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Eight novel serine proteases inhibitors from a water bloom of the cyanobacterium *Microcystis* sp.

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ABSTRACT

Eight new secondary metabolites, micropeptin MM836 (**1**), micropeptin MM850 (**2**), micropeptin MM916 (**3**), micropeptin MM932 (**4**), micropeptin MM978 (**5**), anabaenopeptin MM823 (**6**), anabaenopeptin MM850 (**7**), and anabaenopeptin MM913 (**8**), as well as the known anabaenopeptin B (**9**) were isolated from the hydrophilic extract of the cyanobacterium *Microcystis* sp. that was collected from a fishpond in Kibbutz Ma'agan Michael, Israel, in September 2006. The structure of the pure natural products was established by spectroscopic methods including 1D and 2D NMR, UV, and MS techniques. The absolute configuration of the chiral centers of the compounds was determined using Marfey's method. The inhibitory activity of the compounds was determined against the serine proteases, trypsin, chymotrypsin, thrombin and elastase.

1. Introduction

Freshwater cyanobacteria produce a diverse mixture of toxic and non-toxic metabolites. In most cases, the non-toxic secondary metabolites are cyclic and non-cyclic modified peptides, usually members of five distinct families: micropeptins,[1] anabaenopeptins,[2] aeruginosins,[3] microginins,[4] and microviridins,[5] which inhibit proteolytic enzymes. Proteases are involved in a variety of biological processes and are implicated in the pathogenesis of many human diseases, including cancer and Alzheimer's disease. Thus, there are many proteases that are validated drug targets and the discovery of new protease modulators is important for the development of pharmacological tools, as well as potential therapeutics.[6] The micropeptins are the most diverse group of cyclic depsipeptides in cyanobacteria and are composed of more then 115 members[7] that inhibit various serine proteases. They are characterized by a lactone ring consisting of six amino acid residues, of which one is 3-amino-6-hydroxy-2-piperidone (Ahp). Anabaenopeptins are cyclic hexapeptides, which are characterized by the cyclization of the C-terminal amino acid carboxyl to the ϵ-amine residue of the N-terminal-D-lysine, while the α-amine of this lysine moiety is linked through an ureido-bridge to the side chain of another amino acid.[8] All anabaenopeptins described to date from cyanobacteria, contain D-lysine, have an L-absolute configuration for the other amino acids and exhibit mainly carboxypeptidase inhibition.[8]

2. Results and discussion

As part of our continuing interest in the chemical ecology of cyanobacterial water blooms and the search for novel drugs for human diseases, we investigated the extracts of a *Microcystis* sp. bloom material, collected from a fishpond in Kibbutz Ma'agan Michael, Israel, in September 2006. The crude extract was found to inhibit the serine protease, chymotrypsin. The active extract was flash-chromatographed on an ODS column to afford three fractions, which exhibited chymotrypsin inhibitory activity and were thus further purified on a reversed phase HPLC column. Eight new protease inhibitors, micropeptin MM836 (**1**), micropeptin MM850 (**2**), micropeptin MM916 (**3**), micropeptin MM932 (**4**), micropeptin MM978 (**5**), anabaenopeptin MM823 (**6**), anabaenopeptin MM850 (**7**), and anabaenopeptin MM913 (**8**) along with the known anabaenopeptin B (**9**)[2,9] were isolated and characterized from the extract of this cyanobacterium. The structure elucidation and biological activity of these metabolites are discussed below (Scheme 1).

Micropeptin MM836 (**1**) was isolated as a glassy material. The molecular formula of **1**, $C_{43}H_{60}N_6O_{11}$, was deduced from a high-resolution ESI mass measurement of its sodiated molecular ion cluster at *m*/*z* 859.4249. Examination of the 1D NMR spectra in DMSO-d_6 (see Tables 1 and 2) revealed its peptide nature and similarity to the micropeptin family of cyanobacteria natural

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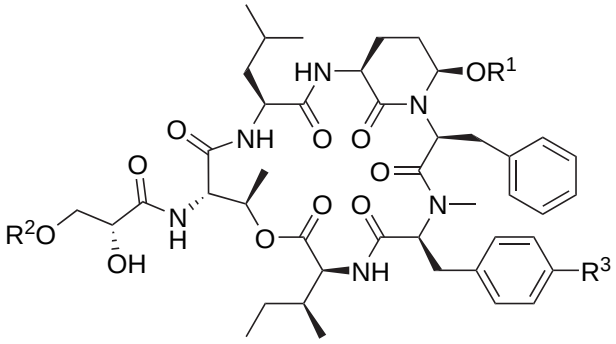
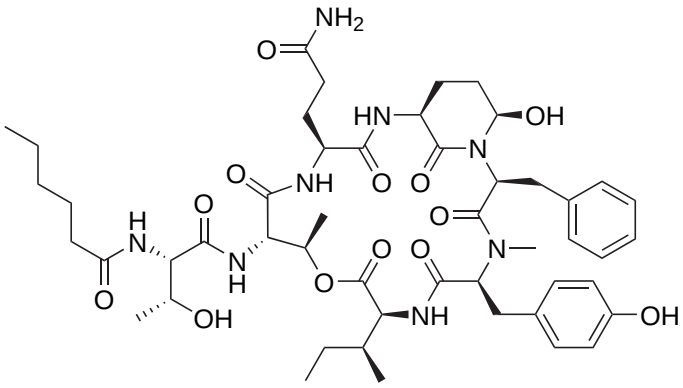
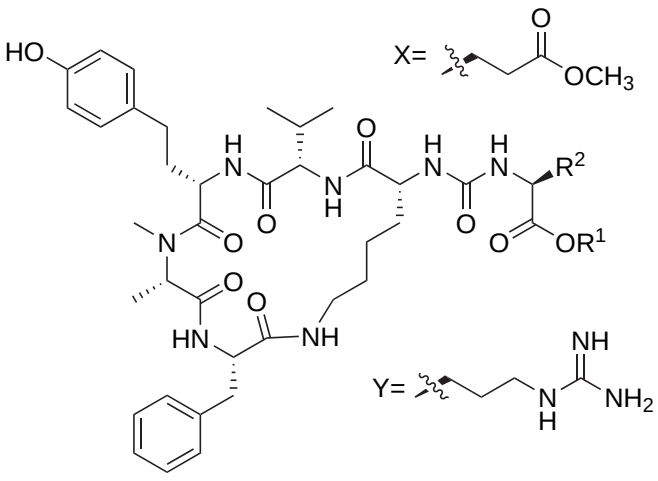


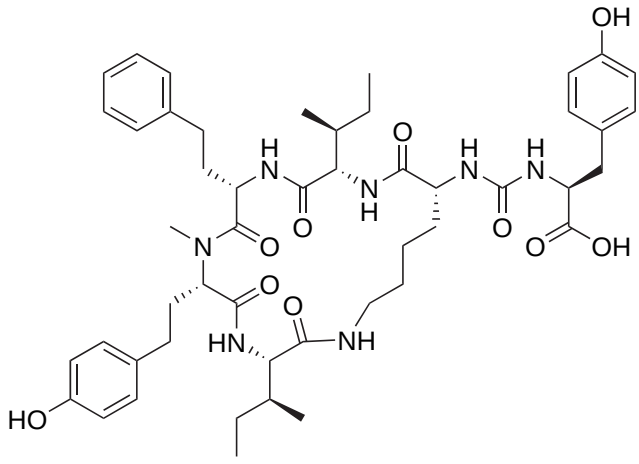
Micropeptin MM836 (**1**) R^1=R^2=R^3=H
Micropeptin MM850 (**2**) R^1=CH_3, R^2=R^3=H
Micropeptin MM916 (**3**) R^1=R^3=H, R^2=SO_3^-
Micropeptin MM932 (**4**) R^1=H, R^2=SO_3^-, R^3=OH



