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Protease inhibitors from a Slovenian Lake Bled toxic waterbloom of the cyanobacterium *Planktothrix rubescens*

Olga Grach-Pogrebinsky,[a] Bojan Sedmak[b] and Shmuel Carmeli[a,*]

[a]*School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel*
[b]*National Institute of Biology, Vecna pot 111, POB 141, 1001 Ljubljana, Slovenia*

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Abstract—Three new protease inhibitors, planktopeptin BL1125, planktopeptin BL843 and planktopeptin BL1061 were isolated, along with three known compounds, anabaenopeptin A, anabaenopeptin B and anabaenopeptin F from the hydrophilic extract of *Planktothrix rubescens*. The planar structure of the new compounds was determined by homonuclear and inverse-heteronuclear 2D NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was studied using Marfey's method for HPLC and by comparison of the acid hydrolysate with authentic samples on a chiral HPLC column. The new peptides were found to be elastase and chymotrypsin inhibitors.

Planktothrix (formerly *Oscillatoria*) (Oscillatoriles, Oscillatoriaceae) is one of the genera of cyanobacteria that produce toxic freshwater blooms. Two species of this genus, *Planktothrix agardhii* (Gomont) Anagnostidis and Komarek and *Planktothrix rubescens* (DC. ex Gomont) Anagnostidis and Komarek, occasionally produce hepatotoxic blooms. The hepatotoxicity of these cyanobacteria, is associated with the toxic cyclic peptides, microcystins,[1] which are usually accompanied by a considerable amount of protease inhibitors.[2] All five classes of protease inhibitors that have been described from toxic genera of cyanobacteria are found in *Planktothrix* spp., namely—micropeptins,[3] aeruginosins,[4] microginins,[5] anabaenopeptins[6] and microveridins.[7] Serine protease inhibitors of the micropeptins type, are the most abundant protease inhibitors from cyanobacteria, with more than fifty described compounds,[8] nine of which were isolated from extracts of *Planktothrix* spp.[3,9a–d] Twelve of the seventeen known anabaenopeptin type proteases/protein phosphatases inhibitors, were described from extracts of *Planktothrix* spp.[9c,10a–c] Four of the nine published microviridins were isolated from *Planktothrix* spp.[9c,11] Only two aeruginosins[12] and two microginin[13] type protease inhibitors were found in *Planktothrix* spp. We hereby report the structure elucidation and biological activity of three new micropeptin-type serine protease inhibitors.

As part of our ongoing research on the chemistry of cyanobacterial blooms in water bodies, a toxic strain of the cyanobacterium *Planktothrix rubescens* was collected, in the summer of 1999, from Lake Bled, Slovenia. The sample of the cyanobacterium was freeze-dried and extracted with 70% MeOH in H_2O. The extract was found to inhibit several serine proteases. The active extract was flash-chromatographed on an ODS column. Three fractions eluted from the column, with 40–60% MeOH in H_2O, exhibited protease inhibitory activity and were further separated on a reversed-phase HPLC column. Three new protease inhibitors, planktopeptin BL1125 (**1**, 380.0 mg), planktopeptin BL843 (**2**, 9.5 mg) and planktopeptin BL1061 (**3**, 15.6 mg) were isolated, along with three known compounds, anabaenopeptin A[10d] (**4**, 10.7 mg), anabaenopeptin B[10d] (**5**, 252.0 mg) and anabaenopeptin F[10a] (**6**, 322.0 mg).

Examination of the ^1H and ^{13}C NMR spectra, of the planktopeptins, revealed that they are structurally related to micropeptin SD944[8] and oscillapeptin G.[3]

Planktopeptin BL1125 (**1**) was isolated as an amorphous white solid. Its molecular formula, $C_{54}H_{79}N_9O_{17}$, was deduced from high-resolution FAB MS measurements. The ^1H NMR spectrum, in DMSO-d_6, revealed six doublet NH proton signals and two singlet proton signals between δ_H 6.6 and 8.5 ppm, pointing to eight amino acid residues (taking into account the NMe-aromatic amino acid and the

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* Corresponding author. Tel.: +972-3-6408550; fax: +972-3-6409293; e-mail: carmeli@post.tau.ac.il

