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Protease inhibitors from three fishpond water blooms of *Microcystis* spp.

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ABSTRACT

Four new natural products, micropeptin GH979 (**1**), microginin GH787 (**2**), micropeptin HM978 (**3**), and micropeptin HA983 (**4**), as well as 10 known protease inhibitors and hepatotoxins, were isolated from the hydrophilic extract of three samples of cyanobacteria (*Microcystis* spp.) that were collected from fishponds in Kibbutz Giva'at Haim, Kibbutz Hama'apil, and Kibbutz Gan Shmuel. The structures of the pure natural products were elucidated using spectroscopic methods, including UV, 1D and 2D NMR, 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 for the serine proteases: trypsin, chymotrypsin, thrombin and elastase, and the metalloprotease, aminopeptidase N.

1. Introduction

The environmental, toxicological, biological, and chemical properties of toxic and nontoxic *Microcystis* spp. water blooms have been extensively studied.[1–4] Extracts of strains of *Microcystis* have been shown to be a rich source of unique modified peptides, such as microginins,[5] micropeptins,[6] aeruginosins,[7] anabaenopeptins,[8] and microviridines,[9] all of which exhibit protease inhibitory activity. Some of these secondary metabolites have been recently shown to be toxic to aquatic organisms.[10] The variation in structure within each one of the five groups of protease inhibitors and in the relative abundance of members of these groups in bloom material is enormous. The micropeptins are the most abundant group of serine protease inhibitors from cyanobacteria, composed of more than 130 different variants.[11] Second to the micropeptins in abundance are the aeruginosins followed by the anabaenopeptins, microginins while the least abundant are the microviridins. As part of our ongoing research on the chemistry and chemical ecology of cyanobacterial blooms in water bodies, biomasses from three collections of bloom material (TAU strains IL-332, IL-337, and IL-359) of *Microcystis* spp. collected from geographical sites located close to each other, during November 2003 (first two) and August 2007, were chemically investigated. The isolation and structure elucidation of the new secondary metabolites isolated from these cyanobacterial bloom biomasses, as well as their biological activity, is discussed below.

2. Results and discussion

Bloom material (IL-337, *Microcystis* sp.) collected from a commercial fishpond in Kibbutz Giva'at Haim on November 2003 was stored in a deep-freezer immediately after collection and freeze-dried. The freeze-dried cyanobacterium biomass was extracted with 70% MeOH in H_2O. The extract, which inhibited trypsin and chymotrypsin was flash-chromatographed on a reversed-phase C_{18} column. The fractions that exhibited protease inhibitory activity were further separated by gel filtration on Sephadex LH-20 and reversed-phase HPLC to afford 11 pure natural products. Three of which, micropeptin GH979 (**1**), microginin GH787 (**2**), and aeruginosin GH553,[12] were found to be new natural products while the rest were found to be the known microcystin LR,[13] microcystin YR,[14] micropeptin EI992,[15] aeruginosins 98A and 98B,[16] aeruginazole A,[17] anabaenopeptin F,[18] and oscillamide Y.[19] From a bloom material (IL-332, *Microcystis* sp.) collected from a commercial fishpond in Kibbutz Hama'apil on November 2003 and processed in a similar fashion, a new natural product, micropeptin HM978 (**3**) and five known natural products, micropeptin SF909,[20] aeruginosin 98A,[16] microcin SF608,[20] anabaenopeptin F,[18] and aeruginazole A,[17] were isolated. The third bloom material (IL-359, *Microcystis* sp.) was collected from a commercial fishpond of Kibbutz Gan Shmuel on August 2007. It afforded two micropeptins, the new micropeptin HA983 (**4**) and the known cyanopeptolin S (Scheme 1 and Fig. 1 in Supplementary Data).[21]

2.1. Micropeptin GH979 (1)

Micropeptin GH979 (**1**) was isolated as a colorless glassy solid. The molecular formula of **1**, $C_{49}H_{69}N_7O_{14}$, was deduced from the

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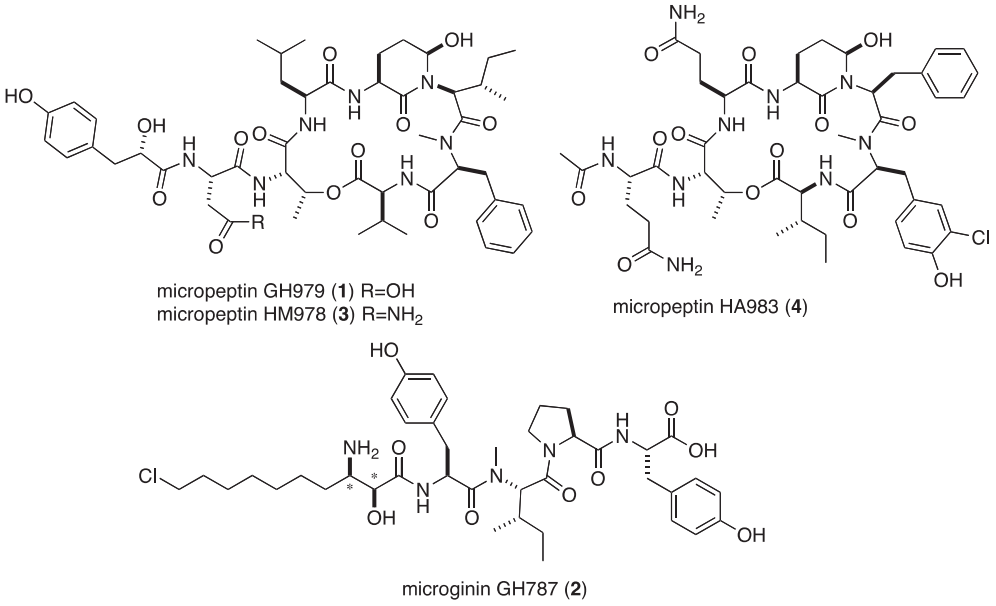