Micropeptin MM978 (**5**)



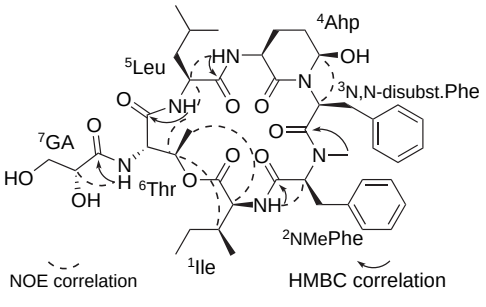
Anabaenopeptin MM823 (**6**) R^1=H, R^2=X
Anabaenopeptin MM850 (**7**) R^1=CH_3, R^2=Y
Anabaenopeptin B (**9**) R^1=H, R^2=Y



Anabaenopeptin MM913 (**8**)

Scheme 1.

products. In particular, seven carbonyl carbons at δ_C 168–173, four amide protons δ_H 7.0–8.5, and seven α-protons of amino acids at δ_H 3.6–5.0 were observed, in addition to a broad singlet attributed to the hydroxyl function of 3-amino-6-hydroxy-2-piperidinone (Ahp, δ_H 6.04), an amide NMe (δ_H 2.76; δ_C 29.9), and an oxymethine quartet due to a threonine ester (δ_H 5.39). Analysis of the COSY, TOCSY, and HSQC 2D NMR experiments (see Table 5 in the Supplementary data) allowed the assignment of the side chains of an isoleucine, a leucine, a *O*-acylated threonine, a glyceric acid, two aliphatic spin systems of aromatic amino acids, and the spin system of an Ahp moiety. Further analysis of the 2D HMBC spectrum allowed the full assignment of the *N*,*N*-disubstituted phenylalanine, the *N*-Me-phenylalanine and the Ahp moiety as well as the ascription of the carbonyl signals to the individual amino acids. The sequence of the amino acids in **1** was deduced from the HMBC and ROESY correlations. Key HMBC correlations (see Fig. 1), observed from the carbonyl of one amino acid to the NH or *N*Me of the neighboring amino acid residues included *N*MePhe and Ile, *N*,*N*-disubstituted Phe and *N*MePhe, Leu and Ahp, Thr and Leu, and glyceric acid and Thr. The remaining connectivities were established based on correlations observed between the oxymethine (H-6) of Ahp and H-2 of *N*,*N*-disubstituted Phe, and between the oxymethine (H-3) of Thr and H-2 of Ile in the ROESY spectrum. The amino acid sequence could also be fully assembled from the ROESY data (see Fig. 1 and Table 5 in the Supplementary data).



Micropeptin MM836 (**1**)

Fig. 1. Key HMBC and ROESY correlations in **1**.

Table 1
Comparison of ^{1}H NMR data of micropeptins **1**–**5** in DMSO-d_6[a]

Compound		**1**[c]	**2**[b]	**3**[c]	**4**[b]		**5**[b]
Position		δ_H, mult., J(Hz)	δ_H, mult., J(Hz)	δ_H, mult., J(Hz)	δ_H, mult., J(Hz)	Position	δ_H, mult., J(Hz)
^{1}Ile	2	4.60 dd 9.7,5.3	4.61 m	4.62 dd 9.6, 5.6	4.62 dd 9.5, 5.1	^{1}Ile	2 4.62 dd 9.5, 5.2
	3	1.73 m	1.73 m	1.76 m	1.74 m		3 1.77 m
	4a	1.23 m	1.28 m	1.23 m	1.23 m		4a 1.22 m
	4b	1.03 m	1.08 m	1.03 m	1.03 m		4b 1.02 m
	5	0.81 t 7.4	0.83 t 6.6	0.81 t 7.5	0.80 t 7.7		5 0.78 t 7.0
	6	0.82 d 6.7	0.85 d 7.2	0.83 d 7.5	0.82 d 6.6		6 0.82 d 7.5
	NH	7.54 d 9.7	6.81 d 9.6	7.50 d 9.6	7.49 d 9.5		NH 7.50 d 9.5
^{2}*N*MePhe/	2	5.00 br d 12.5	5.03 dd 11.5, 2.5	5.02 dd 12.3, 2.2	4.89 dd 12.1, 2.2	^{2}*N*MeTyr	2 4.87 br d 11.5
^{2}*N*MeTyr	3a	3.24 br d 14.5	3.23 dd 14.1, 2.5	3.24 m	3.10 dd 13.9, 2.2		3a 3.10 br d 13.5
	3b	2.84 dd 14.5, 12.5	2.89 dd 14.1, 11.5	2.83 m	2.70 dd 13.9, 12.1		3b 2.67 dd 13.5, 11.5
	5,5′	7.23 d 7.5	7.24 d 7.8	7.24 d 7.3	7.00 d 8.1		5,5′ 6.99 d 7.7
	6,6′	7.40 t 7.5	7.40 t 7.8	7.38 t 7.3	6.76 d 8.1		6,6′ 6.76 d 7.7
	7	7.31 t 7.5	7.30 t 7.8	7.29 t 7.3	—		7
	*N*Me	2.76 s	2.77 s	2.76 s	2.73 s		*N*Me 2.72 s
	OH	—	—	—	9.32 s		OH 9.31 br s
^{3}*N*,*N*-disubst. Phe	2	4.71 m	4.75 dd 11.1, 4.2	4.72 dd 10.8, 3.1	4.73 dd 11.7, 4.4	^{3}*N*,*N*-2 disubst. Phe	4.74 dd 12.0, 3.0
	3a	2.85 dd 13.7, 11.1	2.86 dd 13.9, 11.1	2.81 m	2.85 dd 15.4, 11.7		3a 2.84 dd 14.5, 12.0
	3b	1.67 dd 14.5, 2.6	1.59 dd 13.9, 4.2	1.67 m	1.79 dd 15.4, 4.4		3b 1.79 dd 14.5, 3.3
	5,5′	6.76 d 7.4	6.78 d 7.0	6.76 d 7.2	6.83 d 7.3		5,5′ 6.82 d 7.5
	6,6′	7.17 t 7.4	7.15 t 7.0	7.16 t 7.2	7.16 t 7.3		6,6′ 7.16 dd 7.5, 7.0
	7	7.12 t 7.4	7.12 t 7.0	7.12 t 7.2	7.12 m		7 7.12 t 7.0
^{4}Ahp/Amp	3	3.59 m	3.56 m	3.60 m	3.60 m	^{4}Ahp	3 3.59 m
	4_{pax}	2.41 br q 14.3	2.21 br q 12.2	2.40 br q 12.8	2.42 br q 14.2		4_{pax} 2.37 br q 13.4
	4_{peq}	1.57 m	1.57 m	1.58 m	1.57 m		4_{peq} 1.60 m
	5_{pax}	1.50 m	1.33 m	1.48 m	1.54 m		$_{5pax}$ 1.53 m
	5_{peq}	1.67 m	2.00 ddt 14.3, 2.5, 3.0	1.66 m	1.68 m		$_{5peq}$ 1.67 m
	6	5.02 br s	4.59 br s	5.02 br s	5.04 br s		6 5.03 br s
	NH	7.03 d 9.0	6.96 d 8.9	7.02 d 8.8	7.03 d		NH 7.04 d 8.7
	OH/OMe	6.09 br s	3.04 s	6.04 d 3.1	6.04 br d 2.9		OH 6.04 br s
^{5}Leu	2	4.19 m	4.19 ddd 12.0, 8.8, 3.7	4.20 ddd 10.5, 9.1, 2.0	4.19 ddd 12.6, 8.8, 3.6	^{5}Gln	2 4.12 ddd 12.2, 8.6, 4.1
	3a	1.71 m	1.70 m	1.70 m	1.69 m		3a 2.00 m
	3b	1.30 m	1.29 m	1.32 m	1.29 m		3b 1.60 m
	4	1.43 m	1.43 m	1.48 m	1.45 m		4a 1.98 m
	5	0.84 d 6.6	0.84 d 6.5	0.83 d 6.5	0.83 d 5.9		4b 1.96 m
	6	0.73 d 6.4	0.72 d 6.6	0.74 d 6.4	0.73 d 6.2		NH_2 7.12 br s, 6.64 br s
	NH	8.41 d 8.7	8.41 d 8.8	8.39 d 9.1	8.41 d 8.8		NH 8.43 d 8.6
^{6}Thr	2	4.59 br d 10.0	4.61 br d 9.9	4.59 br d 9.8	4.59 br d 9.5	^{6}Thr	2 4.59 br d 9.0
	3	5.39 br q 6.7	5.39 br q 6.7	5.39 br q 6.6	5.38 br q 6.6		3 5.37 br q 6.6
	4	1.17 d 6.7	1.16 d 6.7	1.17 d 6.6	1.16 d 6.6		4 1.17 d 6.6
	NH	7.58 d 10.0	7.58 d 9.9	7.65 d 9.1	7.65 d 9.5		NH 7.73 d 9.0
^{7}GA	2	4.04 dt 4.0, 5.4	4.03 ddd 6.0, 5.5, 4.3	4.26 ddd 8.2, 5.1, 2.6	4.26 ddd 8.1, 5.1, 2.6	^{7}Thr	2 4.38 dd 8.9, 4.2
	3a	3.56 m	3.57 m	3.99 dd 10.8, 2.6	3.99 dd 11.7, 2.6		3 3.99 ddq 4.2, 4.6, 6.2
	3b	3.44 m	3.43 dt 11.1, 5.8	3.66 dd 10.8, 8.2	3.65 dd 11.7, 8.1		4 1.04 d 6.2
	2-OH	5.74 d 5.4	5.69 d 5.5	5.98 d 5.1	5.96 d 5.1		NH 7.73 d 8.9
	3-OH	4.70 t 6.0	4.67 t 5.8	—	—		3-OH 4.83 br d 4.6
		—	—	—	—	^{8}Hex	2 2.18 t 7.2
		—	—	—	—		3 1.49 tt 7.2 7.2
		—	—	—	—		4 1.22 m
		—	—	—	—		5 1.25 m
		—	—	—	—		6 0.84 t 7.3

