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Micropeptins from *Microcystis aeruginosa* collected in Dalton reservoir, Israel

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ABSTRACT

Three new micropeptins, micropeptin DR1056, DR1060 and DR1006 and three known metabolites, micropeptin SF909, aeruginosins 298A and B were isolated from the extracts of a *Microcystis aeruginosa* bloom material collected in Dalton reservoir, Israel. The planar structure of the compounds was determined by homonuclear and inverse-heteronuclear 2D NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers of the amino acids was studied using Marfey's method for HPLC. The inhibitory activity of the compounds was determined for the serine proteases: trypsin, chymotrypsin, and elastase and for amino peptidase N.

1. Introduction

Natural blooms of cyanobacteria in water bodies frequently produce a large array of toxic and non-toxic metabolites. The toxic metabolites most frequently produced by certain genera of water-bloom forming cyanobacteria including the genus Microcystis, are the microcystins. Eighty different microcystin variants are known, exhibiting potent protein phosphatase (PP's) inhibitory activity[1] and mammal hepatotoxicity.[2] The non-toxic metabolites, that accompany the microcystins, are usually members of five discrete families of protease inhibitors: micropeptins,[3] aerugenosins,[4] microginins,[5] anabaenopeptins,[6] and microviridins.[7] Cyanobactrial blooms, toxic and non-toxic, usually contain one or several of these groups of protease inhibitors and in certain cases also other groups of non-toxic metabolites.[8] In most cases the latter non-toxic secondary metabolites are cyclic and non-cyclic modified peptides. Modification of proteinogenic amino acids, by cyanobacteria, results in new amino acid mimics, i.e., 3-amino-6-hydroxy-2-piperidone (Ahp) in the micropeptins. The acid residue sequence of this family of cyclic peptolides is highly variable, as can be figured from the ca. 125 members of the family.[9] Some of the positions present high tendency and frequency for acid variation while others vary only between two closely related acids. The fifth position from the C-termini of the peptolide has attracted most of the attention in this respect, since the nature of the amino acid at this position selects between trypsin and chymotrypsin types of enzyme inhibition.[10] Recently, we have shown that also the second position might alter this selectivity.[11] The side chain of the micropeptins is also highly variable in the length (0–3, most frequently two amino acids) and acid composition and is always terminated by either hydroxy acid (hydroxyphenyllactic acid or glyceric acid derivatives) or short chain fatty acid (1 to 8 carbons). As part of our continuing interest in the chemical ecology of cyanobacterial water-blooms and the search for novel drugs for human diseases, we examined the extracts of a *Microcystis aeruginosa* bloom material collected in the summer of 2001 from Dalton reservoir, Israel. The extract of this bloom (Samples IL-237 and IL-238) afforded six non-toxic secondary metabolites. Three of the compounds are new natural products: micropeptins DR1056 (**1**), DR1060 (**2**), and DR1006 (**3**) and three are the known metabolites, micropeptin SF909 (**4**)[12] and aeruginosins 298A and B.[13] The structure elucidation and the biological activity of the compounds are discussed.

2. Results and discussion

Micropeptin DR1056 (**1**), a glassy material, presented an ESIMS pseudo-molecular $[MNa]^+$ ion at *m*/*z* 1079.5071, corresponding to the molecular formula $C_{54}H_{73}N_8O_{14}Na$. Examination of the NMR spectra of **1**, in DMSO-d_6, revealed its peptide nature, i.e., nine carboxylic carbons in the ^{13}C NMR spectrum and five amide doublet protons in the ^1H NMR spectrum. Some characteristic signals suggested that it was a micropeptin type of compound.[14] Taking into account the *N*Me-aromatic amino acid and the *N*,*N*-disubstituted-amino acid of the micropeptins, suggested that this

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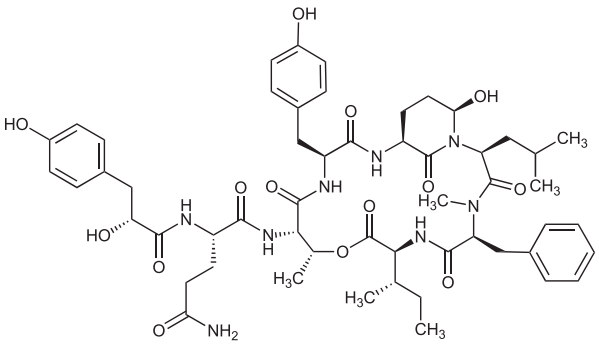
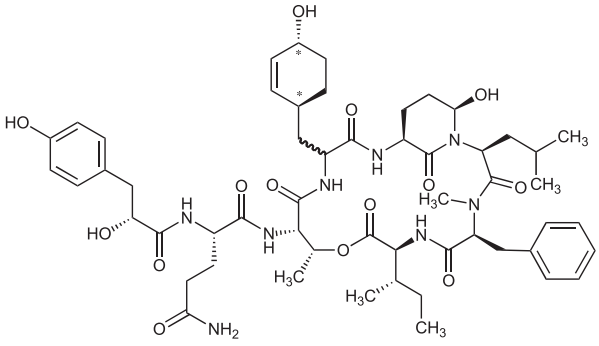

Micropeptin DR1056 (**1**)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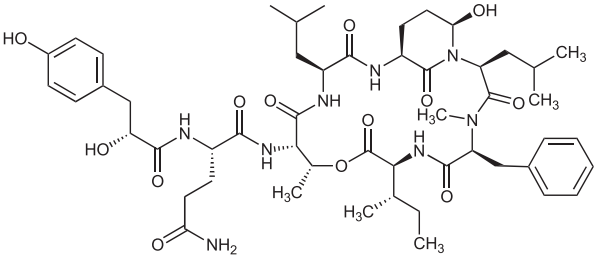
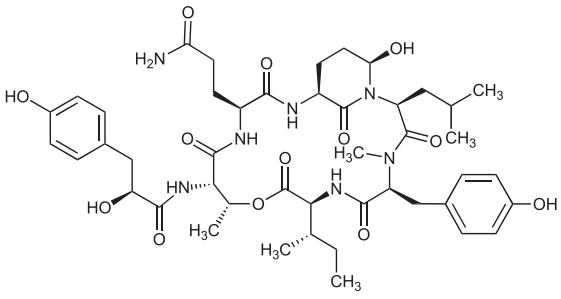

