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Protease inhibitors from a water bloom of the cyanobacterium *Microcystis aeruginosa*

Vered Reshef and Shmuel Carmeli*

School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv Tel Aviv 69978, Israel

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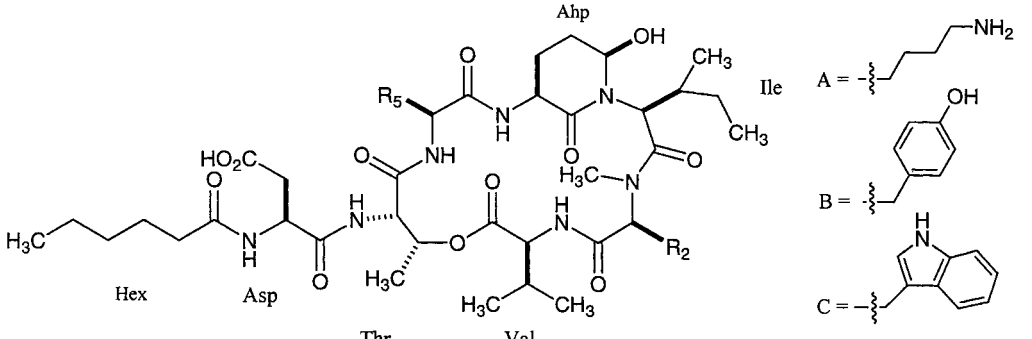
Abstract—Five new protease inhibitors, micropeptins SD944 (**1**), SD979 (**2**), SD999 (**3**) and SD1002 (**4**) and microginin SD755 (**5**) were isolated along with two known inhibitors, micropeptin SF995 (**6**) and microcin SF608 (**7**), from the hydrophilic extract of *Microcystis aeruginosa*. The planar structure of compounds **1**–**5** was determined by homonuclear and inverse-heteronuclear 2D-NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was studied using Marfey's method for HPLC. Compounds **1**–**4**, **6** and **7** are serine-protease inhibitors while compound **5** was found to inhibit amino-proteases.

Cyanobacteria strains that produce massive water blooms and have the capacity to synthesize hepatotoxins of the microcystin family (*Microcystis*, *Anabaena*, *Nostoc*, *Oscillatoria* and *Nodularia* spp.)[1] usually contain protease inhibitors. The synthesis of these protease inhibitors in the cyanobacterial cell does not necessarily correlate with the synthesis of the toxic microcystins, although the protease inhibitors were shown to enhance the activity of the microcystins in mice.[2] Only about 50% of the *Microcystis* water blooms show hepatotoxicity to mammals and other animals but almost all contain protease inhibitors.[3] Recently, several groups of inhibitors, of serine and amino proteases, were isolated from toxic and non-toxic *Microcystis* species.[1] The most abundant are depsipeptides, such as cyanopeptolin A,[4] which contain the modified amino acid, 3-amino-6-hydroxy-2-piperidone (Ahp). The modified linear peptides, aeruginosins (such as aeruginosin 298-A[5]) and the linear peptides of the microginin type (such as microginin[6]) are less frequently found. The trideca-peptides of the microviridin type are rarely found.[7]

A non-toxic strain of the cyanobacterium *Microcystis aeruginosa* (strain IL-215) was collected, in the summer of 1998, from a pond in the Dan district sewage treatment



Micropeptin SD944 (**1**) R_2=B, R_5=A
Micropeptin SD979 (**2**) R_2=B, R_5=B
Micropeptin SD999 (**3**) R_2=D, R_5=E
Micropeptin SD1002 (**4**) R_2=C, R_5=B
Micropeptin SF995 (**6**) R_2=C, R_5=E

Keywords: cyanobacteria; *Microcystis*; protease inhibitors.
* Corresponding author. Tel.: +972-3-6408550; fax: 972-3-6409293; e-mail: carmeli@ccsg.tau.ac.il.

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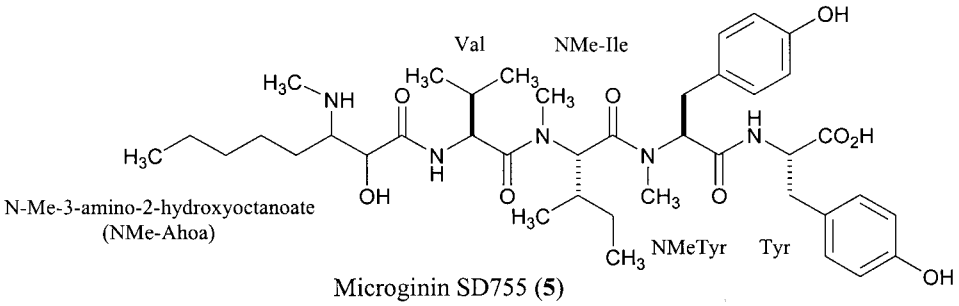
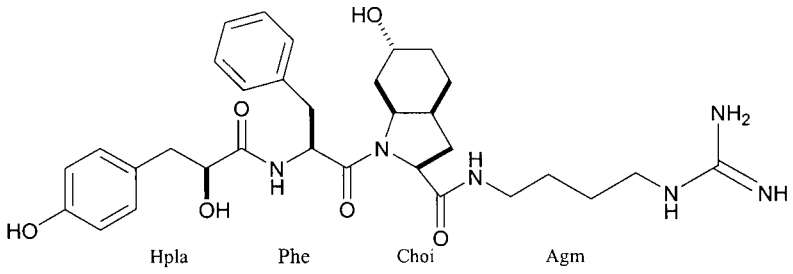


Microginin SD755 (**5**)



Microcin SF608 (**7**)

plant, the Shofdan. The sample of the cyanobacterium was freeze-dried and extracted with 70% MeOH in H_2O. The extract was found to inhibit several serine and amino proteases. The active extract was flash-chromatographed on an ODS column. Two fractions eluted from the column, with 60 and 70% MeOH in H_2O, exhibited protease inhibitory activity and were further purified on a reversed-phase HPLC column. Five new protease inhibitors: micropeptin SD944 (**1**, 11.1 mg), micropeptin SD979 (**2**, 12.0 mg), micropeptin SD999 (**3**, 6.5 mg), micropeptin SD1002 (**4**, 3.5 mg) and microginin SD755 (**5**, 5.6 mg) were isolated, along with two known inhibitors, micropeptin SF995 (**6**, 14.7 mg) and microcin SF608 (**7**, 25.3 mg). The latter two, **6** and **7**, were recently isolated in our laboratory from a *Microcystis* sp.[8]

Examination of the ^1H and ^{13}C NMR spectra, of compounds **1**–**4**, reveal that they are related in structure to micropeptin SF995 (**6**)[8] and to other known micropeptins.[4] Several proton signals in the ^1H NMR spectra are indicative of the micropeptin structure. (i) The presence of at least one aromatic amino acid. (ii) An *N*-methyl at ~2.70 ppm. (iii) A broad quartet methine at ~5.45 ppm and a doublet methyl at ~1.15 ppm, characteristic of the bridging threonine moiety of these peptolides. (iv) The two broad singlets of the amino-hydroxy-piperidone (Ahp) moiety, at ~5 and 6 ppm, characteristic of the methine at position 5 and its adjacent hydroxyl proton, respectively. In the ^{13}C NMR spectra, the presence of two methine carbons, adjacent to an oxygen at ~74 and 72 ppm, are indicative of the micropeptin skeleton.