[a] Complete NMR data is available in the Supplementary data.
[b] 500 MHz.
[c] 400 MHz.

The relative configuration of Ahp as 3*S**,6*R** was established by comparison of its ^{1}H and ^{13}C NMR data with those of micropeptin MZ845[7] and the arguments described below for compound **3**. The signals observed for H_2-5 of the Ahp moiety in **1** (as well as in **3** and **4**) appeared at a higher field (1.67 ppm and 1.50 ppm) than in most of the known micropeptins,[1] most probably due to the weak anisotropic affect of the neighboring Phe aromatic ring. Based on the arguments presented above planar structure, **1**, was assigned to micropeptin MM836.

Micropeptin MM850 (**2**) was isolated as a glassy colorless material. The molecular formula of **2**, $C_{44}H_{62}N_6O_{11}$, was deduced from a high-resolution MALDI-TOF-MS measurement of its sodiated molecular ion cluster at m/z 873.4405. Comparison of the ^{1}H NMR data (see Table 1) of **2** and **1** revealed that they differ in the substitution of C-6 of the Ahp moiety; a methoxyl (δ_H 3.03, 3H, s) in **2** instead of hydroxyl (δ_H 6.04, 1H, br s), in **1**. Analysis of the 2D NMR experiments (see Table 6 in the Supplementary data) allowed the assignment of the full planar structure of **2**. The proton and carbon chemical shifts of positions 2, 3, and 4 of the Ahp of **1** and Amp of **2** were almost identical (see Tables 1 and 2) while those of carbons 5 and 6 were considerably different. C-5 was shifted upfield by 6 ppm and C-6 was shifted downfield by 9 ppm, in **2** relative to **1**. H-5_{peq} (pseudoeqatorial) was shifted downfield by 0.33 ppm while H-5_{pax} (pseudoaxial) and H-6 were upfield shifted by 0.17 and 0.44 ppm, respectively, in **2** relative to **1**. In the ROESY spectrum, H-4_{pax} displayed NOE's with H-5_{peq} and 3-NH, 6-*O*Me correlated with H-5_{peq}, while H-5_{pax} showed NOEs with H-3_{pax} and H-4_{peq} (see Fig. 2). This established the relative configuration of the Amp to be identical to that of the Ahp moiety in **1**, 3*S**,6*R**. The possibility that **2** is an isolation artifact was excluded by us in the past, for cyanopeptolin

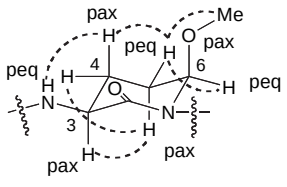
Table 2
Comparison of the ^{13}C NMR data of micropeptins **1**–**5** in DMSO-d_6[a]

Position		**1**[c]	**2**[b]	**3**[c]	**4**[b]	Position		**5**[b]
[1]Ile	1	172.4 s	172.1 s	172.3 s	172.1 s	[1]Ile	1	172.3 s
	2	54.2 d	55.2 d	55.3 d	55.2 d		2	55.5 d
	3	37.4 d	38.0 d	37.7 d	37.7 d		3	37.5 d
	4	24.5 t	24.8 t	24.3 t	24.6 t		4	24.6 t
	5	11.3 q	11.2 q	11.3 q	11.4 q		5	11.4 q
	6	16.0 q	16.0 q	16.0 q	16.1 q		6	16.1 q
[2]*N*MePhe/	1	168.7 s	168.8 s	168.9 s	169.0 s	[2]*N*MeTyr	1	169.3 s
[2]*N*MeTyr	2	60.7 d	60.8 d	60.7 d	61.0 d		2	61.0 d
	3	33.5 t	33.8 t	33.8 t	33.0 t		3	32.9 t
	4	137.6 s	137.9 s	138.0 s	127.9 s		4	127.7 s
	5,5′	129.7 d	129.6 d	129.6 d	130.5 d		5,5′	130.6 d
	6,6′	128.6 d	128.8 d	128.7 d	115.4 d		6,6′	115.5 d
	7	126.4 d	126.9 d	126.7 d	156.3 s		7	156.4 s
	*N*Me	29.9 q	30.4 q	30.4 q	30.4 q		*N*Me	30.4 q
[3]*N*,*N*-disubst.-Phe	1	170.3 s	170.3 s	170.4 s	170.5 s	[3]*N*,*N*-disubst.-Phe	1	170.6 s
	2	50.3 d	50.0 d	50.2 d	49.7 d		2	50.5 d
	3	35.3 t	34.7 t	35.3 t	35.4 t		3	35.4 t
	4	136.2 s	136.5 s	136.7 s	136.8 s		4	136.9 s
	5,5′	129.5 d	129.4 d	129.4 d	129.5 d		5,5′	129.6 d
	6,6′	127.9 d	127.9 d	127.9 d	127.7 d		6,6′	127.9 d
	7	126.0 d	126.4 d	126.3 d	126.3 d		7	126.4 d
[4]Ahp/Amp	2	168.0 s	168.7 s	169.0 s	169.0 s	[4]Ahp	2	168.9 s
	3	48.5 d	51.3 d	48.8 d	51.3 d		3	48.9 d
	4	21.5 t	21.5 t	21.5 t	21.2 t		4	21.7 t
	5	29.2 t	23.3 t	29.4 t	29.4 t		5	29.5 t
	6	73.8 d	82.8 d	73.9 d	73.9 d		6	73.9 d
	*O*Me	—	55.4 q	—	—			—
[5]Leu	1	169.9 s	170.4 s	170.3 s	170.5 s	[5]Gln	1	169.8 s
	2	50.3 d	50.5 d	50.5 d	50.6 d		2	52.0 d
	3	38.9 t	39.2 t	39.0 t	39.0 t		3	26.5 t
	4	24.3 d	24.3 d	24.6 d	24.2 d		4	31.7 t
	5	23.4 q	23.3 q	23.3 q	23.3 q		5	173.8 s
	6	21.1 q	21.1 q	21.2 q	21.2 q		6	—
[6]Thr	1	169.0 s	169.0 s	169.1 s	169.1 s	[6]Thr	1	169.4 s
	2	54.0 d	54.1 d	54.2 d	54.2 d		2	54.9 d
	3	71.9 d	72.3 d	72.2 d	72.2 d		3	72.1 d
	4	18.0 q	17.7 q	17.9 q	17.9 q		4	18.0 q
[7]GA	1	172.4 s	172.7 s	171.1 s	171.7 s	[7]Thr	1	171.2 s
	2	72.8 d	72.8 d	70.8 d	70.8 d		2	58.0 d
	3	64.0 t	64.0 t	68.5 t	68.5 t		3	66.9 d
	4	—	—	—	—		4	19.8 q
		—	—	—	—	[8]Hex	1	172.8 s
		—	—	—	—		2	35.3 t
		—	—	—	—		3	31.0 t
		—	—	—	—		4	25.2 t
		—	—	—	—		5	22.1 t
		—	—	—	—		6	14.1 q