Micropeptin DR1006 (**3**)


MicropeptinSF909 (**4**)

micropeptin was assembled from seven amino acid moieties. In addition, a hydroxy group of a *p*-hydroxyphenyl lactic acid residue, resonating at 5.48 ppm (br s) and the hydroxy proton of Ahp at 5.99 ppm were evident in the ^{1}H NMR spectrum. Analysis of the COSY, TOCSY, and HSQC 2D NMR experiments (see Table 1) allowed the assignment of the side chains of isoleucine, an *N*,*N*-disubstituted leucine, a threonine, a glutamine, three short fragments in agreement with *N*,*N*-disubstituted aromatic amino acid, aromatic amino acid and aromatic hydroxy acid moieties, two *para*-substituted phenol rings, a phenyl ring, and an amino hydroxy piperidone (Ahp) moiety. The entire structure of the latter fragments, and the assignment of the carboxamide carbons to the side chains, was achieved by analysis of the correlation map of an ^{1}H–^{13}C HMBC experiment (see Table 1). This procedure established the structure of Ile, *N*MePhe, *N*,*N*-disubstituted Leu, Ahp, Tyr, 3-*O*-substituted-Thr, Gln, and *p*-hydroxyphenyl lactic acid residues. The sequence of the amino acids of the peptide: Ile, *N*MePhe, *N*,*N*-disubstituted-Leu, Ahp, Tyr, Thr, Gln, and Hpla was assigned on the basis of HMBC and ROESY correlations as follows: HMBC correlations between the carboxyl of *N*MePhe and Ile NH, the carboxyl of *N*,*N*-disubstituted-Leu and the *N*Me of *N*MePhe, the carboxyl of Tyr and NH of Ahp, the carboxyl of Thr and NH of Tyr, the carboxyl of Gln and the NH of Thr and the carboxyl of Hpla and NH of Gln, and the NOE correlation between H-2,3,4 of *N*,*N*-disubstituted-Leu and Ahp H-3 and H-6. The ester-linkage between Thr and Ile was established through the HMBC correlation of Thr H-3 and the carboxyl of Ile. Acid hydrolysis of **1** and derivatization with Marfey's reagent,[15] followed by HPLC analysis, demonstrated the L-configuration of the isoleucine, *N*Me-phenylalanine (by comparison with synthetic material), tyrosine, threonine glutamic acid residues. Jones oxidation[16] of **1**, followed by a similar hydrolysis, derivatization and HPLC analysis, determined the 3*S*-configuration for the Ahp residue (oxidation and subsequent hydrolysis liberated L-glutamic acid from Ahp). The configuration of C-6 of the Ahp was determined as *R* on the basis of the *J*-values of H-6, <1 Hz, which points to an equatorial orientation of this proton, the chemical shift of the pseudoaxial H-4, δ_H 2.52 br q, which is down-field shifted by the axial 6-hydroxy-group, the trans diaxial relation ship with H-3 and NOE correlation between H-4pax and the 6-OH. The configuration of the *p*-hydroxyphenyl lactic acid was determined to be D (*R*), by the comparison of the retention time of the *p*-hydroxyphenyl lactic acid L-menthyl ester from the hydrolysate with the retention time of authentic samples of D- and L-*p*-hydroxyphenyl lactic acid L-menthyl ester on a RP HPLC column.[17] Based on these findings the structure of micropeptin DR1056 was established as **1**.

Examination of the NMR spectra of micropeptin DR1060 (**2**) and micropeptin DR1006 (**3**) revealed that their ^{1}H NMR spectra are similar to that of **1**, except for missing one of the *para*-substituted phenol moieties. The molecular formula of **2**, $C_{54}H_{76}N_8O_{14}$, was deduced from the high-resolution ESIMS measurements of its sodiated molecular cluster ion at *m*/*z* 1083.5365. Comparison of the NMR data of micropeptin DR1056 (**1**) and micropeptin DR1060 (**2**) (see Tables 1 and 2, respectively) revealed that the difference between them is located in the amino acid inhabiting the fifth position of the peptide. The amide proton of the amino acid in the fifth position from the C-terminus of the peptide resonates in all micropeptins around δ_H 8.50 ppm.[9] COSY correlation of the amide proton at δ_H 8.47 ppm assigned H-2 of this amino acid at δ_H 4.33 ppm, but the remaining proton and carbon signals that belong to this spin system did not match those of any proteogenic amino acid. Full assignment of this amino acid was achieved using COSY, TOCSY, ROESY, HSQC, and HMBC 2D NMR correlations (see Table 2). Using the COSY and TOCSY correlations the α-methine was connected to a methylene, which in turn was connected to a methine that was part of a 4-hydroxy-cyclohex-2-enyl moiety. This amino acid moiety was thus established as the rare 3-(7-hydroxycyclohex-5-enyl)-alanyl (HcAla) previously identified in two cases in micropeptins.[18] Assuming a twisted boat conformation for the cyclohexenyl moiety, the pseudoaxial H-8 (H-8pax), and H-9 (H-9pax) were identified by their shift to higher field. NOE correlation of H-9pax with H-7 and of H-8pax with H-4, as well as, the rest of the NOE's of this spin system shown in Figure 1, established both as pseudoaxial,