Micropeptin SD944 (**1**) was isolated as an amorphous white solid. The molecular formula of **1**, $C_{46}H_{72}N_8O_{13}$, was deduced from high-resolution FAB MS measurements of its protonated molecular cluster ion (*m/z* 945.5318). The ^1H NMR, in DMSO-d_6 revealed five doublet NH proton signals and one broad singlet (2H) signal between δ 7.3 and 8.6 ppm, pointing to seven amino acid residues (taking into account the NMe-aromatic amino acid and the Ahp residue that count as two amino acids). One of the latter amino acids contains a primary amine. The two two-proton doublets, at 6.98 and 6.63 ppm, indicate that the aromatic amino acid of this peptolide is a NMe-tyrosine. Analysis of the 1D (^1H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 1) revealed the seven amino acid units and the fatty acid that constitute compound **1**, namely: valine, NMe-tyrosine, *N*,*N*-disubstituted isoleucine, amino-hydroxy-piperidone (Ahp), lysine, threonine, aspartic acid and hexanoic acid. The ester linkage of **1** arises from the carbonyl of valine and the hydroxyl of threonine. All the proton and carbon signals of the latter residues, except the lysine carbonyl, were assigned by the COSY, TOCSY, HMQC and HMBC data. No HMBC correlation was observed between the carbonyl of lysine and the lysine side chain protons. The assignment of the carbonyl at δ_C 170.4 ppm to lysine is based on the NOE correlation between the proton at position 2 of the lysine and Ahp-NH as well as the HMBC correlation of the Ahp-NH proton with this carbonyl. The amino acid sequence of micropeptin SD944 (**1**) was determined from the HMBC correlations (see Table 1) of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Val-NMe-Tyr, Ahp-Lys, Lys-Thr, Thr-Asp and Asp-Hex), the NMe protons with Ile-carbonyl and the Ile-H-2 with C-5 of the Ahp residue. The ester bond was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine. The amino acid sequence could also be assembled from the ROESY data (see Table 1). Acid hydrolysis of **1** and derivatization with Marfey's reagent,[9] followed by HPLC analysis, demonstrated the L-stereochemistry of the valine, NMe-tyrosine, isoleucine, lysine, threonine and aspartic acid residues. Jones oxidation[10] of **1**, followed by a similar hydrolysis, derivatization and HPLC analysis, demonstrated an L-stereochemistry for the Ahp residue (the oxidation and subsequent hydrolysis liberated glutamic acid from Ahp).

The gross structures and absolute stereochemistry of **2**, **3** and **4** were elucidated using similar arguments and

Table 1. NMR data of micropeptin SD944 (**1**)

Position		δ_C, mult.[a]	δ_H, mult., J (Hz)	LR H-C correlations[b]	NOE correlations[c]
Val	1	172.5 s		Val-4, Thr-3	
	2	56.5 d	4.59 m	Val-3,4,5	Val-3,4,5, Thr-3
	3	30.3 d	2.01 m	Val-4,5	Val-2,NH
	4	17.9 q	0.75 d 6.7	Val-3,5	Val-2,NH, Ahp-OH, Thr-3
	5	19.3 q	0.85 d 6.6	Val-3,4	Val-2,NH, Ahp-OH
	NH		7.66 d 9.2		Val-3,4,5, Tyr-2, Ahp-OH
NMeTyr	1	169.6 s		Val-NH, Tyr-2	
	2	60.8 d	5.02 brd 11.8	Tyr-NMe	Ile-2,Val-NH, Tyr-3,5,5′,NMe
	3	33.3 t	2.65 dd 14.0,11.8	Try-5,5′	Tyr-2,5,5′
			3.15 dd 14.0,5.6		Tyr-2,5,5′
	4	127.5 s		Tyr-3,6,6′	
	5,5′	130.5 d	6.98 d 8.3	Tyr-3	Ile-2,4, Tyr-2,3,6,6′,NMe
	6,6′	115.5 d	6.63 d 8.2		Ile-2,4, Tyr-5,5′
	7	156.4 s		Tyr-5,5′,6,6′	
	NMe	30.7 q	2.71 s		Tyr-2,5,5′
Ile	1	169.9 s		Tyr-NMe, Ile-2	
	2	54.3 d	4.44 m	Ile-3,6	Ile-3,4,5,6, Tyr-2, 5,5′,6,6′
	3	33.3 d	1.75 m	Ile-2,6	Ile-2,4,5,6
	4	23.9 t	0.62 m	Ile-5,6	Ile-2,3, Tyr-5,5′,6,6′
			1.08 m		Ile-2,3, Tyr-5,5′,6,6′
	5	10.4 q	0.62 t 2.6		Ile-2,3,6
	6	14.0 q	-0.11 d 5.2	Ile-5	Ile-2,3,4,5, Tyr-6,6′
Ahp	1	169.4 s		Ahp-3′,5	
	2	49.1 d	4.45 m		Ahp-3,4
	3	21.8 t	2.56 m	Ahp-2	Ahp-2,4,5,NH, Thr-NH
			1.75 m		Ahp-2,4,5,NH, Thr-NH
	4	29.9 t	1.73 m (2H)		Ahp-2,5,NH, Thr-NH
	5	74.1 d	4.92 brs	Ahp-2, Ile-2	Ahp-3,4,OH
	OH		6.12 brs		Ahp-5, Val-4,5,NH
	NH		7.32 d 9.1		Ahp-2,3,4, Lys-2,NH, Thr-3
Lys	1	170.4 s		Ahp-NH	
	2	52.3 d	4.23 brs		Ahp-NH, Lys-3,4,5,NH
	3	29.6 t	1.48 m		Lys-2,6,NH,NH_2
			1.99 m		Lys-2,6,NH,NH_2
	4	22.5 t	1.28 m	Lys-3,5	Lys-2,6,NH,NH_2
	5	26.5 t	1.50 m	Lys-3	Lys-2,3,6,NH,NH_2
	6	38.8 t	2.76 m	Lys-4,5	Lys-3,4,5,NH_2
	NH_2		7.70 brs		Lys-3,4,5,6
	NH		8.52 d 8.1		Lys-2,3,4,Thr-2,3, Ahp-NH
Thr	1	169.2 s		Lys-NH, Thr-2	
	2	55.0 d	4.58 d 4.3	Thr-4	Lys-NH, Thr-4,Hex-2
	3	72.0 d	5.47 brq 6.3	Thr-4	Thr-4, Ahp-NH, Val-4, Lys-NH
	4	17.9 q	1.19 d 6.5		Val-2, Thr-2,3 Asp-NH
	NH		7.62 d 9.2		Thr-2,3, Asp-2,NH, Ahp-3,4
Asp	1	171.7 s		Asp-3, Thr-NH	
	2	49.6 d	4.65 q 5.6	Asp-3,NH	Asp-3,NH, Thr-NH
	3	35.7 t	2.76 dd 5.3,17.1	Asp-2	Asp-2,NH
			2.51 dd 8.6,17.1		Asp-2,NH
	4	171.9 s		Asp-2,3	
	NH		8.25 d 7.6		Asp-2,3, Thr-4,NH,Hex-2
Hex	1	172.9 s		Hex-2, Asp-NH	
	2	35.3 t	2.13 t 7.4	Hex-3	Hex-3,4,5,NH, Thr-2
	3	24.9 t	1.52 m	Hex-2	Hex-2,4,5,6
	4	30.9 t	1.26 m	Hex-2,3,4,6	Hex-2,3,5,6
	5	22.4 t	1.26 m	Hex-3,4,6	Hex-2,3,4,6
	6	13.9 q	0.84 t 7.1		Hex-3,4,5