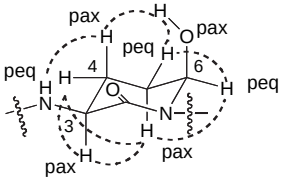
[a] Complete NMR data is available in the Supplementary data.
[b] 125 MHz.
[c] 100 MHz.

SS, by verifying its stability in conditions similar to those used in the isolation procedure.[7] Based on these arguments planar structure, **2**, was assigned to micropeptin MM850.

Micropeptin MM916 (**3**) was isolated as a glassy colorless material. Its molecular formula, $C_{43}H_{60}N_6O_{14}S$, was determined by high-resolution ESIMS (*m*/*z* 939.3826, $[M+Na]^+$). Comparison of the ^{1}H and ^{13}C NMR data (see Tables 1 and 2) of **3** and **1** revealed that the difference between them was located in the GA moiety. In **3**, C-1 and C-2 were shifted downfield by 1.3 and 2.0 ppm, respectively, while C-3 was shifted upfield by 4.5 ppm, relative to **1**, indicating the presence of a 3-sulfo-glyceric acid moiety in **3**, as in the case of micropeptin MZ845.[7] Analysis of COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (see Table 7 in the Supplementary data) allowed the assignment of all signals to the individual amino- and hydroxy-acid residues in **3**. The sequence of the amino acids was deduced from HMBC and ROESY correlations in a similar manner as described above for **1**, with the exception that the ester linkage between Thr and Ile was evident from the HMBC correlation of the oxymethine H-3 of Thr to the carbonyl of Ile. The relative



Amp



Ahp

pax - pseudo axial, peq - pseudo equatorial

Fig. 2. NOE correlations of Ahp moiety in **3** and Amp moiety in **2**.

Table 3
Comparison of ^{1}H and ^{13}C NMR data of anabaenopeptins **6**–**8** in DMSO-d_6[a]

Compound			**6**[c]		**7**[b]				**8**[b]
Position		δ_C, mult.	δ_H, mult. *J* (Hz)	δ_C, mult.	δ_H, mult. *J* (Hz)	Position		δ_C, mult.	δ_H, mult. *J* (Hz)
[1]Phe	1	171.0 s	—	171.1 s	—	[1]Ile	1	170.7 s	
	2	55.1 d	4.37 ddd 12.7, 8.3, 3.2	55.1 d	4.38 ddd 12.3, 8.9, 2.8		2	58.1 d	4.17 dd 9.5, 7.0
	3a	37.7 t	3.32 m	37.7 t	3.31 m		3	36.1 d	2.01 m
	3b	—	2.78 m	—	2.77 m		4a	24.3 t	1.30 m
	4	138.4 s	—	138.5 s	—		4b		0.96 m
	5,5′	129.0 d	7.05 d 7.2	129.1 d	7.06 d 7.4		5	11.5 q	0.74 t 7.4
	6,6′	128.4 d	7.18 t 7.2	128.5 d	7.19 t 7.4		6	16.1 q	0.76 d 6.4
	7	126.2 d	7.14 t 7.2	126.3 d	7.15 m		NH		8.10 d 9.5
	NH	—	8.68 d 8.3	—	8.67 d 8.9	[2]*N*MeHt	1	169.5 s	
[2]*N*MeAla	1	170.0 s	—	170.0 s	—		2	59.5 d	4.66 dd 7.7, 5.9
	2	54.4 d	4.79 q 6.8	54.5 d	4.79 q 6.6		3a	30.9 t	2.04 m
	3	14.0 q	1.06 d 6.8	14.0 q	1.06 d 6.6		3b		1.79 m
	NMe	27.3 q	1.76 s	27.2 q	1.77 s		4a	28.8 t	2.31 dd 13.1, 4.5
[3]Hty	1	171.1 s	—	171.0 s	—		4b		2.24 dd 13.1, 4.2
	2	48.9 d	4.72 dt 8.0, 4.8	48.9 d	4.73 dt 4.2, 6.0		5	131.6 s	
	3a	33.4 t	1.86 m	33.4 t	1.86 m		6,6′	129.1 d	6.96 d 6.6
	3b	—	1.71 m	—	1.70 m		7,7′	115.4 d	6.66 d 6.6
	4a	30.7 t	2.62 ddd 13.9, 9.9, 4.0	30.7 t	2.61 ddd 13.4, 12.2, 2.5		8(OH)	155.7 s	9.18 s
	4b	—	2.42 ddd 13.9, 10.8, 6.6	—	2.42 ddd 13.4, 11.5, 6.1		NMe	28.2 q	2.57 s
	5	131.2 s	—	131.2 s	—	[3]Hph	1	172.4 s	
	6,6′	129.2 d	7.00 d 8.3	129.2 d	7.00 d 8.3		2	48.5 d	4.71 m
	7,7′	115.3 d	6.66 d 8.3	115.3 d	6.66 d 8.3		3a	33.1 d	2.00 m
	8(OH)	155.7 s	9.17 s	155.7 s	9.18 s		3b		1.78 m
	NH	—	8.91 d 4.8	—	8.92 d 4.2		4a	31.7 t	2.81 m
[4]Val	1	172.8 s	—	172.8 s	—		4b		2.66 m
	2	58.1 d	3.89 dd 8.8, 7.8	58.1 d	3.92 m		5	141.3 s	
	3	30.2 d	1.96 m	30.2 d	1.99 m		6,6′	130.0 d	7.08 d 7.8
	4	19.4 q	0.93 d 6.8	19.1 q	1.03 d 7.2		7,7′	128.6 d	7.23 t 7.8
	5	19.1 q	1.03 d 6.8	19.2 q	0.92 d 6.6		8	126.3 d	7.18 t 7.8
	NH	—	7.01 d 7.8	—	7.00 m		NH		8.95 d 4.6
[5]Lys	1	172.3 s	—	172.2 s	—	[4]Ile	1	173.1 s	
	2	54.9 d	3.92 dt 5.2, 7.2	55.0 d	3.91 m		2	55.9 d	4.20 dd 5.0, 5.9
	3	31.9 t	1.61 m	31.9 t	1.62 m		3	36.4 d	1.78 m
	4a	20.5 t	1.33 m	20.5 t	1.28 m		4a	25.7 t	1.34 m
	4b	—	1.16 m	—	1.17 m		4b		1.12 m
	5	28.3 t	1.44 m	28.3 t	1.43 m		5	11.8 q	0.84 t 7.5
	6a	38.4 t	3.57 m	38.4 t	3.58 m		6	14.6 q	0.79 d 7.0
	6b	—	2.79 m	—	2.79 m		NH		6.72 d 5.9
	ϵ-NH	—	7.13 m	—	7.18 t 7.4	[5]Lys	1	172.4 s	
	NH	—	6.42 d 7.2	—	6.46 d 7.2		2	55.0 d	3.85 dt 5.2, 6.1
Urea	CO	157.3 s	—	157.3 s	—		3	30.9 t	1.62 m
[6]OMeGlu/1		174.0 s	—	173.5 s	—		4	20.6 t	1.22 m
[6]OMeArg/2		51.9 d	4.07 dt 4.1, 8.0	52.3 d	4.15 dt 5.4, 8.2		5	29.3 t	1.22 m
	3a	27.2 t	1.96 m	29.0 t	1.66 m		6	38.2 t	3.47 m
	3b	—	1.77 m	—	1.53 m		ϵ-NH		7.15 dd 8.1, 4.8
	4	29.9 t	2.32 m	25.2 t	1.47 m		NH		6.56 d 6.1
	5	172.8 s	—	40.4 t	3.08 m	Urea	CO	157.3 s	
	6	—	—	156.8 s	—	[6]Tyr	1	173.8 s	
	OMe	51.5 q	3.57 s	52.0 q	3.58 s		2	54.2 d	4.26 dt 4.8, 7.6
	NH	—	6.38 d 8.0	—	6.55 d 8.1		3a	37.1 t	2.86 dd 13.7, 4.8
	5-NH	—	—	—	7.54 t 5.5		3b		2.75 dd 13.7, 7.3
							4	127.5 s	
							5,5′	130.3 d	6.94 d 6.2
							6,6′	115.2 d	6.65 d 6.2
							7 (OH)	156.2 s	9.23 s
							NH		6.22 d 7.9