Scheme 1.

high-resolution MALDI-TOF-MS measurements of its sodiated molecular cluster ion at *m*/*z* 1002.4862. Examination of the NMR spectra of **1** in DMSO-d_6 (see Table 1 and Table 4 in Supplementary data) revealed its peptide nature and belonging to the micropeptins; i.e., nine carboxylic carbons in the ^{13}C NMR spectrum, five amide doublet protons in the ^{1}H NMR spectrum, a hydroxy group at δ_H 6.04 (br s), and an *N*Me group δ_H 2.80 (s). Taking into account the *N*Me-aromatic amino acid and the *N*,*N*-disubstituted-amino acid of the micropeptins, the five amide doublet protons revealed that this micropeptin was composed of seven amino acids. The existence of the amino hydroxy piperidone (Ahp) and the *p*-hydroxy-phenyllactic acid (Hpla) were suggested on the basis of the COSY and HMQC spectra, which showed a broad singlet of a hydroxyl group at δ_H 6.04 coupled to the aminal proton at δ_H 4.91 (br s) [δ_C 74.1 (d)] of the Ahp moiety, and a characteristic oxymethine at δ_H 4.00 (dd) and δ_C 72.8 (d) for Hpla. Analysis of the COSY, TOCSY, and HMQC 2D NMR spectra allowed the assignment of the side chains of a valine, an isoleucine, a leucine, a threonine, an aspartic acid, and three short fragments consistent with a phenylalanine moiety, *p*-hydroxy-phenyllactic acid, and an Ahp moiety. The structure of the side chains of the latter two amino acids and the assignment of the carboxyamide carbons to the side chains were achieved by analysis of the results of an ^{1}H–^{13}C HMBC experiment (see Table 4 in Supplementary data). The sequence of the amino acids of the peptide: Val, *N*MePhe, Ile, Ahp, Leu, Thr, Asp, and Hpla was assigned on the basis of HMBC correlations between the carbonyl of *N*MePhe and Val NH, the carbonyl of Ile and the *N*Me of *N*MePhe, Ahp C-6 with H-2 of Ile, the carbonyl of Thr and NH of Leu, the carboxyl of Val and H-3 of Thr (the lactone linkage), the carbonyl of Asp and both H-2 and NH of Thr, the carbonyl of Hpla and the NH of Asp and by the NOE correlations of Ahp NH and H-2 of Leu. The amino acid sequence could also be assembled from the ROESY data (see Table 4 in Supplementary data). The relative configuration of Ahp, (3*S**,6*R**), was based on the *J*-values of H-6 (<1 Hz), which pointed to an equatorial orientation of this proton, the chemical shift of the pseudoaxial H-4 (δ_H 2.60), which was downfield shifted by the axial hydroxy-group and the NOE correlation observed between H-4$_{pax}$ and the 6-OH. Jones' oxidation[22] followed by Marfey's analysis[23] established the L-configuration of Asp, Glu (from Ahp), Ile, Leu, *N*MePhe, Thr, and Val, while analysis on a chiral-HPLC-column established the absolute configuration of Hpla as L. The assignment of the absolute configuration of Glu, resulting from oxidation of Ahp, as L(*S*) by Marfey's analysis established the absolute configuration of Ahp C-6 as *R*. Marfey's analysis using 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) as Marfey's reagent[23] fails to distinguish L-threonine from L-*allo*-threonine and L-isoleucine from L-*allo*-isoleucine and thus additional evidence was needed to support their establishment. The observed *J*-value (0–1 Hz) between H-2 and H-3 of the *N*,*O*-disubstituted threonine in **1**, suggested that, as in the case of all known micropeptins, it should be L-threonine and not L-*allo*-threonine.[24] In the case of the *N*,*N*-disubstituted-Ile, the carbon chemical shifts measured for compound **1**, 10.4 (C-5) and 13.9 (C-6) ppm, were found similar to those measured for L-Ile, 10.3 (C-5) and 13.9 (C-6) ppm (established for nostopeptins A and B by chiral-GC–MS[25]) while different from those measured for *allo*-Ile, 12.2 (C-5) and 14.1 (C-6) ppm (established for micropeptin KT1042[12]). Based on the results discussed above, the structure of micropeptin GH979 was assigned as **1**.

2.2. Microginin GH787 (2)

Microginin GH787 (**2**) was isolated as a colorless glassy material. Its molecular formula, $C_{40}H_{58}ClN_5O_9$, was deduced from the high-resolution MALDI-TOF-MS measurements of its sodiated molecular ion cluster at *m*/*z* 810.3808. Examination of the NMR spectra of **2** in DMSO-d_6 revealed that it contained two tyrosine moieties (four 2H doublet signals at δ_H 6.61, 6.65, 6.91 and 7.02), an *N*Me-amino acid (δ_H 2.82 s), an isoleucine moiety (δ_H 0.89 d and 0.66 t, 3H's each), and a chloromethyl group (δ_H 3.62 2H, t; δ_C 45.3 t).[26] Analysis of the COSY, TOCSY, HMQC, and HMBC 2D NMR data allowed the assignment of the side chains of the amino acids in **2** as an *N*,*N*-disubstituted isoleucine, a proline, a 3-amino-10-chloro-2-hydroxy-decanoate (chloro-Ahda), two AA′BB′ spin systems of *p*-substituted phenols, and two aliphatic ABMX spin systems, in agreement with two tyrosine moieties (see Table 2). The latter fragments were assembled to the full tyrosine moieties by HMBC correlations of the aliphatic and aromatic protons with the bridging aromatic C-4 and aliphatic protons with the carbonyl carbons (see Table 2). The carbonyls of Pro, *N*MeIle, and chloro-Ahda were assigned based on their correlations with their α-protons. The sequence of the amino acids of the peptide, chloro-Ahda-^{II}Tyr-*N*MeIle-Pro-^{I}Tyr was assigned on the basis of HMBC correlations