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 400 ms.

methodology. Micropeptin SD979 (**2**) was isolated as an amorphous white solid. FAB MS measurements, for micropeptin SD979 (**2**), furnished a molecular formula of $C_{49}H_{69}N_7O_{14}$. The seven amino acid residues were identified as Ahp, NMe-Tyr, *N*,*N*-disubstituted Ile, Val, Tyr, Thr and Asp by NMR spectral analysis, all demonstrating the L-stereochemistry.[9] The presence of hexanoic acid was also indicated by the NMR data. The proton and carbon NMR chemical shifts of these building units were found to be similar to the corresponding units in micropeptin SD944 (**1**) and micropeptin SF995 (**6**). A partial amino acid sequence (Val-NMe-Tyr, Ahp-Tyr, Tyr-Thr and Asp-Hex) of micropeptin SD979 (**2**) was determined from HMBC correlations (see Table 2). The ester linkage between Thr and Val was inferred by the HMBC cross-peak between H-3 of threonine and the carbonyl of valine, leaving two

Table 2. NMR data of micropeptin SD979 (**2**)

Position		δ_C, mult.[a]	δ_H, mult., J (Hz)	LR H-C correlations[b]	NOE correlations[c]
Val	1	172.3 s		Val-2, Thr-3	
	2	56.4 d	4.56 m	Val-4,5, NMeTyr-2	Val-3,4,5,NH
	3	30.7 d	2.00 m	Val-2,4,5	Val-2,4,5,NH, Ahp-OH
	4	17.8 q	0.73 d 6.7	Val-2,3,5	Val-2,NH, Ahp-OH, NMeTyr-NMe
	5	19.3 q	0.84 d 6.3	Val-4	Val-2, NMeTyr-NMe
	NH		7.63 d 9.3		Val-2,3,4,Ahp-OH, NMeTyr-NMe
NMeTyr	1	169.5 s		Val-NH, NMeTyr-2	
	2	60.7 d	5.02 brd 10.7	NMe-Tyr-3,3′,5,5′,NMe, Val-2	Val-NH, NMeTyr-3,5,5′,NMe, Ile-2
	3	33.4 t	3.12 dd 15.0,6.7 2.65 dd 10.7,15.0	NMe-Tyr-2,5,5′	NMeTyr-2,3′,5,5′ NMeTyr-3,5,5′
	4	127.4 s		NMeTyr-3,3′,6,6′	
	5,5′	130.4 d	6.98 d 8.2	NMeTyr-2,3,3′,5,5′	Ile-5,6, NMeTyr-2,3,3′,6,6′,NMe
	6,6′	115.4 d	6.63 d 8.2	NMeTyr-5,5′,6,6′	Ile-5,6, NMeTyr-5,5′
	7	156.4 s		NMeTyr-5,5′6,6′	
	NMe	30.2 q	2.70 s	NMeTyr-2	Val-2,4,5,NH, Ahp-OH, NMeTyr-2,5,5′
Ile	1	169.9 s		NMeTyr-NMe, Ile-2	
	2	54.3 d	4.41 d 10.5	Ile-6	Ile-4′,5,6, NMeTyr-2
	3	33.2 d	1.77 m	Ile-2,4,4′,5,6	Ile-4,4′,5,6
	4	23.8 t	0.68 m 1.06 m	Ile-5,6	Ile-3 Ile-2,3,Ahp-5
	5	10.4 q	0.63 t 6.2	Ile-6	Ile-2,3, NMeTyr-5,5′,6,6′, Ahp-3,3′,4
	6	14.0 q	-0.11 d 6.2	Ile-2,4,5	Ile-2,3, NMeTyr-5,5′,6,6′
Ahp	1	169.4 s		Ahp-3,5	
	2	49.3 d	4.43 m	Ahp-3,4	Ahp-4,NH
	3	21.6 t	1.76 m 2.57 m	Ahp-2,5	Ahp-2,3′,5,OH, Ile-5 Ahp-3,4,NH,OH, Ile-5
	4	29.8 t	1.77 m (2H)		Ahp-2,3′,5, Ile-5
	5	74.1 d	4.92 brs	Ahp-2,3,4, Ile-2	Ahp-3,4,OH, Ile-4′
	OH		6.09 brs		Ahp-3,3′,4,5,Val-3,4,NH, NMeTyr-NMe
	NH		7.32 d 8.2		Ahp-2,3,3′,4, Tyr-NH,Thr-3
Tyr	1	170.1 s		Ahp-NH	
	2	54.3 d	4.40 m	Tyr-3,3′	Tyr-3′,5,5′,NH, Ahp-NH
	3	35.3 t	2.59 dd 3.8,14.5 3.23 dd 3.6,14.5	Tyr-5,5′	Tyr-3′,5,5′,NH Tyr-2,3,5,5′
	4	128.3 s		Tyr-6,6′,3,3′	
	5,5′	129.8 d	6.95 d 8.2	Tyr-3,3′,5,5′	Tyr-2,3,3′,6,6′, Thr-2
	6,6′	115.2 d	6.59 d 8.2	Tyr-5,5′,6,6′	Tyr-5,5′,OH
	7	155.8 s		Tyr-5,5′,6,6′	
	OH		9.19 s		Tyr-6,6′
	NH		8.55 d 8.1		Tyr-2,3,5,5′, Ahp-NH, Thr-2,3
Thr	1	168.9 s		Tyr-NH, Thr-2	
	2	54.5 d	4.54 m	Thr-4	Thr-3,4, Tyr-5,5′,NH
	3	72.2 d	5.45 brq 6.3	Thr-4	Thr-2,4, Ahp-NH, Hex-4,5, Tyr-NH
	4	17.6 q	1.13 d 6.3	Thr-2,3	Thr-2,3,NH
	NH		7.52 d 9.1		Asp-2, Thr-4
Asp	1	171.8 s		Asp-2	
	2	49.9 d	4.58 m	Asp-3	Asp-3,3′, Hex-3, Thr-NH
	3	36.6 t	2.39 dd 5.3,14.5 2.57 dd 3.8,14.5		Asp-2,3′,NH Asp-2,3
	4	169.9 s			
	NH		8.25 d 7.6		Asp-3,Hex-2
Hex	1	172.5 s		Hex-2,3, Asp-NH	
	2	35.5 t	2.10 t 7.4	Hex-3	Asp-NH, Hex-3,4,5
	3	24.9 t	1.51 t 6.9	Hex-2,4,5,6	Asp-2, Hex-2,4,5
	4	31.0 t	1.24 m	Hex-2,3,5,6	Thr-3, Ahp-NH, Hex-2,3
	5	21.9 t	1.24 m	Hex-3,4,6	Thr-3, Ahp-NH, Hex-2,3
	6	13.9 q	0.83 t 6.3	Hex-4,5	