[a] Complete NMR data is available in the Supplementary data (coupling constant, HMBC, and ROESY correlations).
[b] 500 MHz for ^{1}H and 125 MHz for ^{13}C.
[c] 400 MHz for ^{1}H and 100 MHz for ^{13}C.

configuration of the chiral centers of the Ahp moiety, *3S**,*6R**, was based on the NOE correlations (see Fig. 2 and Table 7 in the Supplementary data) between H-4$_{pax}$ and 6-OH, H-4$_{pax}$ and 3-NH, and between H-3$_{pax}$ and H-5$_{pax}$, which are characteristic to this moiety.[7] On the basis of these arguments, planar structure **3** was thus assigned to micropeptin MM916.

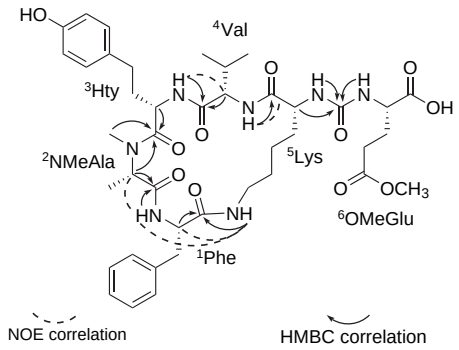
Micropeptin MM932 (**4**) was isolated as a glassy colorless material. Its molecular formula was determined as $C_{43}H_{60}N_6O_{15}S$ by high-resolution negative ESI mass analysis of its molecular ion at *m*/*z* 931.3785 $[M-H]^-$. Comparison of the ^{1}H and ^{13}C NMR data (see Tables 1 and 2) of **4** and **3** revealed that one phenylalanine unit in **3** was substituted by a tyrosine in **4**, as evident from the two doublet protons at 6.76 (2H) and 7.00 (2H) ppm, a phenolic OH at 9.32 ppm, two aromatic methine carbons at 115.4 ppm, and a phenolic quaternary carbon at 156.3 ppm. Correlations from the HMBC spectrum (see Table 8 in the Supplementary data) allowed the full assignment of the phenol ring and its attachment, through the quaternary carbon resonating at 127.9 ppm, to a methylene (δ_C 33.0, δ_H 3.10 and 2.70) and a methine (δ_C 61.0, δ_H 4.89). The correlation of the methine with the *N*-methyl (δ_H 2.73) determined the structure of this unit as *N*Me-tyrosine. This established that the *N*Me-phenylalanine in **3** was replaced by an *N*Me-tyrosine in **4**.

Analysis of COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (see Table 8 in the Supplementary data) allowed the assignment of all signals, the sequence and relative stereochemistry of the chiral centers, in a similar fashion as described for **3**. Consequently, the planar structure, **4**, was assigned to micropeptin MM932.

Micropeptin MM978 (**5**) was isolated as a glassy colorless material exhibiting a molecular formula of $C_{49}H_{70}N_8O_{13}$ as determined by high-resolution ESI mass analysis of its sodiated molecular ion cluster at *m*/*z* 1001.4977. Comparison of the 1D NMR spectra of **4** and **5** suggested that both share the same ^{1}Ile-^{2}*N*MeTyr-*N*,*N*-disubst.^{3}Phe-^{4}Ahp fragment but differ in the rest of the acid sequence. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (see Table 9 in the Supplementary data) allowed the assignment of amino hydroxy piperidone (Ahp), glutamine, hexanoate, isoleucine, *N*,*N*-disubstituted-phenylalanine, *N*Me-tyrosine, *O*-acetylated threonine, and threonine residues. The sequence of the amino acids was deduced from HMBC correlations observed between the carbonyl of one amino acid to the NH or *N*Me of the neighboring amino acid residue. This established the connectivity between *N*MePhe and Ile, *N*,*N*-disubstituted Phe and *N*MePhe, Gln and Ahp, ^{1}Thr and Gln, ^{2}Thr and ^{1}Thr, and hexanoic acid and ^{2}Thr. The carbonyl of Ahp exhibited correlation with H-2 of *N*,*N*-disubstituted-Phe, and the lactone linkage was deduced from the correlation of Ile carboxyl and H-3 of ^{1}Thr. The amino acid sequence could also be exclusively assembled from the ROESY data (see Table 9 in the Supplementary data). The relative configuration of Ahp C-3 and C-6, was assigned as 3*S**,6*R**, as described for compound **3**. The planar structure, **5**, was assigned to micropeptin MM978.

Anabaenopeptin MM823 (**6**) was isolated as a glassy colorless material. Its molecular formula, $C_{41}H_{57}N_7O_{11}$, was deduced by the analysis of high-resolution ESI mass measurements of its sodiated molecular ion at *m*/*z* 846.4056. The 1D NMR spectra of **6** in DMSO-d_6 (see Table 3) revealed the peptide nature of this compound; i.e., carboxamide carbons δ_C 170–174, in the ^{13}C NMR spectrum, amide protons δ_H 6.30–9.00 and protons α to carbonyl in amino acids δ_H 3.90–4.70. The alliance of **6** with the anabaenopeptins was evident from its NMR data, i.e., the chemical shifts of the *N*Me at δ_H 1.76 (s), δ_C 27.3 (q) and of the urea bridge, a carbon at δ_C 157.3 and amide protons at δ_H 6.38 and 6.42. Analysis of the COSY, TOCSY, HSQC, and HMBC 2D NMR experiments (see Table 10 in the Supplementary data) allowed the assignment of valine, *N*MeAla, lysine, phenylalanine, homo-tyrosine, and δ-*O*Me-glutamic acid. The sequence of the amino acids in **6** was established through HMBC correlations (see Fig. 3) of the carbonyl of an amino acid with the amide proton or *N*Me of the adjacent amino acid, for the following pairs: Lys-Val, Val-Hty, Hty-*N*MeAla, and *N*MeAla-Phe. The correlation between the carbonyl of Phe and ϵ-NH of Lys established the closure of the peptide cycle, while correlations of the urea carbonyl (δ_C 157.3) and the α-amide protons of Lys and *O*MeGlu established the urea bridge. The amino acid sequence could also be assembled from the NOE data (see Fig. 3 and Table 10 in the Supplementary data). Based on this evidence the planar structure, **6**, was assigned to anabaenopeptin MM823.