Table 1
NMR data of micropeptin DR1056 (**1**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H-C correlations[c]	NOE correlations[d]
Ile-1	172.5 s		Ile-2, Thr-3	
2	55.5 d	4.74 dd 9.3,4.6	Ile-6	Thr-NH, Ile-3,4,4′,6,NH, *N*MePhe-*N*Me, Tyr-2
3	37.8 d	1.79 m	Ile-2,4′,5,6	Ile-2,3,4,4′,6,NH, *N*MePhe-*N*Me
4	24.5 t	1.24 m	Ile-2,5,6	Thr-2, Ile-2,3,4′,5,NH
		0.98 m		Thr-2, Ile-2,3,4,NH
5	11.6 q	0.78 t 7.3	Ile-4,4′	Thr-2,3, Ile-4,NH
6	16.2 q	0.81 d 7.0	Ile-2,4,4′	Ile-2,3, *N*MePhe-*N*Me
NH		7.49 d 9.4		Thr-2, Ile-2,3,4,4′,5, *N*MePhe-2,*N*Me, Ahp-6,6-OH
*N*MePhe-1	169.1 s		Ile-NH, *N*MePhe-2	
2	60.7 d	5.00 br d 12.6	*N*MePhe-3′,*N*Me	Ile-NH, *N*MePhe-3,5,5′,*N*Me, Leu-2
3	33.8 t	3.20 br d 13.0	*N*MePhe-5,5′	*N*MePhe-2,3′,5,5′,*N*Me
		2.81 dd 13.0,12.6		*N*MePhe-3,5,5′,*N*Me
4	137.7 s		*N*MePhe-3,3′,6,6′	
5,5′	129.4 d×2	7.13 d×2, 7.1	*N*MePhe-3,3′,7	*N*MePhe-2,3,3′,6,6′,*N*Me, Leu-2,6
6,6′	128.7 d×2	7.25 t×2 7.1		*N*MePhe-5,5′,7, Leu-3′,5,6
7	126.6 d	7.20 t 7.1	*N*MePhe-5,5′	*N*MePhe-6,6′, Leu-6
*N*Me	30.5 q	2.71 s	*N*MePhe-2	Ile-2,3,6,NH, *N*MePhe-2,3,3′,5,5′, Leu-4, Ahp-6-OH
Leu-1	170.9 s		*N*MePhe-*N*Me, Leu-2	
2	47.8 d	4.56 m		*N*MePhe-2,5,5′, Leu-3′,4,5,6, Ahp-6
3	38.5 t	1.52 m	Leu-5,6	Leu-3′,4,5, Ahp-6
		0.27 ddd 10.3,10.0,3.0		*N*MePhe-6,6′, Leu-2,3,4,5,6
4	23.7 d	0.96 m	Leu-3,3′,5,6	Ile-NH, *N*MePhe-*N*Me, Leu-2,3,3′,5,6, Ahp-3,6,6-OH
5	24.0 q	0.66 d 6.4		*N*MePhe-6,6′, Leu-2,3,3′,4,6, Ahp-3,6
6	21.9 q	0.43 d 6.3		*N*MePhe-5,5′,6,6′,7, Leu-2,3′,4,5, Ahp-3,6
Ahp-2	169.4 s		Ahp-3,6	
3	49.3 d	4.38 dt 7.1,9.0		Leu-4,5,6, Ahp-4′,6,NH
4	21.9 t	2.52 m	Ahp-3	Ahp-4′,6-OH,NH
		1.72 m		
5	28.6 t	1.71 m		Ahp-6,6-OH
6	73.6 d	4.87 br s		Ile-NH, Leu-2,3,4,5,6, Ahp-3,5,6-OH
6-OH		5.99 br s		Ile-NH, *N*MePhe-*N*Me, Leu-4, Ahp-4,5,6
NH		7.33 d 9.0		Ahp-3,4, Tyr-2,NH, Thr-3
Tyr-1	170.1 s		Ahp-NH, Tyr-3	
2	53.9 d	4.44 m	Tyr-3′	Ile-2, Ahp-NH, Tyr-3,3′,5,5′,NH, Thr-2
3	35.4 t	3.22 dd 14.6,3.3		Tyr-2,3′,5,5′
		2.56 m		Tyr-2,3,5,5′,NH
4	128.4 s		Tyr-3,3′,6,6′	
5,5′	129.8 d×2	6.93 d 8.1	Tyr-3,3′	Tyr-2,3,3′, Thr-2
6,6′	115.2 d×2	6.58 d 8.1	Tyr-5,5′	Thr-2, Gln-4
7	155.7 s		Tyr-5,5′,6,6′	
7-OH		9.08 br s		
NH		8.50 d 8.8		Ahp-NH, Tyr-2,3′, Thr-2,3
Thr-1	169.0 s		Tyr-NH, Thr-2	
2	54.7 d	4.57 d 9.3	Thr-4	Tyr-2,5,5′,6,6′,NH, Thr-NH, Ile-4,4′,5,NH, Gln-3′,5-NH_2(b), Hpla-2
3	72.1 d	5.48 q 6.3	Thr-4	Ahp-NH, Tyr-NH, Thr-4,NH, Ile-5
4	17.7 q	1.14 d 6.3	Thr-3	Thr-3,NH, Gln-2, Hpla-6,6′
NH		8.02 d 9.3		Thr-2,3,4, Gln-2,3,3′,4, Ile-2
Gln-1	171.8 s		Thr-NH, Gln-2	
2	51.6 d	4.44 dt 4.7,8.0	Gln-3,3′,NH	Thr-NH, Gln-3,3′,4,NH
3	29.9 t	1.81 m	Gln-2,4	Thr-NH, Gln-2,NH
		1.75 m		Thr-2,NH, Gln-2,NH, Hpla-3′
4	31.5 t	2.03 q 7.4	Gln-6-NH	Tyr-6,6′, Thr-NH, Gln-2,NH,5-NH_2(a,b), Hpla-5,5′,6,6′
5	174.4 s		Gln-4	
5-NH_2 (a)		6.80 s		Gln-4
(b)		7.20 s		Thr-2, Gln-4
NH		7.77 d 8.0		Gln-2,3,3′,4, Hpla-2,3,3′
Hpla-1	173.5 s		Gln-NH, Hpla-2	
2	72.7 d	4.02 dd 8.4,3.0	Hpla-3,3′	Thr-2, Gln-NH, Hpla-3,3′,5,5′
3	39.8 t	2.88 dd 13.8,3.0	Hpla-5,5′	Gln-NH, Hpla-2,3′,5,5′,6,6′
		2.54 m		Gln-3′,NH, Hpla-2,3,5,5′,6,6′
4	128.9 s		Hpla-2,3,3′,6,6′	
5,5′	130.4 d×2	7.00 d×2 8.0	Hpla-3,3′	Gln-4, Hpla-2,3,3′,6,6′
6,6′	114.9 d×2	6.62 d×2 8.0	Hpla-5,5′	Thr-4, Gln-4, Hpla-3,3′,5,5′
7	155.8 s		Hpla-5,5′,6,6′	
7-OH		9.08 br s		