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 400 ms.

Table 3. NMR data of micropeptin SD999 (**3**)

Position		δ_C, mult.[a]	δ_H, mult., J (Hz)	LR H-C correlations[b]	NOE correlations[c]
Val	1	172.2 s		Thr-3	
	2	56.6 d	4.58 m	Val-4,5	Val-3,4,5
	3	30.8 d	2.06 m	Val-4,5	Val-2,4,5,NH
	4	17.7 q	0.75 d 6.4	Val-2,3,5	Val-2,3,NH
	5	19.3 q	0.85 d 6.6	Val-4	Val-2,3,NH
	NH		7.61 d 9.1		Val-3,4,5, Kyn-2,NMe
NMeKyn	1	169.4 s		Val-NH, Kyn-2	
	2	57.0 d	5.60 brd 9.3	Kyn-3	Val-NH, Kyn-3,3′,10,NMe
	3	37.9 t	3.08 dd 16.9,2.5		Kyn-2,3,10
			3.39 dd 16.9,9.3		Kyn-2,3′,NMe
	4	199.0 s		Kyn-3,10	
	5	116.5 s		Kyn-7,9	
	6	151.6 s		Kyn-8,10	
	7	117.1 d	6.72 d 8.4	Kyn-9	Kyn-8,9
	8	134.6 d	7.24 t 7.7	Kyn-10	Kyn-7,9
	9	114.7 d	6.53 t 7.5	Kyn-7	Kyn-7,8,10
	10	131.5 d	7.82 d 8.1	Kyn-8	Kyn-2,3,3′,9
	NMe	30.9 q	2.67 s		Val-NH, Kyn-2,3,3′
Ile	1	170.5 s		Ile-2, Kyn-NMe	
	2	54.0 d	5.46 d 10.5	Ile-6	Ile-3,4,,5,6, Ahp-NH
	3	34.0 d	2.03 m	Ile-5,6	Ile-2,5,6, Ahp-2,5
	4	24.1 t	0.85 m	Ile-5,6	Ile-2,4′
			1.24 m		Ile-4,5
	5	10.5 q	0.77 t 6.6	Ile-6	Ile-2,3,4′
	6	15.4 q	0.60 d 6.6	Ile-5	Ile-2,3, Ahp-4
Ahp	1	169.6 s		Ahp-5	
	2	49.4 d	4.44 d 5.6	Ahp-3′	Ahp-3′,4,NH, Ile-4′
	3	21.9 t	1.78 m	Ahp-2,5	Ahp-2,3
			2.52 m		Ahp-3′,NH
	4	30.0 t	1.78 m (2H)		Ahp-2,3,3′,5, Ile-6
	5	74.2 d	5.05 brs	Ile-2	Ahp-4,OH, Ile-3
	OH		6.00 brs		Ahp-5
	NH		7.37 d 9.1		Ahp-2,3′,Ile-2, Arg-2,NH
Arg	1	170.2 s		Ahp-NH	
	2	52.1 d	4.26 m		Arg-3,3′,4,5,NH, Ahp-NH
	3	27.6 t	1.45 m		Arg-2,5,6(NH)
			2.02 m		Arg-2,4,5,6(NH)
	4	25.3 t	1.52 m (2H)	Arg-3	Arg-2,3′,5,6(NH),NH
	5	40.0 t	3.08 m (2H)		Arg-2,3,3′,4,6(NH)
	6(NH)		7.59 t 6.6		Arg-3,3′,4,5
	7	156.9 s			
	NH		8.58 d 7.7		Arg-2,4, Ahp-NH, Thr-2,3
Thr	1	169.0 s		Thr-2	
	2	54.8 d	4.61 brd 10.4	Thr-4	Thr-3,4,NH, Arg-NH
	3	72.2 d	5.49 brd 6.4	Thr-4	Thr-2,4,NH, Arg-NH
	4	17.9 q	1.18 d 6.7	Thr-2,3	Thr-2,3,NH, Asp-3,NH
	NH		7.61 d 7.8		Thr-2,3,4, Asp-2,3′,NH
Asp	1	171.6 s		Thr-NH, Asp-2	
	2	49.6 d	4.62 m	Asp-3,3′,NH	Asp-3,3′,NH, Thr-NH
	3	35.6 t	2.50 m		Asp-2,NH, Thr-4
			2.75 dd 5.1,16.5		Asp-2,NH, Thr-NH
	4	171.8 s		Asp-3,3′	
	NH		8.25 d 7.6		Asp-2,3,3′, Thr-4,NH, Hex-2,3
Hex	1	172.8 s		Asp-NH, Hex-2,3	
	2	35.2 t	2.12 t 7.4	Hex-3	Asp-NH, Hex-3,4,5,6
	3	24.9 t	1.45 m	Hex-2	Asp-NH, Hex-2
	4	30.9 t	1.25 m	Hex-2,3,5,6	Hex-2
	5	21.9 t	1.25 m	Hex-2,3,4,6	Hex-2
	6	13.9 q	0.83 t 6.8	Hex-4,5	Hex-2

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 400 ms.

connections to be made by Ahp-isoleucine and threonine-aspartic acid. A ROESY experiment provided the Ahp-Ile connection, by correlating the α proton of Ile to the H-5 of Ahp, and the Thr-Asp connection by correlating the NH of Thr to the α proton of Asp.