Table 1
^{1}H and ^{13}C NMR data of micropeptins GH979 (**1**) and HM978 (**3**) in DMSO-d_6[a]

Position		Micropeptin GH979 (**1**)			Micropeptin HM978 (**3**)		
		δ_C	mult.	δ_H, mult. *J* (Hz)	δ_C	mult.	δ_H, mult. *J* (Hz)
Val	1	172.5	s		172.4	s	
	2	56.4	d	4.60, m	56.4	d	4.67, dd (5.4, 9.3)
	3	30.7	d	1.97, m	30.7	d	2.01, dqq (5.4, 6.8, 6.9)
	4	17.0	q	0.70, d (6.8)	17.8	q	0.70, d (6.8)
	5	19.1	q	0.78, d (6.4)	19.3	q	0.79, d (6.9)
	NH			7.62, d (10.3)			7.65, d (9.3)
*N*MePhe	1	169.3	s		169.4	s	
	2	60.8	d	5.13, dd (12.5, 2.9)	60.6	d	5.14, dd (11.6, 2.6)
	3a	34.3	t	2.76, dd (13.9, 12.5)	34.3	t	2.79, dd (14.2, 11.6)
	3b			3.29, dd (13.9, 2.9)			3.27, dd (14.2, 2.6)
	4	137.8	s		137.8	s	
	5,5′	129.7	d	7.21, m	129.7	d	7.21, d (7.2)
	6,6′	128.7	d	7.24, m	128.7	d	7.25, t (7.2)
	7	126.7	d	7.17, m	126.7	d	7.19, t (7.2)
	*N*Me	30.3	q	2.80, s	30.3	q	2.73, s
Ile	1	169.8	s		169.9	s	
	2	54.3	d	4.37, d (10.7)	54.3	d	4.38, d (10.6)
	3	33.2	d	1.76, m	33.2	d	1.72, m
	4a	23.7	t	1.07, m	23.7	t	1.06, m
	4b			0.60, m			0.59, m
	5	10.4	q	0.60, br d (3.8)	10.4	q	0.59, br d (2.9)
	6	13.9	q	−0.24, d (6.4)	14.0	q	−0.26, d (6.4)
Ahp	2	169.5	s		169.6	s	
	3	49.0	d	4.41, m	49.1	d	4.43, ddd (11.6, 9.3, 6.6)
	4peq	21.8	t	1.80, m	21.8	t	1.80, m
	4pax			2.60, m			2.56, m
	5peq	29.9	t	1.70, m	29.9	t	1.72, m
	5pax			1.70, m			1.72, m
	6	74.1	d	4.91, br s	74.1	d	4.91, br s
	NH			7.31, d (9.2)			7.35, d (9.3)
	OH			6.04, br s			6.09, br s
Leu	1	170.7	s		170.8	s	
	2	50.9	d	4.30, br t (8.4)	51.0	d	4.28, m
	3a	39.5	t	1.39, m	39.6	t	1.38, m
	3b			1.83, m			1.82, m
	4	24.3	d	1.50, m	24.3	d	1.51, m
	5	23.3	q	0.89, d (6.5)	23.4	q	0.88, d (6.6)
	6	21.1	q	0.78, d (6.4)	21.1	q	0.77, d (6.6)
	NH			8.40, d (8.4)			8.42, d (8.5)
Thr	1	168.9	s		169.1	s	
	2	54.9	d	4.64, d (9.2)	55.1	d	4.58, br d (9.3)
	3	72.1	d	5.48, q (6.5)	72.1	d	5.48, q (6.4)
	4	17.8	q	1.20, d (6.5)	17.9	q	1.23, d (6.4)
	NH			7.64, d (9.2)			7.73, d (9.3)
Asp/Asn	1	171.3	s		171.7	s	
	2	49.2	d	4.73, m	49.3	d	4.71, dt (7.8, 6.4)
	3a	35.7	t	2.63, dd (16.7,7.4)	36.9	t	2.54, t (6.4)
	3b			2.78, m			
	4	172.1	s		172.0	s	
	NH			8.24, m (8.2)			8.20, d (7.8)
	4-NHa						7.39, s
	4-NHb						6.89, s
Hpla	1	174.1	s		174.0	s	
	2	72.8	d	4.00, dd (9.7, 3.0)	72.8	d	3.99, br d (9.3)
	2-OH			5.60, br s			5.59, br s
	3a	40.0	t	2.55, dd (13.9, 9.7)	40.0	t	2.53, m
	3b			2.95, dd (13.9, 3.0)			2.94, dd (11.2, 2.7)
	4	128.9	s		129.0	s	
	5,5′	130.3	d	7.02, d (8.0)	130.3	d	7.02, d (8.5)
	6,6′	114.9	d	6.65, d (8.0)	114.9	d	6.64, d (8.5)
	7	155.5	s		155.5	s	
	7-OH			9.08, br s			9.10, br s

[a] 500 MHz for H and 125 MHz for C.

between the carbonyl of an amino acid and the amide proton of the adjacent acid for the pairs Pro-^{l}Tyr, *N*MeIle-Pro, and chloro-Ahda-^{ll}Tyr, between the carbonyl of ^{ll}Tyr and the *N*Me of *N*MeIle and ROESY correlations between Pro H-5a and H-5b and *N*MeIle H-2. Marfey's analysis[23] established the absolute configuration of *N*Me-isoleucine, proline, and tyrosine as L. Comparison of the chemical shifts of H-2 and H-3 and C-2 and C-3 of Ahda in **2** ($\delta_{H\text{-}2}$ 4.03, $\delta_{C\text{-}2}$ 69.7, $\delta_{H\text{-}3}$ 3.12, $\delta_{C\text{-}3}$ 53.2) with those of microginin[5] ($\delta_{H\text{-}2}$ 4.05, $\delta_{C\text{-}2}$ 69.4, $\delta_{H\text{-}3}$ 3.22, $\delta_{C\text{-}3}$ 53.0) and microginin 299-D[27] ($\delta_{H\text{-}2}$ 4.21, $\delta_{C\text{-}2}$ 70.6, $\delta_{H\text{-}3}$ 3.36, $\delta_{C\text{-}3}$ 52.9) allowed the assignment of the 2*S**,3*R**