Anabaenopeptin MM850 (**7**) was isolated as a glassy material exhibiting a molecular formula of $C_{42}H_{62}N_{10}O_9$ that was determined from analysis of its high-resolution ESIMS protonated molecular ion at *m*/*z* 851.4794. The 1D NMR spectra of **7** in DMSO-d_6 were similar to that of **6**, suggesting its association with the anabaenopeptins. Comparison of the NMR data (see Table 3) of anabaenopeptin MM850 (**7**) and anabaenopeptin MM823 (**6**) revealed that the difference between them consisted in the substitution of the urea bridge with an *O*MeArg in **7**, instead of the *O*MeGlu in **6**. Analysis of the one and two-dimensional NMR experiments (see Table 11 in the Supplementary data) revealed that



Anabaenopeptin MM823 (**6**)

Fig. 3. Key HMBC and NOE correlations in **6**.

the cyclic portion of **7** was identical with that of **6**, and that **7** is the methyl ester of anabaenopeptin B (**9**), which was also isolated from the extract of this bloom material. Thus, the planar structure, **7**, was assigned to anabaenopeptin MM850.

Anabaenopeptin MM913 (**8**) was isolated as a glassy material with a molecular formula of $C_{49}H_{67}N_7O_{10}$ deduced from HR ESI mass measurement of its molecular ion ($[M+H]^+$) at *m*/*z* 914.5031. Comparison of its 1D NMR spectra in DMSO-d_6 to those of compounds **6** and **7** (see Table 3) revealed that **8** belonged to the anabaenopeptins since it contained the urea bridge but its characteristic *N*Me group (δ_H 2.57) resonate downfield of the expected chemical shift (δ_H ~1.77). Analysis of the 1D and 2D NMR experiments (see Tables 3 and 12 in the Supplementary data) allowed the full assignment of five amino acid residues, two isoleucines, an *N*Me-homotyrosine, a lysine, a tyrosine as well as the side chain of homophenylalanine. The remaining carbonyl still unaccounted for was assigned as C-1 of the latter amino acid. The amino acid sequence of **8** was deduced from the analysis of the HMBC and ROESY 2D NMR experiments. HMBC correlations observed between the carbonyl of an amino acid and the amide proton or *N*Me of the adjacent amino acid, for the following pairs: *N*MeHty and ^{1}Ile, Hph and *N*MeHty and, ^{4}Ile and Hph. In addition ROESY correlations were observed between the α-protons of *N*MeHty and Hph and the amide proton of ^{4}Ile and the α-proton of Lys. The ring closure was based on the NOEs between ϵ-NH of Lys and the amide and α-protons of ^{1}Ile. The urea bridge was assigned through HMBC correlation of the α-proton of Lys with the urea carbonyl (δ_C 157.3) and NOE correlations of Lys-α-NH with Tyr amide and α protons. Based on these arguments the planar structure, **8**, was assigned to anabaenopeptin MM913.

The absolute configuration of the chiral centers of compounds **1**–**8** was determined using Marfey's method. This involved acid hydrolysis that liberated the amino acids, followed by derivatization with Marfey's reagent[10] and HPLC analysis. A small amount of material of micropeptins **1**–**5** was initially oxidized with Jones' reagent,[11] in order to liberate a glutamic acid from the Ahp/Amp residues, followed by Marfey's procedure. The described procedures established the D-absolute configuration of Lys in the anabaenopeptins **6**–**8** and L-stereochemistry of all the remaining amino acids in compounds **1**–**8**. The Ile units in **1**–**5** were established by their carbon chemical shifts to be Ile (contrary to *allo*Ile).[7] Diethyl-ether extraction of the acid hydrolyzate of micropeptins **1**–**4**, followed by chiral HPLC analysis, demonstrated a D-absolute configuration for the glyceric acid moieties. This established the full absolute configuration for compounds **1**–**8**.

The inhibitory activity of **1**–**9** that was determined against the serine proteases chymotrypsin, elastase, trypsin, and thrombin

is summarized in Table 4. Comparison of the IC_{50}'s of the micropeptins revealed that the affinity of the micropeptins to the catalytic binding pocket of chymotrypsin decreases when the hydroxy group at position 3 of ^{7}GA is substituted with a sulfate group, and when the aromatic ring, of residue-2, is substituted by a *para*-hydroxy group. The opposite is observed for the affinity to the catalytic binding pocket of elastase, which increased when residue-2 is a tyrosine instead of a phenylalanine. The inhibition of serine proteases by the anabaenopeptins is much milder then those of the micropeptins. Comparison of the inhibitory activity of the anabaenopeptins revealed that an *O*MeArg in the ureido-bridge of **7** decreased the affinity to the catalytic binding pocket of chymotrypsin (when compared to *O*MeGlu in **6** and Arg in **9**) and increased the affinity to the catalytic binding pocket of elastase (compared to *O*MeGlu in **6**).

Table 4
Summary of the IC_{50}'s of compounds **1**–**9** against serine proteases[a]

Compound	Chymo-trypsin	Elastase	Trypsin	Thrombin
1	1.4	45.5	—	—
2	1.7	—	—	—
3	3.0	—	—	—
4	5.4	4.4	—	—
5	4.6	19.1	—	52.9
6	16.0	50.0	—	—
7	45.0	14.3	45.0	—
8	—	—	—	—
9	—	—	—	—

[a] In μM.

3. Conclusions

The study describes the isolation of five new micropeptins together with three new and one known anabaenopeptins from the water bloom material of the cyanobacterium *Microcystis* sp. This research expandes the library of the micropeptins and anabaenopeptins, and illuminates 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. Anabaenopeptin MM913 (**8**), belongs to a relatively rare subgroup of the anabaenopeptins, presenting an *N*-methyl-homoaromatic amino acid at the second position. There are nine more members of this subgroup of anabaenopeptins: nodulapeptins A and B,[12] anabaenopetins G, H,[13] and T,[14] oscillamide C,[15] anabaenopeptins 908 and 915,[16] and anabaenopeptin HU892.[17]

4. Experimental section

4.1. General experimental procedures

Low and high-resolution MALDI mass spectra were recorded on an Applied Biosystems Voyager System 4312 instrument and low- and high-resolution ESI mass spectra were recorded on a Waters Synapt 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 DRX-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. COSY-45, gTOCSY, gROESY, gHSQC, gHMQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were preformed on a Jasco HPLC system (model PU-2080-plus pump and model MD-2010-plus diode array detector), a Merck Hitachi (model L-6200 intelligent pump, model L-4200 UV–vis) and an Agilent 1100 series HPLC system. The proteases inhibition assays were preformed on an ELx808, BIO-TEK Instruments Eliza reader.

4.2. Biological material

Microcystis sp., TAU strain IL-362, was collected in September 2006, from a fishpond at Kibbutz Ma'agan Michael, Israel. The cell mass was frozen immediately after collection and lyophilized. A sample of the cyanobacterium is deposited at the culture collection of Tel Aviv University.

