this group of linear peptides.[10] The isolation of aeruginosins GE686 (**1**) and GE766 (**2**) emphasized again how versatile the biosynthetic machinery of cyanobacteria is, allowing the substitution of the same aromatic ring with chlorine and bromine. Such versatility of biosynthetic enzymes is seen only for the selective halogenases of red and brown algae, which produce halogenated terpenes[17] and polyketides,[18] and rarely by bacteria for the production of halogenated phenols such as 2,4-dibromo-6-chlorophenol.[19]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). 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, gHMQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. Mass spectra were recorded on a Waters MaldiSynapt instrument. 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. An Eliza EL_x808 reader (BIO-TEK Instruments, Inc.) was used for protease inhibition assays.

Biological Material. *Microcystis aeruginosa*, TAU strain IL-377, was collected in August 2007 from a commercial fishpond in Kibbutz Geva, Israel. Samples of the cyanobacteria are deposited at the culture collection of Tel Aviv University.

Isolation Procedure. The freeze-dried cells (IL-377, 473 g) were extracted with 7:3 MeOH/H_2O (3 × 3L). The crude extract (74 g) was evaporated to dryness and separated on an ODS (YMC-GEL, 120A, 4.4 × 6.4 cm) flash column with an increasing concentration of MeOH in H_2O. Fraction 6 (1:1 MeOH/H_2O, 1.553 g) was subjected to a Sephadex LH-20 column in 1:1 $CHCl_3$/MeOH to obtain seven fractions. Fractions 5−7 from the Sephadex LH-20 column (45 mg) were separated on a reversed-phase HPLC (Cosmosil 5C-8 MS, 250 mm × 20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 78:22 0.1% aqueous TFA/CH_3CN to obtain five pure compounds, aeruginosin DA688 (5.8 mg, retention time 19.3 min, 0.0013% yield based on the dry weight of the bacteria), aeruginosin 98C (5.1 mg, retention time 21.3 min, 0.0011% yield based on the dry weight of the bacteria), aeruginosin 101 (4.3 mg, retention time 25.1 min, 0.0009% yield based on the dry weight of the bacteria), aeruginosin GE766 (**2**) (4.6 mg, retention time 27.5 min, 0.0010% yield based on the dry weight of the bacteria), and aeruginosin GE810 (**4**) (4.3 mg, retention time 30.1 min, 0.0009% yield based on the dry weight of the bacteria). Fractions 7 and 8 from the RP separation (6:4 and 7:3 MeOH/H_2O, 1.43 g) were separated on a reversed-phase HPLC (Cosmosil 5C-8 MS, 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 aeruginosin GE642 (**5**) (1.9 mg, retention time 18.7 min, 0.0004% yield based on the dry weight of the bacteria), aeruginosin KY642 (2.2 mg, retention time 19.4 min, 0.0005% yield based on the dry weight of the bacteria), aeruginosin GE686 (**1**) (3.6 mg, retention time 21.6 min, 0.0008% yield based on the dry weight of the bacteria), and aeruginosin GE730 (**3**) (4.6 mg, retention time 27.5 min, 0.0010% yield based on the dry weight of the bacteria).

Aeruginosin GE686 (1): $[\alpha]^{25}_D$ −23.8 (*c* 0.42, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.32), 248 (3.53), 291 (3.23), 308 (3.23) nm; IR (KBr) 2932, 1675, 1203, 1138 cm^{-1}; for NMR data see Tables 1 and S1 (in the Supporting Information); HRESIMS *m*/*z* 687.2285/689.2256/691.2254 (5:8:3) $[M + H]^+$ (calcd for $C_{29}H_{45}{}^{81}Br^{35}ClN_6O_6$, 689.2252). Retention times of AA Marfey's derivatives: D-Br,Cl-Hpla 3.3 min (L-Br,Cl-Hpla 3.0, D-Br,Cl-Hpla 3.3 min), D-*allo*Ile 47.4 min (L-Ile 44.6, D-Ile/D-*allo*Ile 47.4 min), L-Choi-6α-OH 38.8 min (L-Choi-6α-OH 38.8, L-Choi-6β-OH 37.7 min).