Micropeptin SD999 (**3**) was isolated as an amorphous white solid. Mass spectral analysis (HR FAB MS) of **3** showed a pseudomolecular ion (*m*/*z* 1000.5473) consistent with the molecular formula $C_{47}H_{73}N_{11}O_{13}$. The aromatic region of the ^{1}H NMR spectrum revealed four aromatic protons of a

Table 4. NMR data of micropeptin SD1002 (**4**)

Position		δ_C, mult.[a]	δ_H, mult., J (Hz)	LR H-C correlations[b]	NOE correlations[c]
Val	1	172.2 s		Val-2, Thr-3	
	2	56.2 d	4.66 m	Val-4,5	Val-3,5, Trp-6
	3	31.3 d	2.04 m	Val-4,5	Val-4,5, Thr-2, Trp-6
	4	17.7 q	0.80 d 6.8	Val-5	Val-2
	5	19.2 q	0.89 d 6.8	Val-4	Val-2, Thr-3
	NH		6.90 d 8.6		Val-2,4,5,Trp-2,NMe, Ahp-2
NMeTrp	1	169.8 s		Val-NH, Trp-NMe	
	2	59.8 d	5.25 dd 2.4,11.6	Trp-NMe,3	Val-NH, Trp-3′,5,6,NMe
	3	24.2 t	3.00 dd 11.6,12.7		Trp-5,9
			3.30 brd 12.7		Trp-2,5,9
	4	109.1 s		Trp-3,3′,5,9,NH	
	4a	127.6 s		Trp-3,3′,5,6,8,9	
	5	118.1 d	7.57 d 7.8	Trp-7	Ile-2,4′,5, Trp-2,3,3′,6
	6	118.6 d	6.96 t 6.0	Trp-8	Val-2,3, Trp-2,5,NMe
	7	121.0 d	7.03 t 7.6	Trp-5	Ile-4,4′,5, Trp-8
	8	111.5 d	7.28 d 8.1	Trp-6	Trp-7,NH
	8a	136.7 s		Trp-5,7,9,NH	
	9	124.5 d	7.12 s	Trp-3,3′,NH	Trp-NH,7
	NH		10.82 s		Trp-8,9
	NMe	30.7 q	2.73 s		Tyr-2,5,9
Ile	1	170.0 s		Trp-NMe, Ile-2	
	2	53.6 d	4.40 d 10.6	Ile-6	Ile-3,4,4′,5,6, Trp-5
	3	32.7 d	1.64 m	Ile-2,6	Ile-2, Ahp-5
	4	23.6 t	0.92 m	Ile-5,6	Ile-2, Trp-7
			0.39 m		Ile-2, Trp-5,7
	5	10.1 q	0.38 t 6.4		Ile-2, Trp-5,7
	6	12.6 q	-0.72 d 6.5	Ile-2	Ile-2
Ahp	1	169.0 s		Ahp-2,3	
	2	49.4 d	4.37 m		Ahp-3′,4,NH
	3	21.9 t	2.45 m	Ahp-2,4	Ahp-4
			1.78 m		Ahp-2, 5
	4	30.0 t	1.70 m (2H)		Ahp-2,3
	5	73.2 d	4.86 brs		Ahp-3′,OH, Ile-3
	OH		5.97 brs		Ahp-5
	NH		7.21 d 8.7		Ahp-2, Tyr-5,5′,6,6′
Tyr	1	170.7 s		Tyr-3	
	2	53.9 d	4.42 m	Tyr-3	Tyr-3,3′,5,5′,NH
	3	35.2 t	2.60 m	Tyr-5	Tyr-2,NH
			3.20 m		Tyr-2
	4	128.6 s		Tyr-3,6,6′	
	5,5′	129.8 d	6.97 d 8.4	Tyr-3,3′,5	Tyr-2,3,3′6,6′,NH, Thr-NH, Ahp-NH
	6,6′	115.1 d	6.60 d 8.4	Tyr-5,5′,6	Ahp-NH, Tyr-5,5′, Thr-NH
	7	155.8 s		Tyr-5,5′,6,6′	
	NH		8.55 d 8.6		Tyr-2,3,5,5′, Asp-3, Thr-2,3
Thr	1	168.8 s		Tyr-NH,2, Thr-2	
	2	54.5 d	4.56 m	Thr-4	Thr-4, Val-3, Tyr-NH
	3	72.3 d	5.45 brq 6.5	Thr-4	Thr-4, Val-3,5, Asp-3, Tyr-NH
	4	17.7 q	1.13 d 6.3	Thr-2	Thr-2
	NH		7.57 d 7.8		Asp-NH, Tyr-5,5′,6,6′
Asp	1	171.3 s		Thr-2,NH	
	2	49.6 d	4.60 m	Asp-3,3′,NH	Asp-3,NH
	3	35.6 t	2.71 m	Asp-2,NH	Asp-2,NH, Thr-3, Tyr-NH
			2.49 m		Asp-NH
	4	171.8 s		Asp-2,3,3′	
	NH		8.20 d 7.7		Asp-2,3,3′, Thr-NH, Hex-2
Hex	1	172.7 s		Hex-2,3, Asp-NH	
	2	35.2 t	2.13 t 6.8	Hex-3	Asp-NH
	3	25.0 t	1.51 m	Hex-2	
	4	30.9 t	1.26 m	Hex-2,3,5,6	
	5	21.9 t	1.26 m	Hex-3,4,6	
	6	13.9 q	0.85 t 6.7	Hex-4,5	

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 400 ms.