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

Table 2
NMR data of micropeptin DR1060 (**2**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H-C correlations[c]	NOE correlations[d]
Ile-1	172.6 s		Ile-2, Thr-3	
2	55.4 d	4.79 dd 9.7,4.6	Ile-6	Ile-3,5,6,NH
3	37.9 d	1.79 m	Ile-4′,5,6	Ile-2,4,4′,5,NH
4	24.5 t	1.48 m	Ile-5,6	Thr-2, Ile-3
		1.00 m		Thr-2, Ile-3,5
5	11.6 q	0.80 t 7.2	Ile-4′	Thr-2,3, Ile-2,3,4′
6	16.2 q	0.83 d 6.2	Ile-4′	Ile-2, *N*MePhe-*N*Me
NH		7.51 d 9.4		Thr-2, Ile-2,3, *N*MePhe-2,*N*Me
*N*MePhe-1	169.2 s		Ile-NH, *N*MePhe-2	
2	60.7 d	5.00 d 10.7	*N*MePhe-3′,*N*Me	Ile-NH, *N*MePhe-3,3′,5,5′,*N*Me, Leu-2
3	34.0 t	3.21 br d 12.6	*N*MePhe-5,5′	*N*MePhe-2,3′,5,5′
		2.81dd 13.3,12.6		*N*MePhe-2,3,5,5′
4	137.7 s		*N*MePhe-3,3′,6,6′	
5,5′	129.4 d×2	7.13 d×2, 7.1	*N*MePhe-3,3′,7	*N*MePhe-2,3,3′6,6′,*N*Me, Leu-2
6,6′	128.7 d×2	7.25 t×2 7.1		*N*MePhe-5,5′,7, Leu-6
7	126.6 d	7.20 t 7.1	*N*MePhe-5,5′	*N*MePhe-6,6′, Leu-6
*N*Me	30.5 q	2.71 s	*N*MePhe-2	Ile-6,NH, *N*MePhe-2,5,5′
Leu-1	170.9 s		*N*MePhe-*N*Me	
2	47.8 d	4.54 dd 11.0,3.5		*N*MePhe-2,5,5′, Leu-3′,4,5,6
3	38.5 t	1.52 m	Leu-5,6	Leu-3′,4,5, Ahp-6
		0.26 ddd 10.3,10.7,3.0		Leu-2,3
4	23.7 d	0.91 m	Leu-5,6	Leu-2,3,5,6, Ahp-6
5	24.0 q	0.65 d 6.3	Leu-6	*N*MePhe-6,6′, Leu-2,3′,4,6
6	22.4 q	0.41 d 6.3	Leu-3,5	*N*MePhe-6,6′,7, Leu-2,4,5
Ahp-2	169.4 s		Ahp-3,5,6	
3	49.0 d	4.37 dt 6.9,9.0		Ahp-5,NH,
4	22.2 t	2.51 m		Ahp-4′,NH
		1.72 m		Ahp-4,6
5	29.9 t	1.71 m		Ahp-3,6
6	73.6 d	4.87 br s		Leu-3,4, Ahp-4,5,6-OH
6-OH		5.99 br s		Ahp-5,6
NH		7.33 d 9.0		Ahp-3,4, HcAla-2,NH Thr-3
HcAla-1	170.6 s		Ahp-NH, HcAla-2	
2	49.9 d	4.33 m	HcAla-NH	Ahp-NH, HcAla-3,3′,4,NH
3	36.6 t	1.81 m	HcAla-2,5,NH	HcAla-2,3′,5,6
		1.48 m		HcAla-2,3,4,5,6,NH
4	31.9 d	1.97 m	HcAla-5,6,8pax,9pax	HcAla-2,3′,5,6,8pax,9peq,9pax,NH
5	132.2 d	5.40 br d 9.6	HcAla-6,7,9peq	HcAla-3,3′,4,6,7
6	133.0 d	5.57 br d 9.6	HcAla-5,8peq	HcAla-3,3′,4,5,7
7	65.4 d	3.97 br m	HcAla-5,8peq,8pax,9peq,9pax	HcAla-5,6,8peq,9pax
8	31.6 t	1.80 m (peq)	HcAla-9pax	HcAla-7,8pax,9pax
		1.20 m (pax)		HcAla-4,8peq,9peq,7-OH
9	26.0 t	1.70 m (peq)	HcAla-5,8peq,8pax	HcAla-4,8pax,9pax
		0.96 m (pax)		HcAla-7,8peq,9peq
7-OH		4.62 d 9.0		HcAla-8pax,NH
NH		8.47 d 8.4		Ahp-NH, HcAla-2,3′,4,7-OH, Thr-3
Thr-1	169.5 s		HcAla-NH, Thr-2	
2	55.2 d	4.61 br d 9.3	Thr-4	HcAla-NH, Thr-NH, Ile-4,4′,5,NH, Gln-3′,5-NH_2(b), Hpla-2
3	71.7 d	5.49 q 6.3	Thr-4	Ahp-NH, HcAla-NH, Thr-4,NH, Ile-5
4	17.7 q	1.21 d 6.3	Ile-4′, Thr-3	Thr-3,NH, Hpla-6,6′
NH		8.25 d 9.3		Thr-2,3,4, Gln-2,3,3′,4
Gln-1	172.3 s		Thr-NH, Gln-2	
2	51.7 d	4.49 dt 5.7,7.7	Gln-3,3′	Thr-NH, Gln-3,3′,4,NH
3	28.9 t	1.82 m	Gln-4	Thr-NH, Gln-2,NH
		1.75 m		Thr-2,NH, Gln-2,NH, Hpla-3′
4	31.5 t	2.10 t 7.0	Gln-6-NH	Thr-NH, Gln-2,NH,5-NH_2, Hpla-5,6
5	174.0 s		Gln-4	
5-NH_2(a)		6.70 s		Gln-4
(b)		7.18 s		Thr-2, Gln-4
NH		7.78 d 7.7		Gln-2,3,3′,4, Hpla-2,3,3′
Hpla-1	173.5 s		Gln-NH, Hpla-3	
2	72.7 d	4.00 dd 9.0,3.5	Hpla-3′	Thr-2, Gln-NH, Hpla-3,3′,5,5′
3	39.8 t	2.88 dd 14.1,3.5	Hpla-5,5′	Gln-NH, Hpla-2,3′,5,5′,6,6′
		2.54 m		Gln-3′,NH, Hpla-2,3,5,5′,6,6′
4	128.9 s		Hpla-2,3,3′,6,6′	
5,5′	130.3 d×2	7.00 d×2, 7.7	Hpla-3,3′	Gln-4, Hpla-2,3,3′,6,6′
6,6′	114.9 d×2	6.63 d×2, 7.7	Hpla-5,5′	Thr-4, Gln-4, Hpla-3,3′,5,5′
7	155.7 s		Hpla-5,5′,6,6′	
2-OH		5.48 br s		
7-OH		9.07 s		