Aeruginosin GE766 (2): $[\alpha]^{25}_D$ −8.8 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.00), 247 (3.14), 290 (2.85), 310 (2.73) nm; IR (KBr) 2927, 1677, 1384, 1206, 1138 cm^{-1}; for NMR data see Tables 1 and S2 (in the Supporting Information); HRESIMS *m*/*z* 789.1649/791.1637/793.1608 (5:8:3) $[M + Na]^+$ (calcd for $C_{29}H_{44}{}^{81}Br^{35}ClN_6NaO_9S$ 791.1640). Retention times of AA Marfey's derivatives: D-Br,Cl-Hpla 3.4 min (L-Br,Cl-Hpla 3.0, D-Br,Cl-Hpla 3.4 min), D-*allo*Ile 53.3 min (L-Ile 49.2, D-Ile/D-*allo*Ile 53.3 min), L-Choi-6α-OH 41.9 min (L-Choi-6α-OH 41.9, L-Choi-6β-OH 41.5 min).

Aeruginosin GE730 (3): $[\alpha]^{25}_D$ −39.1 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.29), 247 (3.44), 292 (2.99), 310 (3.05) nm; IR (KBr) 2932, 1675, 1204, 1138 cm^{-1}; for NMR data see Tables 2 and S3 (in the Supporting Information); HRESIMS *m*/*z* 731.1768/733.1755/735.1782 (1:2:1, $[M + H]^+$, calcd for $C_{29}H_{45}{}^{79}Br^{81}BrN_6O_6$ 733.1747). Retention times of AA Marfey's derivatives: D-di-Br-Hpla 4.1 min (L-di-Br-Hpla 3.8, D-di-Br-Hpla 4.1 min), D-*allo*Ile 45.9 min (L-Ile 43.0, D-Ile/D-*allo*Ile 45.9 min), L-Choi-6α-OH 36.2 min (L-Choi-6α-OH 36.2, L-Choi-6β-OH 34.9 min).

Aeruginosin GE810 (4): $[\alpha]^{25}_D$ −39.0 (*c* 2.7, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.95), 289 (2.64), 344 (1.08) nm; IR (KBr) 2929, 1679, 1206, 1139 cm^{-1}; for NMR data see Tables 2 and S4 (in the Supporting Information); HRESIMS *m*/*z* 833.1169/835.1136/837.1133 (1:2:1) $[M + Na]^+$ (calcd for $C_{29}H_{44}{}^{79}Br_2N_6NaO_9S$, 833.1134). Retention times of AA Marfey's derivatives: D-di-Br-Hpla 3.4 min (L-di-Br-Hpla 3.1, D-di-Br-Hpla 3.4 min), D-*allo*Ile 51.3 min (L-Ile 47.5, D-Ile/D-*allo*Ile 51.3 min), L-Choi-6α-OH 40.3 min (L-Choi-6α-OH 40.3, L-Choi-6β-OH 38.7 min).

Aeruginosin GE642 (5): $[\alpha]^{25}_D$ −19.5 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.75), 249 (2.50), 291 (2.95) nm; IR (KBr) 1683, 1383, 1206 cm^{-1}; for NMR data see Tables 2 and S5 (in the Supporting Information); HRESIMS *m*/*z* 643.2772/645.2777/647.2780 (9:6:1) $[M + H]^+$ (calcd for $C_{29}H_{45}{}^{35}Cl_2N_6O_6$, 643.2778).

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Retention times of AA Marfey's derivatives: D-di-Cl-Hpla 3.29 min (L-di-Cl-Hpla 2.99, D-di-Cl-Hpla 3.29 min), D-leucine 46.89 min (L-Leu 43.8, D-Leu 46.9 min), L-Choi-6α-OH 36.3 min (L-Choi-6α-OH 36.3, L-Choi-6β-OH 35.0 min).