Table 2
NMR data of micropeptin GH787 (**2**) in DMSO-d_6[a]

Position		δ_C, mult.	δ_H, mult. J (Hz)	LR H–C correlations	NOE correlations
^{I}Tyr	1	172.9, s		^{I}Tyr-2,3a,3b,NH	
	2	53.9, d	4.28, q (7.5)	^{I}Tyr-3a,3b	^{I}Tyr-5,5′,NH
	3a	36.0, t	2.73, m	^{I}Tyr-2,5,5′	^{I}Tyr-5,5′,NH
	3b		2.89, m		^{I}Tyr-5,5′,NH
	4	127.3, s		^{I}Tyr-2,3a,3b,6,6′	
	5,5′	130.0, d	7.02, d (8.5)	^{I}Tyr-3a,3b	^{I}Tyr-2,3a,3b,NH
	6,6′	114.9, d	6.65, d (8.5)	^{I}Tyr-5,5′,OH	^{I}Tyr-OH
	7	155.9, d		^{I}Tyr-5,5′,6,6′,OH	
	7-OH		9.16, s		^{I}Tyr-6,6′
	NH		7.91, d (7.5)		^{I}Tyr-2,3a,3b,5,5′, Pro-2
Pro	1	171.3, s		Pro-2, ^{I}Tyr–NH	
	2	59.1, d	4.32, dd (8.5, 3.0)		^{I}Tyr-NH, Pro-3a,3b, 4a,4b
	3a	29.1, t	1.78, m		Pro-2,4a,4b
	3b		2.00, m		Pro-2,4a,4b
	4a	24.0, t	1.72, m		Pro-2,3b,5a,5b
	4b		1.79, m		Pro-2,3b,5a,5b
	5a	46.9, t	3.40, m		Pro-4a,4b, *N*Melle-2
	5b		3.44, m		Pro-4a,4b, *N*Melle-2
*N*Melle	1	167.7, s		*N*-Melle-2	
	2	57.1, d	4.93, d (10.5)	*N*-Melle-5, *N*-Me	*N*-Melle-5,6, Pro-5a,5b
	3	32.5, d	1.80, m	*N*-Melle-2,5	*N*-Melle-5,*N*-Me
	4a	23.8, t	1.26, m	*N*-Melle-5,6	*N*-Melle-*N*-Me
	4b		1.38, m		*N*-Melle-*N*-Me
	5	10.5, q	0.66, m		*N*-Melle-2,*N*-Me
	6	14.9, q	0.89, m		*N*-Melle-2
	*N*Me		2 82, s	*N*-Melle-2	*N*-Melle-3,4a,4b,5,
^{II}Tyr	1	171.2, s		TyrII-2,3a,3b, *N*-Melle-*N*-Me	
	2	50.3, d	4.86, q (7.5)	TyrII-3a,3b	^{II}Tyr-5,5′,NH
	3a	36.4, t	2.73, m	TyrII-2,5,5′	^{II}Tyr-5,5′,NH
	3b		2.89, m		^{II}Tyr-5,5′,NH
	4	126.4, s		^{II}Tyr-2,3a,3b,6, 6′	
	5,5′	130.0, d	6.91, d (8.5)	^{II}Tyr-3a,3b	^{II}Tyr-2,3a,3b,NH
	6,6′	114.9, d	6.61, d (8.5)	^{II}Tyr-5,5′,OH	^{II}Tyr-OH
	7	156.1, d		^{II}Tyr-5,5′,6,6′, OH	
	7-OH		9.25, s		^{II}Tyr-6,6′
	NH		8.17, d (7.5)		^{II}Tyr-2,3a,3b,5,5′, Cl-Ahda-2,2-OH
Chloro-Ahda	1	170.1, s		^{II}Tyr-NH, Cl-Ahda-2,2-OH	
	2	69.7, d	4.03, t (5.0)	Cl-Ahda-2-OH	Cl-Ahda-3-NH_2, 4a,4b, 5,6, ^{II}Tyr-NH
	2-OH		6.45, br d (5.0)		Cl-Ahda-3,5,6, ^{II}Tyr-NH
	3	53.2, d	3.12, m	Cl-Ahda-2,2-OH	Cl-Ahda-3-NH_2, 4a,4b,5,6
	3-NH_2		7.77, br s		Cl-Ahda-2,3,4a,4b,5,6
	4	28.6, t	1.42, m; 1.35, m	Cl-Ahda-5,6	Cl-Ahda-2,3; Cl-Ahda-2,3
	5	24.6, t	1.24, m		Cl-Ahda-2,2-OH, 3,3-NH_2
	6	28.4, t	1.24, m	Cl-Ahda-4a,4b	Cl-Ahda-2,2-OH, 3,3-NH_2
	7	27.9, t	1.26, m	Cl-Ahda-8,9	
	8	26.1, t	1.37, m	Cl-Ahda-9,10	Cl-Ahda-10
	9	32.0, t	1.70, m	Cl-Ahda-10	Cl-Ahda-10
	10	45.3, t	3.62, t (6.5)	Cl-Ahda-8,9	Cl-Ahda-8,9

[a] 400 MHz for H and 100 MHz for C.

relative stereochemistry to Ahda in **2**. The structure of microginin GH787 (**2**) was thus assigned to be (2*S**,3*R**)-chloro-Ahda-L-Tyr-L-*N*Melle-L-Pro-L-Tyr.