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

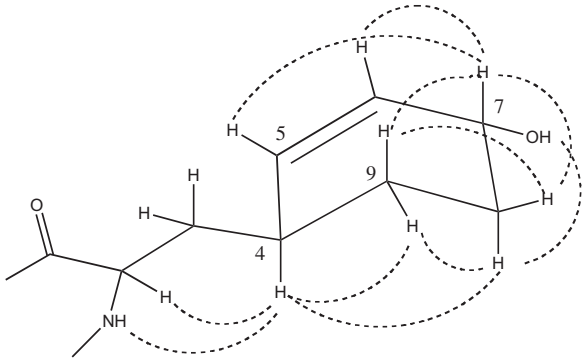


Figure 1. NOE correlations that established the relative configuration of the hydroxycyclohexenyl moiety.

and the relative configuration of the hydroxycyclohexenyl moiety as 4*S**,7*R**. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments allowed the assignment of all the remaining amino acids that composed **2** as: Ile, *N*Me-Phe, Leu, Ahp, Thr, Gln, and Hpla. The Ile-*N*MePhe-Leu sequence could be deduced by both HMBC correlations between the carboxyl of *N*MePhe and Ile NH, the carboxyl of *N*,*N*-disubstituted-Leu and the *N*Me of *N*MePhe and by NOE correlations of Ile NH with both H-2 and *N*Me of *N*MePhe and of H-2 of *N*MePhe with H-2 of *N*,*N*-disubstituted-Leu. NOE correlations between Ahp H-6 and Leu H-3 (δ 1.52 ppm) and H-4 connect the Leu residue to the Ahp moiety. HMBC correlation of Ahp-NH with HcAla-CO established their connectivity. The sequence HcAla-Thr-Gln-Hpla could be deduced by either the HMBC or NOE correlations, namely, HMBC correlation of AcAla-NH with the Thr-carboxyl, Thr-NH with the Gln-carboxyl and Gln-NH with Hpla-carboxyl, and NOE correlations (see Table 2). Finally, the lactone ring was established through the HMBC correlation of Thr H-3 and Ile carboxyl. The relative configuration of C-6 of Ahp was established in the same way as for **1**. Marfey's analysis established the absolute configuration of all proteogenic amino acids as L. The absolute configuration of HcAla was not established due to the unavailability of the appropriate reference amino acid. The D, (*R*), absolute configuration of Hpla was established as described above. On the basis of these arguments the structure of micropeptin DR1060 was established as **2**.

Micropeptin DR1006 (**3**) was isolated as a glassy solid. It presented a high-resolution ESIMS sodiated molecular cluster ion, *m*/*z* 1029.5278, corresponding to the molecular formula, $C_{51}H_{74}N_8O_{13}Na$. Comparison of the ^{1}H NMR spectra of **3** and **1** revealed that the two distinctive phenol doublet signals (δ 6.58 and 6.93 ppm) in **1** were replaced with two doublet aliphatic methyl groups (δ 0.74 and 0.86 ppm) in **3**, suggesting that **3** contains either Val or Leu instead of the Tyr moiety in **1**. COSY correlations established the latter amino acid, in **3**, as leucine. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (see Table 3) allowed the assignment of the side chains of all of the amino acids that composed **3** as: Ile, *N*Me-Phe, 2×Leu, Ahp, Thr, Gln, and Hpla. The carboxy groups of Thr, Gln and Hpla were assigned through their HMBC correlations with the side chain protons. The carboxy groups of Ile, *N*Me-Phe, Leu(I), Ahp, and Leu(II) were assigned through HMBC correlation of the carboxyl of the amino acid with an amide- or α-proton of the adjacent amino acid coupled with an NOE that confirmed their vicinity (see Table 3). The sequence of the amino acids in the peptide chain was assigned through NOE correlations of: Ile-NH and H-2 of *N*Me-Phe, H-2 of *N*Me-Phe and H-2 of Leu(I), H-3′ of Leu(I) and H-6 of Ahp, NH of Ahp and H-2 of Leu(II), NH of Leu(II) and H-3 of Thr, NH of Thr and H-2 of Gln, NH of Gln and H-2 Hpla. The closure of the lactone was confirmed through the NOE of Thr methyl group and Ile NH and Methyl-5. The relative configuration of C-6 of Ahp, in **3**, was established in the same way as for **1**. Marfey's analysis established the absolute configuration of all proteogenic amino acids, in **3**, as L. The D, (*R*), absolute configuration of Hpla was established by comparison of its retention time with that of a racemic mixture on a chiral HPLC column. On the basis of the arguments described above the structure of micropeptin 1006 was established as **3**.