4.3. Isolation procedure

Lyophilization of the first batch of IL-362 cells produced 1 Kg of dry cells. Extraction with 7:3 MeOH/H_2O (4×2L) and evaporation yielded 41 g of crude extract. The crude extract was chromatographed in 5 g portions, on a reversed phase (ODS) flash column (YMC-GEL, 120A, 4.4×6.4 cm) eluted with increasing percentage of MeOH in water. Fraction 7 (3:2 MeOH/H_2O) 484 mg, was further separated on a Sephadex LH-20 column with 1:1 MeOH/$CHCl_3$ to obtain twelve fractions (fractions 7a–l). Fractions 7e–h were combined (a, 169 mg) and separated on a reversed-phase HPLC column (YMC-Pack C-8, 250 mm×20.0 mm, DAD at maximum absorbance, flow rate 5.0 mL/min) eluted with 1:1 H_2O/acetonitrile (MeCN) to obtain seven fractions (a1–a7). Fraction a7, pure compound **1** (4.0 mg, 0.0004% yield based on the dry weight of the bacterium, retention time 32.5 min). Fraction a1 was further separated on the same RP HPLC column with 3:7 MeCN/0.1% TFA in H_2O to obtain four fractions (a1a–d). Fraction a1d, pure compound **7** (2.4 mg, 0.0002% yield, retention time 56.2 min). Fraction 8 from the initial RP separation (7:3 MeOH/H_2O) 480 mg, was further separated on a Sephadex LH-20 column with 1:1 MeOH/$CHCl_3$ to obtain eleven fractions (fractions 8a–k). Fractions 8d–i were combined (b, 97 mg) and separated on a reversed-phase HPLC column (YMC-Pack C-8, 250 mm×20.0 mm, DAD at maximum absorbance, flow rate 5.0 mL/min) with 3:2 H_2O/MeCN to obtain seven fractions (b1–b7). Fraction b3, pure compound **5** (8.8 mg, retention time 17.2 min, it was isolated once more from another fraction, 3.8 mg, total yield 0.0012%). Fraction b1 and b5 were combined (c, 71 mg) and further separated on the same RP HPLC column with 7:13 ACN/0.1% TFA in H_2O to obtain eleven fractions (c1–c11), two of which were found to be pure compounds. Compound **9** (c4, 3.3 mg, 0.0003% yield, retention time 15.6 min). Compound **4** (c6, 3.4 mg, 0.0003% yield, retention time of 17.6 min). Fraction c10 (7.8 mg) was further separated on the same RP HPLC column, with 1:1 MeCN/0.1% TFA in H_2O to obtain five fractions (c10a–e). Fraction c10d, pure compound **2** (4.0 mg, retention time 19.6 min, it was isolated once more from another fraction, 1.0 mg, total yield 0.0005%). A second batch of lyophilized cells (0.5 Kg) was extracted with 7:3 MeOH/H_2O (3×1.5 L) and then evaporated *in vacuo* to produce 42 g of crude extract. It was separated on an ODS Flash column with an increasing amount of MeOH in water. Fraction 6 from the RP column (1:1 MeOH/H_2O) 491 mg was separated on a Sephadex LH-20 column with 1:1 MeOH/H_2O to obtain ten fractions (6a–j). Fractions 6c–e were combined (d, 129 mg) and separated on an RP HPLC column YMC-Pack C-8, with 7:13 MeCN/0.1% TFA in H_2O to obtain 16 fractions (d1–16), one of which was found to be a pure compound. Compound **6** (d9, 3.7 mg, 0.0007% yield, retention time 29.9 min). Fraction 7 from the RP column (1:1 MeOH/H_2O) 256 mg, was separated on a Sephadex LH-20 column with 1:1 MeOH:H_2O to obtain fourteen fractions (7*m*-*z*). Fractions 7r-t were combined (e, 63 mg) and separated on an RP HPLC column YMC-Pack C-8 with 23:27 MeCN: 0.1% TFA in H_2O to obtain twelve

fractions (e1–12) two of which were pure compounds. Compound **3** (e3, 3.8 mg, 0.0008% yield, retention time 24.4 min) and compound **8** (e12, 3.1 mg, 0.0006% yield, retention time 37.3 min).

*4.3.1. Micropeptin MM836 (**1**).* Glassy material; $[\alpha]_D^{23}$ +120 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.12), 230 (3.24), 248 (2.55), 280 (1.29) nm; IR (KBr): 3450, 3021, 2950, 2933, 2875, 1735, 1632, 1546, 1384, 1200, 1036 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); HR ESIMS *m/z* 859.4249 $[M+Na]^+$, (calcd for $C_{43}H_60N_6NaO_{11}$, 859.4218). Retention time of AA Marfey derivatives: L-threonine 30.5 min (L-Tre 30.5, D-Thr 33.0 min), L-glutamic acid 32.7 min (L-Glu 32.7, D-Glu 33.5 min), L-isoleucine 44.4 min (L-Ile 44.4, D-Ile 47.5 min), L-leucine 45.1 min (L-Leu 45.1, D-Leu 47.9 min), L-*N*Me-phenylalanine 45.1 min, and L-phenylalanine 45.5 min (L-Phe 45.5, D-Phe 47.6). Retention time of D-glyceric acid on the chiral column was 3.95 min (L-glyceric acid 3.92 D-glyceric acid 3.95 min).

*4.3.2. Micropeptin MM850 (**2**).* Glassy white material; $[\alpha]_D^{23}$ +10 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.21), 248 (2.88) nm; IR (KBr): 3420, 3029, 2960, 2934, 2874, 1735, 1656, 1527, 1441, 1201, 1054 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); HR MALDI-TOF-MS m/*z* 873.4405 $[M+Na]^+$, (calcd for $C_{44}H_{62}N_6NaO_{11}$, 873.4369). Retention time of AA Marfey derivatives: L-threonine 22.9 min (L-Thr 22.9, D-Thr 26.2 min), L-glutamic acid 24.5 min (L-Glu 24.5, D-Glu 26.2 min), L-isoleucine 39.3 min (L-Ile 39.3, D-Ile 40.0 min), L-phenylalanine 40.0 min (L-Phe 40.0, D-Phe 43.1 min), L-leucine 39.9 min (L-Leu 39.9, D-leucine 43.9 min), and L-NMe-phenylalanine 47.3 min. Retention time of D-glyceric acid on the chiral column was 3.97 min (L-glyceric acid 3.95, D-glyceric acid 3.97 min).

*4.3.3. Micropeptin MM916 (**3**).* Glassy white material; $[\alpha]_D^{23}$ +14 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (4.35), 230 (3.72), 248 (3.36), 280 (3.16) nm; IR (KBr): 3420, 3360, 2961, 2933, 2874, 1735, 1670, 1533, 1451, 1384, 1204, 1138, 1007 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); HR ESIMS *m/z* 939.3826 $[M+Na]^+$, (calcd for $C_{43}H_{60}N_6NaO_{14}S$, 939.3786). Retention time of AA Marfey derivatives: L-threonine 30.8 min (L-Thr 30.8, D-Thr 33.2 min), L-glutamic acid 32.6 min (L-Glu 32.6, D-Glu 33.4 min), L-isoleucine 44.5 min (L-Ile 44.5, D-Ile 47.6 min), L-leucine 45.3 min (L-Leu 45.3, D-Leu 48.1 min), L-*N*Me-phenylalanine 45.3 min, and L-phenylalanine 45.7 min (L-Phe 45.7, D-Phe 47.7). Retention time of D-glyceric acid on the chiral column was 3.92 min (L-glyceric acid 3.90, D-glyceric acid 3.92 min).