Table 5. NMR data of microginin SD755 (**5**)

Position		δ_C, mult.[a]	δ_H, mult., J (Hz)	LR H-C correlations[b]	NOE correlations[c]
Tyr	1	172.9 s		Tyr-2,3,3′	
	2	53.7 d	4.35 ddd 4.8,7.8,9.2	Tyr-3,3′,NH	Tyr-NH,6,6′
	3	35.8 t	2.79 dd 9.2,13.9	Tyr-2,5,5′	Tyr-3′,NH
			2.94 dd 4.8,13.9		Tyr-3,NH
	4	127.6 s		Tyr-3,3′,6,6′	
	5,5′	129.4 d	6.95 d 8.2	Tyr-3,3′,5,5′	Tyr-6,6′,NH
	6,6′	115.0 d	6.62 d 8.2	Tyr-5,5′,6,6′,OH	Tyr-2,5,5′,OH, NMeAhoa-3-NH
	7	156.0 s		Tyr-5,5′,6,6′,OH	
	OH		9.18 s		Tyr-6,6′
	NH		7.94 d 7.8		Tyr-2,3,3′,5,5′, NMeTyr-2
NMeTyr	1	170.1 s		NMeTyr-2,5,5′,6,6′, Tyr-NH	
	2	55.8 d	5.45 dd 4.9,11.0	NMeTyr-2,5,5′	Tyr-NH, NMeTyr-6,6′
	3	33.3 t	3.04 dd 4.9,15.0		Tyr-NH, NMeTyr-3′,5,5′
			2.72 dd 11.0,15.0		Tyr-NH, NMeTyr-3
	4	127.3 s		NMeTyr-3,3′,6,6′	
	5,5′	130.0 d	6.95 d 8.2	NMeTyr-3,3′,5,5′	NMeTyr-6,6′, NMeIle-NMe
	6,6′	115.1 d	6.62 d 8.2	NMeTyr-5,5′,6,6′,OH	NMeTyr-2,5,5′,OH, NMeIle-NMe
	7	156.1 s		NMeTyr-5,5′,6,6′,OH	
	OH		9.19 s		NMeTyr-6,6′
	NMe	30.3 q	2.65 s	NMeTyr-3	NMeIle-5,6, Val-3
NMeIle	1	169.6 s		NMeIle-2, NMeTyr-NMe	
	2	56.9 d	4.90 d 10.6	NMeIle-5,6,NMe	NMeIle-3,4,5,6,NMe
	3	32.6 d	1.90 m	NMeIle-2,4,4′,5	NMeIle-2,4,4′
	4	23.6 t	0.63 m		NMeIle-NMe
			1.06 m	NMeIle-5,6	NMeIle-2,3,NMe, Val-2
	5	11.1 q	0.75 t 6.3	NMeIle-4,6	NMeIle-2,NMe, NMeTyr-NMe
	6	15.9 q	0.71 d 6.4	NMeIle-2,4′	NMeIle-2, NMeTyr-5,5′,6,6′,NMe
	NMe	29.5 q	2.41 s	NMeIle-2	NMeIle-2,3,4,4′,5, Val-2,3, NMeTyr-2,5,5′,6,6′
Val	1	170.8 s		NMeIle-NMe	
	2	53.2 d	4.47 dd 4.6,8.8	Val-4,5,NH	Val-3,4,NH, NMeIle-NMe
	3	29.8 d	1.56 m	Val-4,5	Val-2,4,5,NH, NMeIle-NMe-4′, NMeTyr-3,5,5 ‘,6,6′
	4	20.0 q	0.77 d 6.7	Val-5	Val-2,3, NMeTyr-OH
	5	16.5 q	0.66 d 6.7	Val-4	Val-3,NH
	NH		7.75 d 8.8		NMeAhoa-2,2-OH,3, Val-2,3,5
NMeAhoa	1	170.2 s		NMeAhoa-2, Val-NH	
	2	68.7 d	4.37 brs		Val-NH, NMeAhoa-2-OH,3,3-NMe, 4,5
	2-OH		6.55 d 5.1		Val-NH, NMeAhoa-2
	3	60.2 d	3.35 m	NMeAhoa-3-NMe,4	Val-NH, NMeAhoa-2,3-NH,3-NMe,4,5,6,7
	3-NH		8.60 brs		NMeAhoa-3, Tyr-6,6′
	3-NMe	30.9 q	2.57 s		NMeAhoa-2,3,4
	4	26.3 t	1.46 m	NMeAhoa-5,6	NMeAhoa-2,3,3-NMe,6,7
			1.43 m		
	5	25.0 t	1.25 m	NMeAhoa-4,7	NMeAhoa-2,3
			1.36 m		
	6	31.4 t	1.17 m	NMeAhoa-4,7,8	NMeAhoa-3,4
	7	21.8 t	1.23 m	NMeAhoa-6,8	NMeAhoa-2,3,4,8
	8	13.9 q	0.84 t 7.1	NMeAhoa-6,7	NMeAhoa-7

Carried out on an ARX-500 Bruker instrument.
[a] Multiplicity and assignment from HMQC experiment.
[b] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s.
[c] By ROESY experiment, mixing time 500 ms.

1,2-disubstituted aromatic system, (δ_H 6.53 dd, 6.72 d, 7.24 dd and 7.82 d). The chemical shift of these protons and the aromatic carbons (δ_C 151.6 s, 134.6 d, 131.5 d, 117.1 d, 116.5 s and 114.7 d) pointed to a mono-substituted phenyl-ketone moiety. Indeed, a signal of an unsaturated carbonyl carbon (δ_C 199.0) was observed in the ^{13}C NMR spectrum of **3**. The latter residue was identified as *N*-methyl kynurenine by analysis of the NMR data and comparison of the proton chemical shifts with those of a synthetic material [(δ_H 6.54, dd, J=7.9, 7.1 Hz (H-9), 6.77, d, J=8.3 Hz, 7.27, dd, J=7.1, 8.3 Hz, (H-8), 7.72, d, J=7.79 Hz (H-10)]. Micropeptin SD999 (**3**) also contains two other modified amino acid residues (Ahp and *N*,*N*-disubstituted Ile), four normal amino acid residues (Val, Arg, Thr and Asp), all of the L-stereochemistry, and a fatty acid residue (hexanoic acid). A partial amino acid sequence (Val-NMe-Kyn, Ahp-Arg, Thr-Asp and Asp-Hex) of micropeptin SD999 (**3**) was determined from HMBC

correlations (see Table 3) of the NH protons with vicinal carbonyl carbons, the NMe protons with Ile-carbonyl and the Ile-H-2 with C-5 of the Ahp residue. The ester bond was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine, leaving the last connection to be assigned by ROESY correlatins between the NH of arginine and the α and β protons of threonine.