The crude cyanobacteria extracts exhibited significant inhibition of the serine proteases trypsin and chymotrypsin and amino peptidase N at a concentration of 1 mg/ml. The activity-guided purification of the proteases inhibiting components of the extract revealed that the aeruginosins 298A and B[4,13] were responsible for the inhibition of trypsin, while micropeptins **1**–**4** were responsible for the inhibition of chymotrypsin. This finding was in accordance with the known activity of these two groups of compounds; the aeruginosins, which contain arginine or its derivative, are usually potent trypsin inhibitors while micropeptins that contain aromatic or aliphatic amino acid next to the Ahp moiety are usually potent chymotrypsin inhibitors.[10] The inhibitory activity of **1**–**4** was determined for serine protease chymotrypsin, elastase and trypsin. Micropeptins **1**–**4** did not inhibit trypsin at a concentration of 45.5 μM. Chymotrypsin was inhibited by micropeptin DR1056 (**1**) with an IC_{50} of 1.6 μM, by micropeptin DR1060 (**2**) with an IC_{50} of 5.3 μM, by micropeptin DR1006 (**3**) with an IC_{50} of 2.7 μM and by micropeptin SF909 (**4**) with an IC_{50} of 4.4 μM. Elastase was inhibited by micropeptin DR1006 (**3**) with an IC_{50} of 13.0 μM, but not by micropeptin DR1056 (**1**), and micropeptin DR1060 (**2**) at a concentration of 50 μM. The relative potency of **1**–**4** to chymotrypsin is in accordance with the published data.[10] The inhibition of elastase by **3**, correlates as well with the published data for micropeptins that contain leucine in the fifth position from the C-terminus.[19] Micropeptin SF909 (**4**) inhibited amino peptidase N (APN) with an IC_{50} of 4.2 μM and cytosolic AP with an IC_{50} of 4.7 μM.

3. Conclusions

In conclusion, the study described here the isolation of three new micropeptins together with three known metabolites from the water-bloom material of the cyanobacterium *M. aeruginosa*. The research expanded the library of the micropeptins and illuminated the variety of the metabolites produced by these fresh water cyanobacteria. In particular, the protease inhibition assays showed that several of the micropeptins possess significant biological activity and selectivity toward different serine proteases. The inhibition of amino peptidases by micropeptin SF909 (**4**) is unprecedented and to the best of our knowledge, this is the only known case where micropeptin inhibits amino peptidase.

4. Experimental section

4.1. General experimental procedures

Mass spectra were recorded on a Waters MaldiSynapt instrument. UV spectra were recorded on an Agilent 8453 spectrophotometer. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). 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, 100.62 MHz for ^{13}C. DEPT, COSY-45, gTOCSY, gROESY, gHSQC, gHMQC, gHMBC, gNHHSQC, and gNHHMBC spectra were recorded using standard Bruker pulse sequences. 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.