2.3. Micropeptin HM978 (3)

Micropeptin HM978 (**3**) was isolated as a colorless glassy solid. The molecular formula of **3**, $C_{49}H_{70}N_8O_{13}$, was deduced from the high-resolution MALDI-TOF-MS measurements of its molecular cluster ion $[M+K]^+$ at m/z 1017.4695. The difference between micropeptin HM978 (**3**) and micropeptin GH979 (**1**), a NH_2 instead of OH, respectively, was located in the side chain of the aspartyl residue, as evident from the two additional (relative to **1**) singlet protons at δ_H 7.39 and 6.89 (see Table 1, and Table 5 in Supplementary data). The remaining ^{1}H and ^{13}C NMR signals were comparable in terms of chemical shifts and multiplicities. Analysis of the COSY, TOCSY, HMQC, and HMBC 2D NMR data allowed the assignment of the planar structure of **3**, with the only difference from **1** being the substitution of the Asp, in **1**, with Asn, in **3**. A similar procedure to that applied for **1** established the absolute configuration of all acid residues, in **3**, to be L. Based on the results discussed above the structure of micropeptin HM978 was assigned as **3**.

2.4. Micropeptin HA983 (4)

Micropeptin HA983 (**4**) was isolated as a glassy solid. The molecular formula of **4**, $C_{46}H_{62}ClN_9O_{13}$, was deduced from the high-resolution MALDI-TOF-MS measurements of its potassium adduct ion $[M+K]^+$ at m/z 1022.3838/1024.3380 (3:1 intensity ratio). The 3:1 intensity ratio indicated the presence of a chlorine atom due to the isotopic ratio of $^{35}Cl/^{37}Cl$. The NMR spectra of **4** in DMSO-d_6 revealed its micropeptin nature; i.e., 10 carboxylic carbons in the ^{13}C NMR spectrum, 5 doublet and 4 singlet amide protons in the ^{1}H NMR spectrum (see Table 3), a hydroxy group at δ_H 6.06 (br s) and an *N*Me group δ_H 2.73 (s). Analysis of the COSY, TOCSY, HSQC, and

Table 3
NMR data of micropeptin HA983 (**4**) in DMSO-d_6[a]

Position		δ_C, mult.	δ_H, mult. J (Hz)	LR H–C Correlations	NOE correlations
Ile	1	172.5, s		Thr-3, Ile-2	
	2	55.7, d	4.71, m	Ile-6	Ile-3, NH
	3	37.9, d	1.81, m	Ile-2,4	Ile-2,5,6
	4	24.8, t	1.20, m	Ile-2,5,6	Thr-3, Ile-3,5,6
			1.42, m		
	5	11.8, q	0.78, t (6.0)	Ile-4	Ile-2,3
	6	16.4, q	0.83, d (5.2)	Ile-2	Ile-2,3
	NH		7.46, d (7.2)		Ile-2, *N*Me,Cl-Tyr-2
*N*Me-*m*-Cl-Tyr	1	169.3, s		Ile-NH *N*Me,Cl-Tyr-2	
	2	61.0, d	4.68, d (8.4)	*N*Me,Cl-Tyr-3a,3b	Ile-NH, *N*Me,Cl-Tyr-2,9
	3a	32.4, t	2.79, m	*N*Me,Cl-Tyr-5,9	*N*Me,Cl-Tyr-5,9
	3b		3.12, m		*N*Me,Cl-Tyr-5,9
	4	121.5, s		*N*Me,Cl-Tyr-5,8,9	
	5	130.9, d	7.14, m	*N*Me,Cl-Tyr-9	*N*Me,Cl-Tyr-3a,3b
	6	120.0, s		*N*Me,Cl-Tyr-5,8	
	7	152.4, s		*N*Me,Cl-Tyr-5,8,9	
	7-OH		10.2, s		
	8	115.3, d	6.97, d (6.4)	*N*Me,Cl-Tyr-9	*N*Me,Cl-Tyr-9
	9	129.5, d	6.95, d (6.4)	*N*Me,Cl-Tyr-5,8	*N*Me,Cl-Tyr-8
	*N*Me	32.4, q	2.73, br s		*N*Me,Cl-Tyr-2,3a,3b,5,9
Phe	1	170.7, s		*N*Me,Cl-Tyr-*N*Me, Phe-2	
	2	50.8, d	5.71 m	Phe-3b	Phe-5,6
	3a	35.5, t	1.83, m	Phe-5	Ile-4a,4b,5, Ahp-3,6, Val-3
	3b		2.87, br t (10.0)		Phe-5
	4	120.0, s		Phe-6,6′	
	5,5′	129.8, d	6.79, d (5.6)	Phe-6,6′,7	Phe-6,6′,7
	6,6′	128.2, d	7.14, m	Phe-7	Phe-5,5′
	7	126.6, d	7.14, m	Phe-5,5′,6,6′	
Ahp	2	169.3, s		Phe-2, Ahp-3,6	
	3	49.0, d	3.60, m		Ahp-4eq,5ax
	4peq	22.0, t	1.55, m	Ahp-3,6	Ahp-3,4pax
	4pax		2.37, q (10.5)		Ahp-4peq,OH
	5peq	24.7, t	1.56, m		Ahp-4peq,4pax
	5pax		1.68, m		Ahp-3,4peq
	6	74.2, d	5.05, br s	Ile-2	Ahp-5,OH
	NH		7.07, d (6.8)		Ahp-3,4peq
	OH		6.06, br s		Ahp-4pax,6
^{I}Gln	1	170.1, s		Ahp-NH, ^{I}Gln-2	
	2	51.0, d	4.14, br t (9.1)	^{I}Gln-NH	Ahp-NH, ^{I}Gln-3b,NH
	3a	26.7, t	1.55, m	^{I}Gln-4	^{I}Gln-4,5-NH_2
	3b		2.01, m		^{I}Gln-2
	4a	31.6, t	2.03, m	^{I}Gln-3	^{I}Gln-3,5-NH_2
	4b		2.03, m		
	5	174.2, s		^{I}Gln-4,5-NH_2	
	5-NH_2		6.67, s; 7.18, s		^{I}Gln-5-NH_2 ^{I}Gln-4
	NH		8.45, d (6.4)		Ahp-NH, Thr-2,3
Thr	1	169.6, s		^{I}Gln-NH, Thr-2,3	
	2	55.2, d	4.54, d (6.8)	Thr-3	^{I}Gln-NH, Thr-3,4,NH
	3	72.2, d	5.37, br q (6.2)	Thr-2,4	^{I}Gln-NH, Thr-2,4,NH
	4	18.0, q	1.17, d (6.2)	Thr-3	Thr-2,3,NH
	NH		7.96, d (6.8)		Thr-2, ^{II}Gln-NH
^{II}Gln	1	172.9, s		Thr-2, NH, ^{II}Gln-2,	
	2	52.4, d	4.36, br q (5.9)	^{II}Gln-NH	^{II}Gln-3a,4a,4b,NH, Ac-2
	3a	28.2, t	1.68, m	^{II}Gln-2,3,NH	^{II}Gln-2,4,NH
	3b		1.84, m		^{II}Gln-2,NH
	4a	31.3, t	2.14, m	^{II}Gln-2,NH,5-NH_2	^{II}Gln-2,3b,NH
	4b		2.14, m		
	5	174.4, s		^{II}Gln-4,5-NH_2	
	NH		8.07, d (5.9)		^{II}Gln-2,3a,3b,4a
	5-NHa		6.73, s		^{II}Gln-5-NHb
	5-NHb		7.46, s		^{II}Gln-3b
Ac	1	169.8, s		^{II}Gln-2,NH	
	2	22.8, q	1.84, s		^{II}Gln-2,NH