Micropeptin SD1002 (**4**) was isolated as an amorphous white solid. FAB MS measurements furnished a molecular formula of $C_{51}H_{70}N_8O_{13}$ for micropeptin SD1002 (**4**). Micropeptin SD1002 (**4**) contains three modified amino acid residues (L-Ahp, L-NMe-Trp and *N*,*N*-disubstituted L-Ile, four normal amino acid residues (L-Val, L-Tyr, L-Thr and L-Asp) and a fatty acid residue (hexanoic acid). The amino acid sequence of micropeptin SD1002 (**4**) was assembled by HMBC correlations (see Table 4) of the NH protons with the vicinal carbonyl carbons (Val-NMe-Trp, Tyr-Thr, Thr-Asp and Asp-Hex) and the Trp-NMe protons with Ile-carbonyl. The ester bond was assigned from an HMBC correlation between H-3 of threonine and the carbonyl of valine, leaving two connections to be made by Ahp-isoleucine and Ahp-tyrosine. Since compound **2** is very similar to compound **4** (NMe-Trp in compound **4** instead of NMe-Tyr in compound **2**), it was especially helpful in the determination of the Tyr-Ahp-Ile portion of compound **4** which did not connect by HMBC correlation. A ROESY experiment provided the Ahp-Ile connection by correlating H-3 of Ile to the H-5 of Ahp and provided the Ahp-Tyr connection by correlating the NH of Ahp to the H-5,5′ of Tyr.

Microginin SD755 (**5**) was isolated as an amorphous white solid. FAB MS measurements, for microginin SD755 (**5**), furnished a molecular formula of $C_{40}H_{61}N_5O_9$. Microginin SD755 (**5**) is a linear peptide that contains a β-amino acid residue, *N*-methyl-3-amino-2-hydroxy octanoic acid (NMe-Ahoa), two *N*-methylated amino acids, NMe-Ile and NMe-Tyr, and two normal amino acid residues (Val, Tyr). The structures of the acid residues were determined by analysis of the 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 5). The amino acid sequence of Microginin SD755 (**5**) was assembled from HMBC correlations (see Table 5) of the NH protons and vicinal carbonyl carbons (Tyr-NMe-Tyr, Val-NMeAhoa), and of the NMe protons of NMeIle and carbonyl of Val and of the NMe protons of NMeTyr and the carbonyl of NMeIle. A ROESY experiment provided correlations between the two terminal amino acids NMe-Ahoa and Tyr. Integration of the proton signals, of the amine at position 3 of NMeAhoa, shows a value equivalent to two protons. The last two findings suggested that compound **5** is situated in a cyclic zwitterionic structure. Acid hydrolysis of **5** and derivatization with Marfey's reagent,[9] followed by HPLC analysis, demonstrated the L-stereochemistry of the valine, tyrosine, NMe-isoleucine and NMe-tyrosine. The stereochemistry of the chiral centers of NMeAhoa was not determined.

The inhibitory activity of **1**–**4** was determined for two enzymes, the serine proteases trypsin and chymotrypsin. Micropeptin SD944 (**1**) and Micropeptin SD999 (**3**) inhibited trypsin with IC_{50} values of 8.0 and 4.0 μg/mL, respectively, but not chymotrypsin at 45.0 μg/mL. Micropeptin SD979 (**2**) and Micropeptin SD1002 (**4**) inhibited chymotrypsin with IC_{50} values of 2.4 and 3.2 μg/mL, respectively, but not trypsin at 18.0 μg/mL. Microginin SD755 (**5**) inhibits Bovine Amino Peptidase N (APN) with a IC_{90} value of 14.0 μg/mL,[11] but does not inhibit Bovine neutral endo peptidase (NEP) and Angiotensin converting enzyme (ACE) at a concentration of 48 μg/mL.

1. Experimental

1.1. Instrumentation

High resolution MS were recorded on a Fisons VG Auto-SpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^{1}H and 125.76 MHz for ^{13}C. DEPT, COSY-45, HMQC and HMBC 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.

1.2. Isolation procedure

The freeze-dried cells (345 g) were extracted with 7:3 MeOH/H_2O. The crude extract (44.5 g) was evaporated and separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash column with increasing amounts of MeOH in water. Fraction 7 (6:4 MeOH/H_2O) was separated on a Sephadex LH-20 gel-filtration column with 1:1 $CHCl_3$/MeOH. The combined fractions, from the Sephadex LH-20 column, were subjected to a reversed-phase HPLC (C-18 5 μm, 250 mm×20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 6:4 0.1% TFA in water/acetonitrile to obtain two semi-pure compounds in fraction 6 (retention time of 20.0 min) and fraction 7 (retention time of 21.8 min). Fraction 6 was subjected to a reversed-phase HPLC (C-18 5 μm, 250 mm×20.0 mm, DAD at 238 nm, 40:30:30 0.1% TFA in water/methanol/acetonitrile, flow rate 5.0 mL/min). Compound **1** (11.1 mg), 0.0011% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 39.0 min. Fraction 7 was subjected to a reversed-phase HPLC (YMC C-18 5 μm, 300 mm×10 mm, DAD at 238 nm, 7:3 methanol/water, flow rate 2.0 mL/min). Compound **5** (5.6 mg), 0.0016% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 10.5 min. Fraction 8 (7:3 MeOH/H_2O), from the original flash column, was separated on a Sephadex LH-20 size exclusion column with 1:1 $CHCl_3$/MeOH to obtain two fractions. One of the fractions was twice subjected to a reversed-phase HPLC (C-18 5 μm, 250 mm×20.0 mm, DAD at 238 nm, 6:4 0.1% TFA in water/acetonitrile, 1:1 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **3** (6.5 mg), 0.0019% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 42.8 min. The other fraction, obtained from the Sephadex LH-20 gel-filtration column, was subjected to a reversed-phase HPLC (Econosil C-18 10 μm, 250 mm×22.5 mm, DAD at 238 nm, 7:3 methanol/water, flow rate 5.0 mL/min).

Compound **6** (14.7 mg), 0.0042% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 63.0 min. Another semi-pure compound (**7**) was eluted from the same column and subjected to a reversed-phase HPLC (C-18 5 μm, 250 mm×20.0 mm, DAD at 238 nm, 7:3 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **7** (25.3 mg), 0.0073% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 35.5 min. A second batch of freeze-dried cells (131 g) was extracted with 7:3 MeOH/H_2O. The crude extract (21.2 g) was evaporated and separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash column with increasing amounts of MeOH in water. Fraction 7 (6:4 MeOH/H_2O) was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, 45:55 water/methanol, flow rate 5.0 mL/min). A semi-pure compound (retention time of 15.0 min) was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, 1:1 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **2** (12.0 mg), 0.0092% yield based on the dry weight of the bacteria, was eluted from the column with a retention time of 20.4 min. Fraction 8 (7:3 MeOH/H_2O) from the flash column was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, 40:60 water/methanol, flow rate 5.0 mL/min) to obtain fraction 3 (retention time of 17.9 min). Fraction 3 was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, 35:65 water/methanol, flow rate 5.0 mL/min) to obtain a semi-pure compound (retention time of 22.3 min.). The semi pure compound was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, 1:1 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **4** (3.5 mg), 0.0027% yield based on the dry weight of the bacteria, eluted from the column with a retention time of 26.5 min.