Preparation of *m*-Bromo-*m*′-chloro-D,L-*p*-hydroxyphenyllactic Acid (9) (ref 13). To a stirring solution of hydroxyphenyllactic acid (10 mg) in 2 mL of CH_3CN was added 0.1 equiv of TsOH in CH_3CN (2 mL). After 5 min of stirring 1.1 equiv (8.1 mg) of *N*-chlorosuccinimide in CH_3CN (2 mL) was added in one portion. The reaction was left to stir at room temperature for 6 h. Then 1.1 equiv (10.8 mg) of *N*-bromosuccinimide in CH_3CN (2 mL) was added in one portion. The reaction was left to stir at room temperature for 18 h. For the workup, the CH_3CN solution was diluted with EtOAc (20 mL) and washed three times with a 5% aqueous solution of $Na_2S_2O_3$ (25 mL), followed by three washes with water (25 mL) and then with brine (25 mL). After evaporation of the solvents under vacuum, the solid was subjected to a preparative reversed-phase HPLC column (YMC-pack ODS-A, 5 μm, 250 × 20 mm, DAD at 238 nm, flow rate 5.0 mL/min) eluted with 1:1 0.1% aqueous TFA/MeOH to afford *m*-bromo-*m*′-chloro-D,L-*p*-Hpla (**9**, 7.2 mg, 44% yield, retention time 21.7 min) and *m*,*m*-dichloro-D,L-*p*-Hpla (**11**, 2.9 mg, 21% yield, retention time 23.5 min).

***m*-Bromo-*m*′-chloro-D,L-*p*-hydroxyphenyllactic acid (9):** yellowish solid; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.08 (1H, dd, J = 4.0, 8.0 Hz, H-2), 5.58 (1H, brs, 2-OH), 2.68 (1H, dd, J = 14.0, 8.0 Hz, H-3), 2.84 (1H, dd, J = 14.0, 4.0 Hz, H-3′), 7.33 (1H, d, J = 1.5 Hz, H-5), 7.22 (1H, d, J = 1.5 Hz, H-9), 9.75 (1H, s, 7-OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 175.3 (C, C-1), 71.0 (CH, C-2), 38.7 (CH_2, C-3), 132.4 (C, C-4), 132.9 (CH, C-5), 112.0 (C, C-6), 148.6 (C, C-7), 121.8 (C, C-8), 130.5 (CH, C-9); HRESIMS m/z 292.9211, $[M - H]^-$ (calcd for $C_9H_7{}^{79}Br^{35}ClO_4$, 292.9216).

Preparation of *m*,*m*-Dibromo-D,L-*p*-hydroxyphenyllactic Acid (10) (ref 13). To a stirring solution of hydroxyphenyllactic acid (10 mg) in 2 mL of CH_3CN was added 2.0 equiv (19.6 mg) of *N*-bromosuccinimide in CH_3CN (2 mL) in one portion. The reaction was left to stir at room temperature for 18 h. For the workup, the CH_3CN solution was diluted with EtOAc (20 mL) and washed three times with a 5% aqueous solution of $Na_2S_2O_3$ (25 mL), followed by three washes with water (25 mL) and then with brine (25 mL). After evaporation of the solvents under vacuum, the solid was subjected to a preparative reversed-phase HPLC column (YMC-pack ODS-A, 5 μm, 250 × 20 mm, DAD at 238 nm, flow rate 5.0 mL/min) eluted with 1:1 0.1% aqueous TFA/MeOH to afford *m*-bromo-D,L-*p*-Hpla (1.5 mg, 13% yield, retention time 18.9 min) and *m*,*m*-dibromo-D,L-*p*-Hpla (**10**, 10.9 mg, 58% yield, retention time 25.5 min).