[a] 400 MHz for H and 100 MHz for C.

HMBC 2D NMR spectra allowed the assignment of the side chains of the amino acids of this micropeptin, an isoleucine, a threonine, two glutamine moieties, an Ahp, an *N*,*N*-disubstituted phenylalanine, an *N*Me-*m*-Chloro-tyrosine, and an *N*-acetyl residue. The sequence of the amino acids was determined by HMBC 2D NMR experiment. The HMBC correlations were observed between the carbonyl of *N*Me-*m*-Cl-Tyr and the amide proton Ile, the carbonyl of *N*,*N*-disubstituted Phe and *N*Me of *N*Me-*m*-Cl-Tyr, carbonyl of Ahp and H-2 of *N*,*N*-disubstituted Phe, the carbonyl of ^{I}Gln and the amide proton of Ahp, the carbonyl of Thr and the amide proton of ^{I}Gln, the

carbonyl of ^{II}Gln and C-2 and the amide proton of Thr, and the carbonyl of the acetyl group and the amine proton of ^{II}Gln. The lactone linkage was established on the basis of the HMBC correlation of the carbonyl of Ile and H-3 of Thr. The relative configuration of C-6 of Ahp was established as R^* based on the same arguments present above for **1**, i.e., ROESY correlation between H-4_{pax} and the 6-OH and between H-3 and H-5_{pax}. Marfey's analysis,[23] preceded by Jones' oxidation[22] and with no oxidation, established the L-configuration of Ahp (as Glu), Asp, Gln (as Glu), Ile, Leu, *N*MePhe, Thr, and Val as L. Determination of the absolute configuration of Glu, resulting from oxidation of Ahp, as L(*S*) by Marfey's analysis established the absolute configuration of Ahp C-6 as *R*. The observed *J*-value (0–1 Hz) between H-2 and H-3 of the substituted threonine in **4**, suggested that, as in the case of all known micropeptins, it should be threonine and not *allo*-threonine.[24] The possibility that L-*allo*-Ile is present in carboxylic end of **4** was ruled out by the comparison of the ^{13}C chemical shifts of C-5 and C-6 of the isoleucine residues in known micropeptins. The measured chemical shifts in **4**, 11.8 (C-5) and 16.4 (C-6) ppm, were similar to those measured for L-Ile (established for nostopeptins A and B by chiral-GC–MS[18]) 11.3 (C-5) and 16.1 (C-6) ppm, while different from those measured for L-*allo*-Ile (established for oscillamide J by chiral-GC–MS[28]) 11.4 (C-5) and 14.3 (C-6) ppm. Based on the results discussed here, the structure of micropeptin HA983 was assigned as **5**.

2.5. Biological activity

Micropeptins **1**, **3**, and **4** were isolated through bioassay-guided fractionation, targeting their serine protease inhibitory properties against trypsin and chymotrypsin. The inhibitory activities of pure **1**, **3**, and **4** were determined for the serine proteases trypsin, thrombin, chymotrypsin, and elastase while that of **2** against amino peptidase N. Micropeptin GH979 (**1**) inhibited chymotrypsin with an IC_{50} of 15.2 μM and partially elastase at a concentration of 45.5 μM. Microginin GH787 (**2**) inhibited bovine amino peptidase N (APN) with an IC_{50} of 7.7 μM. Micropeptin HM978 (**3**) inhibited chymotrypsin with an IC_{50} of 3.6 μM and partially elastase at a concentration of 45.2 μM. Micropeptin HA983 (**4**) inhibited chymotrypsin with an IC_{50} of 0.94 μM.