1.2.1. Micropeptin SD944 (1). $[\alpha]_D^{25}=-23.0$ (*c* 2.0, MeOH); UV λ_{max} (MeOH) 215 nm (ϵ 15950), 281 nm (ϵ 3290); for NMR data see Table 1. HRFABMS *m/z* 945.5318 (MH^+, calcd for $C_{46}H_{72}N_8O_{13}$, −2.3 ppm error).

1.2.2. Micropeptin SD979 (2). $[\alpha]_D^{25}=-53.6$ (*c* 6.5, MeOH); UV λ_{max} (MeOH) 225 nm (ϵ 11400), 281 nm (ϵ 2000); for NMR data see Table 2. HRFABMS *m/z* 1002.4792 (MNa^+, calcd for $C_{49}H_{69}N_7NaO_{14}$, 0.7 ppm error).

1.2.3. Micropeptin SD999 (3). $[\alpha]_D^{25}=-45.5$ (*c* 3.2, MeOH); UV λ_{max} (MeOH) 226 nm (ϵ 23440), 257 nm (ϵ 7600); for NMR data see Table 3. HRFABMS *m/z* 1000.5473 (MH^+, calcd for $C_{47}H_{73}N_{11}O_{13}$, −0.6 ppm error).

1.2.4. Micropeptin SD1002 (4). $[\alpha]_D^{25}=-70.0$ (*c* 2.0, MeOH); UV λ_{max} (MeOH) 222 nm (ϵ 29560), 281 nm (ϵ 5150); for NMR data see Table 4. HRFABMS *m/z* 1025.4987 (MNa^+, calcd for $C_{51}H_{70}N_8NaO_{13}$, −2.6 ppm error).

1.2.5. Microginin SD755 (5). $[\alpha]_D^{25}=-70.7$ (*c* 2.8, MeOH); UV λ_{max} (MeOH) 225 nm (ϵ 35300), 281 nm (ϵ 6800); for NMR data see Table 5. HRFABMS *m/z* 756.4536 (MH^+, calcd for $C_{40}H_{61}N_5O_9$, 1.4 ppm error).

1.3. Determination of the absolute configuration of the amino acids

0.5 mg portions of compounds **1**–**5** were dissolved in 6N 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**–**4** were first oxidized with Jones reagent (1 drop) in acetone (1 mL) at 0°C for 10 min. Following the usual work-up, the residue was dissolved in 6N HCl (1 mL) and placed in a sealed glass bomb at 108°C for 18 h. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (40 μL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA). The *N*-[(-dinitrophenyl)-5-L-alanine amide]-amino acid (AA) derivatives, from hydrolysates, were compared with similary derivatized standard AA by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10 μm, 4.6 mm× 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. Retention times of the derivatized amino acids were: L-Arg, 24.5 min; D-Arg, 26.0 min; L-Asp, 30.6 min; D-Asp, 32.3 min; L-Glu, 32.0 min; D-Glu, 34.5 min; L-Ile, 51.9 min; D-Ile, 58.1 min; L-Lys, 53.9 min; D-Lys, 56.7 min; L-Thr, 29.7 min; D-Thr, 35.0 min; L-Tyr, 63.0; D-Tyr, 67.6 min; L-Val, 45.0 min; D-Val, 51.6 min; L-NMe-Ile, 58.5 min; D-NMe-Ile, 59.3 min; L-NMe-Kyn, 53.6 min; L-NMe-Trp, 46.3 min; D-NMe-Trp, 47.0 min; L-NMe-Tyr, 62.5 min; D-NMe-Tyr, 63.2 min. HPLC analysis of derivatized hydrolysates of **1** established L-Asp, L-Ile, L-Lys, L-Thr, L-Val, L-NMe-Tyr; that of **2** established L-Asp, L-Ile, L-Thr, L-Tyr, L-Val, L-NMe-Tyr; that of **3** established L-Arg, L-Asp, L-Ile, L-Thr, L-Val; that of **4** established L-Asp, L-Ile, L-Thr, L-Tyr, L-Val, L-NMe-Trp and that of **5** established L-Tyr, L-Val, L-NMe-Ile, L-NMe-Tyr. HPLC analysis of the FDAA derivatives of oxidized **1**–**4** hydrolysates establish L-Glu for all four compounds and thus confirmed the L configuration of the Ahp units in these compounds.

1.4. Determination of the absolute configuration of the NMe-kynurenine

L-Abrine (13.3 mg) in acetic acid (1 ml) was ozonized at 15°C for 2 min. Conc. HCl (5 drops) was added and the reaction mixture allowed to stand for 18 h at room temperature. The HCl was removed by repeated evaporation, in vacuo. The residue was dissolved in 25% aq. NH_4OH and extracted with CH_2Cl_2. The aqueous layer was subjected to a reversed-phase HPLC (YMC Pack ODS-A 10 μm, 250 mm×20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 30:35:35 water/methanol/acetonitrile to obtain L-NMe-Kyn (1.5 mg, yield 12%). ^1H NMR (DMSO-d_6) δ 2.58, brs, (N-Me), 3.54, dd, *J*=18.2, 6.3 Hz (H-3), 3.62, dd, *J*=18.2, 4.1 Hz (H-3′), 3.97, brm, (H-2), 6.50, m, (NH), 6.54, dd, *J*=7.9, 7.1 (H-9), 6.77, d, *J*=8.3 Hz (H-7), 7.20,

m, (NH_2), 7.27, dd, J=7.1, 8.3 Hz (H-8), 7.72, d, J=7.9 Hz (H-10). Following the usual work-up, the residue was resuspended in water (40 μL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA). It was analyzed by HPLC: 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 L-isomer showed a retention time of 53.6 min, and hence the NMe-Kyn was established as L-NMe-Kyn for **3**.

1.5. Protease inhibition assays

Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris–HCl/100 mM NaCl/1 mM $CaCl_2$ to prepare a 1 mg/mL solution. Chymotrypsin was dissolved in 50 mM Tris–HCl/100 mM NaCl/1 mM $CaCl_2$/1 mM HCl to prepare a 1 mg/mL solution. A 2 mM solution of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (for trypsin) and Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) in the appropriate buffer solution was used as substrate solution. The test sample was dissolved in ethanol and diluted with the same buffer solution that was used for the enzyme and substrate. A 100 μL buffer solution, 10 μL enzyme solution and 10 μL of test solution were added to each microtiter plate well and preincubated at 37°C for 5 min. Then, 100 μL of substrate solution was added to begin the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37°C for 30 min. The procedure for the inhibition assays of the amino proteases APN, NEP and ACE was published elsewhere.[11]

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