***m*,*m*-Dibromo-D,L-*p*-hydroxyphenyllactic acid (10):** yellowish solid; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.08 (1H, dd, J = 4.0, 8.0 Hz, H-2), 5.58 (1H, brs, 2-OH), 2.70 (1H, dd, J = 13.5, 8.0 Hz, H-3), 2.84 (1H, dd, J = 13.5, 4.0 Hz, H-3′), 7.36 (2H, s, H-5, H-9), 9.60 (1H, s, 7-OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 175.3 C, C-1), 71.2 (CH, C-2), 38.5 (CH_2, C-3), 132.7 (C, C-4), 133.5 (CH × 2, C-5,5′), 112.0 (C × 2, C-6,6′), 149.4 (C, C-7); HRESIMS m/z 338.8696, $[M - H]^-$ (calcd for $C_9H_7{}^{79}Br^{80}BrO_4$, 338.8691).

Preparation of *m*,*m*-Dichloro-D,L-*p*-hydroxyphenyllactic Acid (11) (ref 13). To a stirring solution of hydroxyphenyllactic acid (10 mg) in 2 mL of CH_3CN was added 2.2 equiv (16.1 mg) of *N*-chlorosuccinimide in CH_3CN (2 mL) in one portion. The reaction was left to stir at room temperature for 18 h. For the workup, the CH_3CN solution was diluted with EtOAc (20 mL) and washed three times with a 5% aqueous solution of $Na_2S_2O_3$, (25 mL) followed by three washes with water (25 mL) and then with brine (25 mL). After evaporation of the solvents under vacuum, the solid was subjected to a preparative reversed-phase HPLC column (YMC-pack ODS-A, 5 μm, 250 × 20 mm, DAD at 238 nm, flow rate 5.0 mL/min) eluted with 1:1 0.1% aqueous TFA/MeOH to afford *m*-chloro-D,L-*p*-Hpla (1.4 mg, 12% yield, retention time 13.8 min) and *m*,*m*-dichloro-D,L-*p*-Hpla (**11**, 5.5 mg, 41% yield, retention time 18.2 min).

***m*,*m*-Dichloro-D,L-*p*-hydroxyphenyllactic acid (11):** yellowish solid; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.01 (1H, dd, J = 4.0, 8.0 Hz, H-2), 5.58 (1H, brs, 2-OH), 2.65 (1H, dd, J = 14.0, 8.0 Hz, H-3), 2.84 (1H, dd, J = 14.0, 4.0 Hz, H-3′), 7.18 (2H, s, H-5, H-9), 9.70 (1H, s, 7-OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ175.3 (C, C-1), 71.1 (CH, C-2), 38.9 (CH_2, C-3), 132.0 (C, C-4), 129.8 (CH × 2, C-5,5′), 122.1 (C × 2, C-6,6′), 147.7 (C, C-7); HRESIMS m/z 248.9725, $[M - H]^-$ (calcd for $C_9H_7{}^{35}Cl_2O_4$, 248.9721).

Determination of the Absolute Configuration of the Amino Acids. Compounds **1**−**5** (0.3 mg each) were hydrolyzed in 6 N 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, 1M) 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 CH_3CN 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 0.1% aqueous TFA buffer, pH 3: CH_3CN to CH_3CN, 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 Hydroxy Phenyl Lactic Acid Derivatives. Extraction of the acid hydrolysates of compounds **1**−**5** with ethyl ether separated the Hpla derivatives 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 277 nm, linear elution with 1:19 1% aqueous triethylamnium acetate buffer, pH 4: MeOH. The Hpla derivative from the aeruginosins was compared with corresponding synthetic standard D,L-Hpla derivatives.

Protease Inhibition Assays. The procedures used to determine the inhibitory activity of the new compounds on trypsin and thrombin were described in a previous paper.[21]

■ ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra and HR MS data of compounds **1**−**5**; tables of full NMR data of **1**−**5**. 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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