3. Experimental section

3.1. General experimental procedures

Optical rotations were determined on a JASCO P-1010 polarimeter. UV spectra were recorded on an Agilent 8453 spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C and a Bruker Avance 400 Spectrometer at 400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C. DEPT, COSY-45, gTOCSY, gROESY, gHSQC, gHMQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. High-resolution MS were recorded on an Applied Biosystems Voyager System 4312 instrument and Waters MALDI Synapt System. HPLC separations were performed on a JASCO HPLC system (model PU-2080 *Plus* pump, model LG-2080-04 Quaternary Gradient unit and model PU-2010 *Plus* Multi-wavelength Detector) and on MERCK HPLC system (model L-6200A pump and model L-4200 UV–vis detector). ELISA for proteases inhibition assay, EL_x808, Bio-Tek Instruments, Inc.

3.2. Biological material

Microcystis sp., TAU strain IL-337, was collected from a fishpond at Kibbutz Giva'at Haim, Israel in November 2003. *Microcystis* sp., TAU strain IL-332, was collected from a fishpond in Kibbutz Hama'apil, Israel in November 2003. *Microcystis* sp., TAU strain IL-359, was collected from a fishpond of Kibbutz Gan Shmuel near Hadera, Israel in August 2007. The cell mass was frozen after collection and lyophilized. Samples of the cyanobacteria are deposited at the culture collection of Tel Aviv University.

3.3. Isolation procedure

Lyophilization of the IL-337 cells produced 500 g of dry cells. Extraction with 7:3 MeOH/H_2O (3×2 L) and evaporation yielded 53 g of extract. The 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 H_2O. Fractions 5–7 (2:3, 1:1, and 3:2 MeOH/H_2O) fully inhibited trypsin and chymotrypsin at a concentration of 1 mg/mL. Fractions 5 (a, 1.2 g) and 6+7 (b, 3.0 g) were further separated on a Sephadex LH-20 column 1:1 MeOH/$CHCl_3$ to obtain 12 and 16 fractions, respectively. The resulting fractions were repeatedly separated on different reversed-phase HPLC columns to afford the pure compounds. The final purification of micropeptin GH979 (**1**) (originating in fractions b6–14) was achieved on a YMC-Pack C-18 HPLC column (5 μm, 250 mm×20 mm, DAD at maximum absorbance, 50:50 0.1% TFA in H_2O/MeCN, flow rate 3.0 mL/min). It was eluted from column with a retention time of 30.5 min (11.0 mg, 0.0022% yield based on the dry weight of the cyanobacteria). Microginin GH787 (**2**) (4.3 mg, 0.0009% yield, originated in fractions b6–14) was eluted, in the final purification step, from a YMC-Pack C-8 column (250 mm×20.0 mm, DAD at maximum absorbance, 50:50 0.1% TFA in H_2O/MeCN, flow rate 3.0 mL/min) with a retention time of 24.5 min accompanied by micropeptin EI992, which eluted from the column with a retention time of 22.7 min (34.0 mg, 0.0068% yield). Aeruginosin GH553 was isolated from fractions b15–16 on a HiBar, Lichrospher 60 RP-select B column (5 μm, 250×25 mm, DAD at maximum absorbance, 3:2 0.1% TFA in H_2O/MeCN, flow rate 5.0 mL/min) with a retention time of 27.5 min (3.0 mg, 0.0006% yield). Anabaenopeptin F was isolated from fractions b2–5 on a YMC-Pack C-8 column (250 mm×20.0 mm, DAD at maximum absorbance, 3:2 0.1% TFA in H_2O/MeCN, flow rate 5.0 mL/min) with a retention time of 21.1 min (18.8 mg, 0.0038% yield). Aeruginosin 98A (t_R 21.1 min, 85.3 mg, 0.0171% yield) and aeruginosin 98B (t_R 19.5 min, 3.4 mg, 0.0007% yield) were isolated from fractions a7–11 on a HiBar, Lichrospher 60 RP-select B column (5 μm, 250×25 mm, DAD at maximum absorbance, 55:45 MeOH/H_2O, flow rate 5.0 mL/min). Oscillamide Y (7.9 mg, 0.0016% yield), microcystin YR (3.0 mg, 0.0006% yield), and microcystin LR (3.1 mg, 0.0006% yield) were isolated from fractions 6+7 of the initial reversed phase separation. Aeruginazole A (4.2 mg, 0.00008% yield) was isolated from the initial fraction 8 eluted from the reversed phase column with 7:3 methanol/water. It was eluted from the column with a retention time of 14.8 min.

Lyophilized cell mass (815 g) of IL-332 were extracted with MeOH/H_2O (3×3 L) and evaporation yielded 89 g of extract. The extract was chromatographed as described for IL-337. Fractions 4+5 (3:7 and 2:3 MeOH/H_2O) from the RP-18 flash chromatography yielded anabaenopeptin F (18.3 mg, 0.0022% yield), aeruginosin 98A (3.2, 0.0004% yield), and micropeptin SF909 (4.0 mg, 0.0005% yield). Fractions 6+7 (1:1 and 3:2 MeOH/H_2O) from the RP-18 flash chromatography yielded micropeptin HM978 (**3**) (13.6 mg, 0.0017% yield). It was eluted from a HiBar, Lichrospher 60 RP-select B column (5 μm, 250×25 mm, DAD at maximum absorbance, 1:1 water/acetonitrile, flow rate 5.0 mL/min) with a retention time of 25.9 min. Fractions 8+9 (7:3 and 8:2 MeOH/H_2O) from the RP-18 flash chromatography yielded microcin SF608 (7.5 mg, 0.0009% yield) and aeruginazole A (18.6 mg, 0.0023% yield).