*4.3.4. Micropeptin MM932 (**4**).* Glassy white material; $[\alpha]_D^{24}$ +26 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.19), 228 (3.68), 248 (3.14), 280 (3.07) nm; IR (KBr): 3400, 2960, 2927, 2874, 1735, 1656, 1544, 1442, 1384, 1204, 1139, 1010 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); Negative HR ESIMS *m/z* 931.3785 $[M-H]^-$, (calcd for $C_{43}H_{59}N_6O_{15}S$, 931.3759). Retention time of AA Marfey derivatives: L-threonine 22.8 min (L-Thr 22.8, D-Thr 26.3 min), L-glutamic acid 24.6 min (L-Glu 24.6, D-Glu 26.3 min), L-isoleucine 39.5 min (L-Ile 39.5, D-Ile 43.7 min), L-leucine 44.2 min (L-Leu 44.2, D-Leu 47.6 min), L-phenylalanine 40.4 min (L-Phe 40.4, D-Phe 43.4), and L-*N*Me-tyrosine 47.2 min. Retention time of D-glyceric acid on the chiral column 3.92 min (L-glyceric acid 3.90, D-glyceric acid 3.92 min).

*4.3.5. Micropeptin MM978 (**5**).* Glassy white material; $[\alpha]_D^{24}$ +57 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.36), 228 (3.87), 248 (3.43), 280 (3.30) nm; IR (KBr): 3420, 3658, 2970, 2935, 2877, 1735, 1656, 1537, 1468, 1384, 1277, 1079, 1036 cm^{-1}; ^1H and ^{13}C NMR (see Table 3); HR ESIMS *m/z* 1001.4977 $[M+Na]^+$, (calcd for $C_{49}H_{70}N_8NaO_{13}$, 1001.4960). Retention time of AA Marfey derivatives: L-threonine 24.4 min (L-Thr 24.4, D-Thr 26.3 min), L-glutamic acid 26.5 min (L-Glu 26.5, D-Glu 27.8 min), L-isoleucine 40.2 min (L-Ile 40.2, D-Ile 44.2 min), L-phenylalanine 39.9 (L-Phe 39.9, D-Phe 42.8) min and L-*N*Me-tyrosine 46.4 min.

*4.3.6. Anabaenopeptin MM823 (**6**).* Glassy white material; $[\alpha]_D^{24}$ +5 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (4.41), 228 (4.41), 248 (3.50), 280 (3.41) nm; IR (KBr): 3200, 2935, 2875, 1735, 1654, 1543, 1458, 1384, 1259, 1106, 1035 cm^{-1}; ^1H and ^{13}C NMR (see Table 4); HR ESIMS *m/z* 846.4056 $[M+Na]^+$, (calcd for $C_{41}H_{57}N_7O_{11}$, 846.4014). Retention time of AA Marfey derivatives: L-glutamic acid 32.7 min (L-Glu 32.7, D-Glu 33.3 min), L-*N*Me-alanine 30.9 min, L-valine 41.0 min (L-Val 41.0, D-Val 44.1 min), L-homo-tyrosine 41.2 min, L-phenylalanine 45.8 min (L-Phe 45.8, D-Phe 48.0 min), and D-lysine 46.6 min (L-Lys 46.2, D-Lys 46.6 min).

*4.3.7. Anabaenopeptin MM850 (**7**).* Glassy material; $[\alpha]_D^{25}$ −54 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.29), 228 (3.82), 248 (3.22) nm; IR (KBr): 3255, 2927, 2862, 1658, 1546, 1204, 1136 cm^{-1}; ^1H and ^{13}C NMR (see Table 4); HR ESIMS *m/z* 851.4794 $[M+H]^+$, (calcd for $C_{42}H_{63}N_{10}O_9$, 851.4779). Retention time of AA Marfey derivatives: L-arginine 29.7 min (L-Arg 29.7, D-Arg 30.7 min), L-*N*Me-alanine 30.9 min, L-valine 41.1 min (L-Val 41.1, D-Val 44.2 min), L-homo-tyrosine 41.0 min, L-phenylalanine 45.5 min (L-Phe 45.5, D-Phe 47.6 min), and D-lysine 46.3 min (L-Lys 45.9, D-Lys 46.3 min).

*4.3.8. Anabaenopeptin MM913 (**8**).* Glassy material; $[\alpha]_D^{25}$ −18 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.57), 228 (4.23), 248 (3.83), 280 (3.73) nm; IR (KBr): 3350, 2975, 2939, 1655, 1544, 1474, 1297, 1129, 1035 cm^{-1}; ^1H and ^{13}C NMR (see Table 5); Positive HR ESIMS *m/z* 914.5031 $[M+H]^+$, (calcd for $C_{49}H_{68}N_7O_{10}$, 914.5028). Retention time of AA Marfey derivatives: L-*N*Me-homo-tyrosine 41.0 min (L-*N*Me-Hty 41.0, D-*N*Me-Hty 44.6 min), L-isoleucine 44.5 min (L-Ile 44.5, D-Ile 47.6 min), D-lysine 46.3 min (L-Lys 45.8, D-Lys 46.3 min), L-homo-phenylalanine 48.3 min (L-Hph 48.3, D-Hph 50.9 min), and L-tyrosine 51.1 min (L-Tyr 51.1, D-Tyr 52.8 min).

*4.3.9. Anabaenopeptin B (**9**).* Glassy white solid; Negative HR ESIMS *m/z* 835.4508 $[M-H]^-$, (calcd for $C_{41}H_{59}N_{10}O_9$, 835.4466); Identical in all respects with the published data.[2,9]

4.4. Determination of the absolute configuration of the amino acids

Compounds **1**–**9** (0.3 mg each) were hydrolyzed in 6 M, HCl (1 mL). The reaction mixture was maintained in a sealed glass vial at 110 °C for 16 h. The acid was removed in vacuo and the residue was re-suspended 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 $NaHCO_3$ (1 M, 120 μL) 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 acids derivatives from hydrolyzate were dissolved in 1 mL ACN 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. For compounds **2**, **4**, and **5**, a linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer, pH 3:ACN, within 60 min, was used. For compounds **1**, **3**, **6**, **7**, and **8**, a linear gradient elution from 100%, 0.1% TFA in H_2O to 1:1, 0.1% TFA in H_2O/ACN, within 60 min, was used. The absolute configuration of each amino acid was determined by spiking the derivatized hydrolyzates with the standard FDAA-amino acids.

4.5. Determination of the absolute configuration of Ahp and Amp derivatives

Compounds **1**–**5** (0.1 mg each) were oxidized with Jones' reagent (1 drop from solution of 1.34 g K_2CrO_7, 1 mL H_2SO_4 in 8 mL

H_2O) in 0.5 mL acetone at 0 °C for 10 min. The mixture was allowed to warm to room temperature and a few drops of MeOH were added. The bluish residue that developed was filtered and the solvent was evaporated *in vacuo*. The resultant products were treated as described above.

4.6. Determination of the absolute configuration of glyceric acid

Extraction of the acid hydrolyzates of compounds **1**–**4** with diethyl-ether separated the glyceric acid 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 Phases, 250×4.6 mm flow rate 1 mL/min, UV detection at 210 nm, linear elution 1:49 1% triethylamine, 1% acetic acid (TEAA) aq buffer, pH 4: MeOH. The absolute configuration of the glyceric acid from the micropeptins was determined by spiking with a mixture of standard L,D-glyceric acids.

4.7. Proteases inhibition assays

Trypsin, thrombin, 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 CaCl2, 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) and thrombin (0.5 g/mL) were 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. For thrombin, 170 μL buffer solution, 10 μL enzyme solution and 10 μL sample solution were added to each micro titer plate well and pre-incubated at 25 °C for 5 min. Then, 10 μL of substrate solution was added and the kinetic of the reaction was measured at 405 nm, 37 °C for 20 min.

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Supplementary data

Tables 5–13, summarizing the ^{1}H and ^{13}C NMR, HMQC or HSQC, HMBC, and ROESY data of **1**–**9**, as well as, the ^{1}H and ^{13}C NMR spectra of compounds **1**–**9**, are available. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2010.09.067. These data include MOL files and InChIKeys of the most important compounds described in this article.

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