Table 3
NMR data of micropeptin DR1006 (**3**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H–C correlations[c]	NOE correlations[d]
Ile-1	172.6 s		Thr-3	
2	55.4 d	4.78 dd 9.7,4.8	Ile-4,6,NH	Ile-3,6,NH
3	37.8 d	1.80 m	Ile-5,6	Ile-2,4,4′,5,6,NH
4	24.5 t	1.22 m	Ile-6	Ile-3,4′,5,6, Ahp-6,6-OH
		1.00 m		Ile-3,4,NH
5	11.6 q	0.80 t 7.3	Ile-6	Thr-4, Ile-3,4,NH
6	16.2 q	0.83 d 6.8	Ile-4′	Ile-2,3,4,NH, *N*MePhe-*N*Me
NH		7.51 d 9.6		Thr-4, Ile-2,3,4′,5,6, *N*MePhe-2,*N*Me, Leu(I)-4
*N*MePhe-1	169.2 s		Ile-NH	
2	60.7 d	5.00 br dd 10.9,1.8	*N*MePhe-*N*Me	Ile-NH, *N*MePhe-3,5,5′,*N*Me, Leu(I)-2
3	33.8 t	3.21 dd 13.0,1.8		*N*MePhe-2,3′,5,5′
		2.80 dd 13.0,10.9		*N*MePhe-3,5,5′,*N*Me
4	137.7 s		*N*MePhe-6,6′	
5,5′	129.4 d×2	7.13 d×2, 7.3	*N*MePhe-3,3′,7	*N*MePhe-2,3,3′,*N*Me, Leu(I)-2,6
6,6′	128.7 d×2	7.25 t×2 7.3	*N*MePhe-5,5′,7	*N*MePhe-*N*Me
7	126.6 d	7.20 t 7.3	*N*MePhe-5,5′	Leu(I)-6
*N*Me	30.6 q	2.71 s	*N*MePhe-3′	Ile-3,6,NH, *N*MePhe-2,3′,5,5′,6,6′, Ahp-6-OH
Leu(I)-1	170.9 s		*N*MePhe-*N*Me	
2	47.7 d	4.56 dd 10.9,3.6	Ahp-3,NH	*N*MePhe-2,5,5′, Leu(I)-3′,4,6
3	38.5 t	1.50 m	Leu(I)-5,6	Leu(I)-3′,4,5
		0.25 dd 13.8,10.0,3.6		Leu(I)-2,3,5,6, Ahp-6
4	22.4 d	0.90 m	Leu(I)-3′,5	Ile-NH, Leu(I)-2,3
5	24.0 q	0.65 d 6.5	Leu(I)-6	Leu(I)-3,3′,6
6	22.0 q	0.41 d 6.5	Leu(I)-5	*N*MePhe-5,5′,7, Leu(I)-2,3′,5, Ahp-4
Ahp-2	169.5 s		Leu(I)-2	
3	49.0 d	4.37 ddd 11.9,9.3,6.9		Ahp-5,NH
4	21.6 t	2.52 m	Ahp-5	Leu(I)-6, Ahp-4′,NH,6-OH
		1.70 m		Ahp-4
5	29.2 t	1.72 m	Ahp-4	Ahp-3,6,6-OH
6	73.6 d	4.87 s		Leu(I)-3′, Ahp-5,6-OH, Ile-4
6-OH		5.99 br s		*N*MePhe-*N*Me, Ahp-4,5, Ile-4, Leu(II)-5
NH		7.33 d 9.3		Ahp-3,4, Leu(II)-2, Thr-3
Leu(II)-1	170.8 s		Ahp-NH	
2	50.7 d	4.30 br dd 10.0,8.8		Ahp-NH, Leu(II)-3,3′,4,6,NH
3	39.5 t	1.48 m	Leu(II)-5,6	Leu(II)-2,5,6, Ile-NH
		1.34 m		Leu(II)-2,4,6,NH
4	24.7 d	1.80 m	Leu(II)-5,6	Leu(II)-2,3′,5,6
5	23.7 q	0.86 d 6.8	Leu(II)-6	Ahp-6-OH, Leu(II)-3,4,6,
6	21.0 q	0.74 d 6.4	Leu(II)-5	Leu(II)-2,3,3′,5,NH, Thr-2
NH		8.40 d 8.8		Leu(II)-2,3′,6, Thr-2,3
Thr-1	169.3 s		Thr-2, Leu(II)-NH	
2	55.1 d	4.63 br d 9.2	Thr-4,NH	Leu(II)-6,NH, Thr-4,NH
3	71.9 d	5.49 q 5.8	Thr-4	Ahp-NH, Leu(II)-NH, Thr-4,NH, Hpla-2
4	17.8 q	1.20 d 5.8		Thr-2,3,NH, Ile-5,NH, Hpla-6,6′
NH		8.20 d 9.2		Thr-2,3,4, Gln-2,3
Gln-1	172.2 s		Gln-2,3,3′, Thr-NH	
2	51.6 d	4.52 dt 5.3,8.1	Gln-3,3′,4	Thr-NH, Gln-3,3′,4,NH
3	28.5 t	1.87 m	Gln-4	Thr-NH, Gln-2,NH
		1.80 m		Gln-2,NH,
4	31.4 t	2.07 t 8.4	Gln-5-NH	Gln-2,NH,5-NH_2(a,b), Hpla-6,6′
5	174.0 s		Gln-4	
5-NH_2 (a)		6.70 s		Gln-4
(b)		7.19 s		Gln-4
NH		7.80 d 8.1		Gln-2,3,3′,4, Hpla-2,3,3′
Hpla-1	173.5 s		Hpla-3′, Gln-3′	
2	72.7 d	4.00 br dd 9.0,6.5	Hpla-3′	Thr-3, Gln-NH, Hpla-3,3′,5,5′
3	39.7 t	2.86 dd 14.2,4.6	Hpla-5,5′	Gln-NH, Hpla-2,3′,5,5′,6,6′
		2.52 m		Gln-NH, Hpla-2,3,5,5′,6,6′
4	128.9 s		Hpla-5,5′,6,6′	
5,5′	130.4 d×2	7.00 d×2, 8.3	Hpla-3,3′	Hpla-2,3,3′,6,6′
6,6′	114.9 d×2	6.62 d×2, 8.3	Hpla-5,5′,7-OH	Thr-4, Gln-4, Hpla-3,3′,5,5′,7-OH
7	155.7 s		Hpla-5,5′,6,6′,7-OH	
2-OH		5.48 d 6.5		
7-OH		9.07 s		Hpla-6,6′

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

4.2. Cyanobacterial material

Microcystis sp., TAU strains IL-237 and IL-238, were collected in August and September 2001, from Dalton water reservoir next to Gush Halav, Israel. Samples of the cyanobacteria are deposited at the culture collection of Tel Aviv University.

4.3. Isolation procedure

The freeze-dried cells (IL-237 and IL-238, 700 g) were extracted with 7:3 MeOH/H_2O (3×3L). The crude extract (50.1 g) was evaporated to dryness and separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash column with increasing amounts of MeOH in water. The combined fractions 5 and 6 (6:4 and 1:1 MeOH/H_2O, 545 mg) were subjected to a Sephadex LH-20 column in 1:1 chloroform/methanol to obtain fifteen fractions. Fraction 8 from the Sephadex LH-20 column (85 mg) was separated on a reversed-phase HPLC (YMC ODS-A, 5 μm, 250 mm×20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 62:38 acetonitrile/water to obtain pure compound **4** (3.9 mg, retention time 23.5 min, 0.0005% yield based on the dry weight of the bacteria). Fractions 9–12 from the Sephadex LH-20 column (227 mg) were separated on a reversed-phase HPLC (YMC ODS-A, 5 μm, 250 mm×20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 25:75 acetonitrile/water to yield pure aeruginosin 298B (4.4 mg, retention time of 12.5 min, 0.0006% yield based on the dry weight of the bacteria), and aeruginosin 298A (10.5 mg, retention time of 17.5 min, 0.0015% yield based on the dry weight of the bacteria). The combined fractions 8–12 (3:7 MeOH/H_2O through MeOH, (2.15 g)) were subjected to a Sephadex LH-20 column in 1:1 chloroform/methanol to obtain thirteen fractions. Fractions 9–13 (987 mg) were subjected to a reversed-phase HPLC (YMC-Pack C-8 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 45:55 acetonitrile/0.1% aq TFA to obtain ten fractions: fraction 8 (29.0 mg, retention time of 23.2 min), contained almost pure **1** and was purified on the same column eluted with 45:55 acetonitrile/0.1% aq TFA to obtain pure compound **1** (6.5 mg, 0.0009% yield based on the dry weight of the bacteria) eluted from the column with retention time of 39 min. Fraction 6 from the initial HPLC separation (15.2 mg, retention time of 20.2 min), was subjected to same column eluted with 48:52 acetonitrile/0.1% aq TFA to afford pure compound **2** (1.5 mg, retention time of 31.6 min, 0.0002% yield based on the dry weight of the bacteria). Fraction 9 from the HPLC separation (9.2 mg, retention time of 26.0 min) was subjected to same column eluted with 48:52 acetonitrile/0.1% aq TFA to afford pure compound **3** (1.9 mg, retention time of 33.0 min 0.0003% yield based on the dry weight of the bacteria).