Lyophilized cell mass (28 g) of IL-359 were extracted with MeOH/H_2O (3×0.5 L) and evaporation yielded 1.6 g of extract. The

extract was chromatographed as described for IL-337. Fractions 5+6 (2:3 and 1:1 MeOH/H_2O) from the RP-18 flash chromatography was separated on a Sephadex LH-20 column eluted with MeOH to afford semipure **4** (44.0 mg), which were purified on a YMC-Pack C-8 HPLC column (250 mm×20.0 mm, DAD at maximum absorbance, 3:7 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min) to afford pure micropeptin HA983 (**4**) (17.0 mg, 0.0607%) with a retention time of 27.4 min. Fraction 7 (3:2 MeOH/H_2O) from the RP-18 flash chromatography afforded after purification on Sephadex LH-20 and RP-18 HPLC column cyanopeptolin S (8.0 mg, 0.0285% yield).

*3.3.1. Micropeptin GH979 (**1**).* Colorless glassy solid; $[\alpha]_D^{20}$ −42(*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 215 (4.60), 278 (3.50) nm; ^1H and ^{13}C NMR (see Table 1); HR MALDI TOF MS *m*/*z* 1002.4862 $[M+Na]^+$ (calcd for $C_{49}H_{69}N_7NaO_{14}$, 1002.4795). Retention time of amino acids (AA) Marfey's derivatives: L-Asp 25.4 min (D-Asp 27.7 min), L-Thr 24.5 min (D-Thr 30.0 min), L-Glu 26.1 min (D-Glu 29.2 min), L-Val 38.0 min (D-Val 44.8 min), L-Ile 44.0 min (D-Ile 50.9 min), L-Leu 44.8 min (D-Leu 51.4 min), and L-*N*Me-Phe 46.5 min. Retention time of L-Hpla on the chiral column 3.1 min (D-Hpla 3.3 min).

*3.3.2. Microginin GH787 (**2**).* Collorless glassy solid; $[\alpha]_D^{22}$ −33(*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 211 (3.90), 223 (4.00), 276 (3.00) nm; ^1H and ^{13}C NMR (see Table 2); HR MALDI TOF MS *m*/*z* 810.3808 $[M+Na]^+$ (calcd for $C_{40}H_{58}{}^{35}ClN_5NaO_9$, 810.3815). Retention time of AA Marfey's derivatives: L-Pro 31.2 min (D-Pro 34.7 min), L-*N*MeIle 44.8 min (D-*N*MeIle 49.7 min), L-Tyr 54.1 min (D-Tyr 60.0 min).

*3.3.3. Micropeptin HM978 (**3**).* Glassy solid; $[\alpha]_D^{26}$ −66(*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 227 (3.98), 278 (3.40) nm; ^1H and ^{13}C NMR (see Table 1); HR MALDI TOF MS *m*/*z* 1017.4695 $[M+K]^+$ (calcd for $C_{49}H_{70}KN_8O_{13}$, 1017.4694). Retention time of amino acids (AA) Marfey's derivatives: L-Asp 24.8 min (D-Asp 27.8 min), L-Thr 24.9 min (D-Thr 31.6 min), L-Glu 26.4 min (D-Glu 29.3 min), L-Val 40.1 min (D-Val 46.8 min), L-Ile 45.8 min (D-Ile 52.3 min), L-Leu 44.9 min (D-Leu 51.5 min), and L-*N*Me-Phe 49.5 min. Retention time of L-Hpla on the chiral column 3.1 min (D-Hpla 3.3 min).

*3.3.4. Micropeptin HA983 (**4**).* Amorphous white solid; $[\alpha]_D^{23}$ −40(*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 227 (4.38), 280 (3.72) nm; ^1H and ^{13}C NMR (see Table 3); HR MALDI TOF MS *m*/*z* 1022.3838/1024.3380 (3:1) $[M+K]^+$ (calcd for $C_{46}H_{62}{}^{35}ClKN_9O_{13}$, 1022.3787). Retention time of AA Marfey's derivatives: L-Thr 21.0 min (D-Thr 23.9 min), L-Glu 22.6 min (D-Glu 24.8 min), L-Ile 37.4 min (D-Ile 42.2 min), L-Phe 38.6 min (D-Phe 41.9 min), and L-*N*Me-Cl-Tyr 48.5 min.

3.4. Determination of the absolute configuration of the amino acids

Compounds **1**–**4** (0.3 mg each) were hydrolyzed in 6 N, HCl (1 mL). The reaction mixture was maintained in a sealed glass bomb at 110 °C for 16 h. The acid was removed in vacuo and the residue was 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 $NaHCO_3$ (120 μL, 1 M) 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 MeCN and compared with standard FDAA-amino acids by HPLC analysis: LiChrospher 60, RP-select B (5 μm), flow rate 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer, pH 3/MeCN, within 60 min. The absolute configuration of each amino acid was determined by spiking the derivatized hydrolyzates with D,L-mixture of the standard derivatized amino acids.

3.5. Determination of the absolute configuration of Ahp

Compounds **1**, **3**, and **4** (0.3 mg each) were oxidized with Jones' reagent[21] (one 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.

3.6. Determination of the absolute configuration of hydroxy phenyl lactic acid

Extraction of the acid hydrolyzates of compounds **1** and **3** with ethyl-ether separated the Hpla from the amino acid salts. The ether was removed in vacuo and the residue was dissolved in MeOH (1 mL). The MeOH solution was analyzed on an Astec, Chirobiotic, LC stationary phase, 250×4.6 mm flow rate 1 mL/min, UV detection at 277 nm, linear elution with 1:19 1% aq triethylamnium acetate (TEAA) buffer, pH 4/MeOH. The Hpla from the micropeptins was compared with standard D,L-Hpla.

3.7. Protease inhibition assays

The procedures used to determine the inhibitory activity of the new compounds on trypsin, thrombin, chymotrypsin, and elastase were described in a previous paper.[29] The procedure for the inhibition assays of the amino protease APN was published elsewhere.[30]

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.04.042. These data include MOL files and InChiKeys of the most important compounds described in this article.

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