*4.3.1. Micropeptins DR1056 (**1**).* Colorless glassy solid; $[\alpha]_D^{25}$ −21.4 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.65), 224 (4.25), 278 (3.49) nm. For NMR data see Table 1; HR ESIMS *m*/*z* 1079.5071 (MNa^+, calcd for $C_{54}H_{72}N_8NaO_{14}$ *m*/*z* 1079.5066).

*4.3.2. Micropeptin DR1060 (**2**).* Colorless glassy solid; $[\alpha]_D^{25}$ −21.0 (*c* 1.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.52), 225 (4.06), 278 (3.37) nm. For NMR data see Table 2; HR ESIMS *m*/*z* 1083.5365 (MNa^+, calcd. for $C_{54}H_{76}N_8NaO_{14}$ *m*/*z* 1083.5379).

*4.3.3. Micropeptin DR1006 (**3**).* Colorless glassy solid; $[\alpha]_D^{25}$ −111 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.31), 226 (3.92), 276 (3.27) nm. For NMR data see Table 3; HR ESIMS *m*/*z* 1029.5278 (MNa^+, calcd. for $C_{51}H_{74}N_8NaO_{13}$ *m*/*z* 1029.5273).

*4.3.4. Micropeptin SF909 (**4**).* Data were found to be identical with reported data.[12]

4.3.5. Aeruginosin 298A. Data were found to be identical with reported data.[4]

4.3.6. Aeruginosin 298B. Data were found to be identical with reported data.[13]

4.4. Determination of the absolute configuration of the amino acids

Compounds **1**–**3** (0.5 mg) were dissolved in 6 N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110 °C for 20 h. In another experiment, 0.25 mg portions of compounds **1**–**3** were first oxidized with Jones reagent (one drop) in acetone (1 mL) at 0 °C for 10 min. Following the usual work-up, the residue was dissolved in 6 M HCl (1 mL) and placed in a sealed glass bomb at 104 °C for 18 h. After the removal of HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (150 μL). A solution of (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA) (3.3 μMol) in acetone (110 μL) and 1 M $NaHCO_3$ (160 μL) was added to each reaction vessel and the reaction mixture was stirred at 40 °C for 2 h. A 2 M HCl solution (80 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 C18, 10 μm, 4.6×300 mm, flow rate: 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/acetonitrile to 1:1 TEAP/acetonitrile within 60 min. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids. HPLC analysis of Marfey's derivatives of **1** and **3** established the following retention times of the derivatized amino acids: L-Thr, 26.8 min; D-Thr, 32.8 min; L-Glu, 32.0 min; D-Glu, 34.5 min L-Ile, 51.9 min; D-Ile, 58.1 min; L-Leu, 53.5 min; D-Leu, 58.0 min; L-NMePhe, 57.3 min;
L-Tyr, 63.0 min; D-Tyr, 67.6 min.

4.5. Determination of absolute configuration of the hydroxy acids

A 0.25 mg portion of **1**–**3** was dissolved in 6 M HCl (1 mL) and the reaction mixture was then placed in a sealed glass bomb at 104 °C for 20 h. The ethereal extract of the acid hydrolysate of **1** and **2** was dried and the residue was treated with (−)-menthol in toluene with *p*-toluenesulfonic acid under reflux for 72 h. The reaction mixture was washed with $NaHCO_3$ solution and water, dried, and analyzed on a Knauer GmbH Eurospher 100 C18, 10 μm, 4.6×300 mm HPLC column using 65:35 water/acetonitrile as eluent at a flow rate of 1 mL/min. The D-isomer showed a retention time of 29.3 min and the L-isomer a retention time of 31.8 min. D-Hpla was determined for compounds **1** and **2**.[17] The ethereal extract of the acid hydrolysate of **3** 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 (TEAA) aq buffer, pH 4: MeOH. The Hpla from the micropeptins was compared with standard L,D-Hpla. Retention time of D-Hpla on the chiral column 4.33 min (L-Hpla 3.97 min).

4.6. Protease inhibition assays

Trypsin, chymotrypsin, and elastase were purchased from Sigma Chemical Co. Trypsin (1 mg/ml) and chymotrypsin (10 mg/ml) were dissolved in 0.05 M Tris–HCl/100 mM NaCl/1 mM $CaCl_2$, pH 7.5 buffer solution. Benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA) the trypsin substrate was dissolved in a solution of 1:9 DMSO: Tris-buffer (0.85 g/ml). Suc-Gly-Gly-*p*-nitroanilide (SGGPNA) the substrate for chymotrypsin was dissolved in Tris-buffer (1 mg/ml). Test samples were dissolved in DMSO (1 mg/ml). A 100 μl buffer solution, 10 μl enzyme solution, and 10 μl sample solution were added to each micro titer plate well and pre-incubated at 37 °C for 10 min. Then, 10 μl of substrate solution was added and the kinetic of the reaction was measured at 405 nm, 37 °C for 30 min. Elastase (75 mg/ml) was dissolved in 0.2 M Tris–HCl, pH 8 buffer solution. *Z*-Gly-Pro-Arg-4MβNA-acetate salt, the thrombin substrate, was dissolved in Tris buffer (0.5 mg/ml). *N*-Suc-Ala-Ala-Ala-*p*-nitroanilide, the elastase substrate, was dissolved in Tris buffer (1 mg/ml). The test samples were dissolved in DMSO (1 mg/ml). For elastase, 150 μl buffer solution, 10 μl enzyme solution, and 10 μl sample solution were added to each micro titer plate well and pre incubated at 30 °C for 20 min. Then, 30 μl of substrate solution was added and the kinetic of the reaction was measured at 405 nm, 37 °C for 20 min. The procedure for the inhibition assays of the amino proteases APN and cytosolic AP was published elsewhere.[20]

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Supplementary data

^{1}H and ^{13}C NMR spectra of compounds **1**–**3** are available. Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2010.06.071. These data include MOL files and InChiKeys of the most important compounds described in this article.

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