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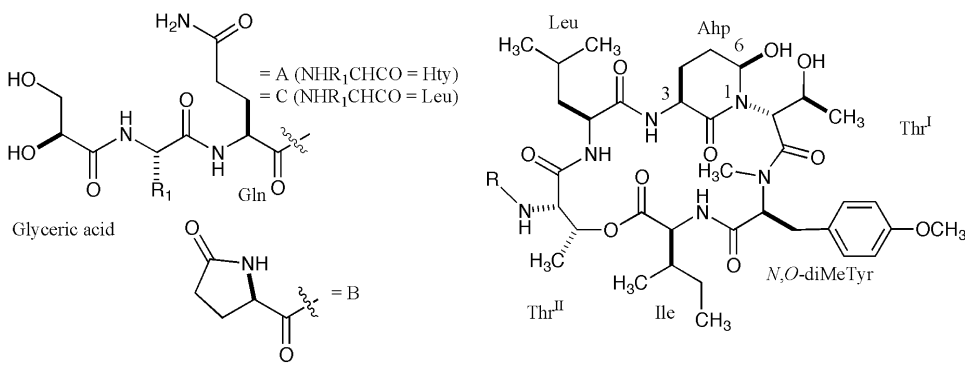
Table 1. NMR data of planktopeptin BL1125 (**1**)[a]

Position		δ_C/δ_N, mult.[b]	δ_H, mult., J (Hz)	LR H−C/H−N correlations[c]	NOE correlations[d]
Ile	1	172.7 s		Thr^{II}-3, Ile-2	
	2	55.6 d	4.77 m	Ile-6	Hty-6,6′, Ile-NH,6
	3	37.7 d	1.81 m	Ile-4,5,6	
	4	24.6 t	1.22 m, 0.99 m		
	5	11.5 q	0.79 t 7.0		
	6	16.3 q	0.83 d 7.0	Ile-3,4	Ile-2
	NH	111.9 d	7.64 d 9.5	Ile-2,6	
N,*O*-diMe Tyr	1	169.3 s		Ile-NH, diMeTyr-2	
	2	60.9 d	5.00 dd 9.0,2.0	diMeTyr-3,NMe	diMeTyr-5,5′
	3	33.3 t	2.75 m, 3.18 m		
	4	129.4 s		diMeTyr-6,6′	
	5, 5′	130.7 d (×2)	7.10 d 8.5 (×2)	diMeTyr-3,7	diMeTyr-2,6,6′, Thr^{I}-2,4
	6, 6′	114.3 d (×2)	6.82 d 8.5 (×2)		diMeTyr-5,5′,OMe, Thr^{I}-4
	7	158.5 s		diMeTyr-6,6′,5,5′	
	*O*Me	55.3 q	3.67 s	diMeTyr-7	MeTyr-6,6′
	*N*Me	30.1 q	2.70 s	diMeTyr-2	
	N	112.0 s		diMeTyr-NMe,3	
Thr^{I}	1	170.6 s		Thr^{I}-2, diMeTyr-NMe	
	2	55.5 d	4.35 d 7.5	Thr^{I}-4	diMeTyr-5,5′
	3	64.5 d	3.58 m		Thr^{I}-4
	4	19.3 q	0.19 d 6.0	Thr^{I}-2,OH	Thr^{I}-3 diMeTyr-5,5′,6,6′
	OH		4.42 d 4.5		
Ahp	N	137.5 s		Ahp-OH	
	2	169.9 s		Ahp-4,6	
	3	49.0 d	4.45 m		
	4	22.0 t	2.54 m, 1.70 m	Thr^{1}-OH, Thr^{1}-OH	Ahp-NH,OH
	5	29.4 t	1.90 m, 1.69 m		Ahp-6
	6	74.8 d	5.04 brs	Thr^{I}-2	Ahp-5
	OH		5.98 d 2.5		Ile-NH
	NH	117.6 d	7.37 d 9.0	Ahp-3	
Leu	1	170.9 s		Ahp-NH, Leu-2	
	2	50.9 d	4.30 m		Ahp-NH, Leu-6
	3	39.6 t	1.37 m, 1.80 m		Leu-NH
	4	24.5 t	1.51 m	Leu-5,6	Leu-NH
	5	23.5 q	0.86 d 6.5		
	6	21.0 q	0.75 d 6.5		Leu-2
	NH	118.3 d	8.38 d 8.5	Leu-2	
Thr^{II}	1	169.4 s		Leu-NH, Thr^{II}-2	
	2	55.1 d	4.64 d 9.0	Thr^{II}-4	Thr^{II}-4, Leu-NH
	3	71.9 d	5.47 q 7.0		Thr^{II}-4, Ahp-NH, Leu-NH
	4	17.8 q	1.18 d 7.0	Thr^{II}-2	Thr^{II}-2,3
	NH	107.6 d	8.11 d 9.0	Thr^{II}-2	
Gln	1	172.3 s		Thr^{II}-NH,2, Gln-2,3	
	2	52.2 d	4.47 m	Gln-3,4	
	3	28.1 t	1.70 m, 1.80 m		
	4	31.6 t	2.13 m	Gln-2,3,5,NH_2	Gln-NH_2
	5	174.1 s		Gln-4,NH_2	
	NH_2	108.2 t	7.22 s, 6.71 s		Gln-4
	NH	117.7 d	8.13 d 8.0	Gln-2,3	
Hty	1	171.3 s		Gln-NH, Hty-2,3	
	2	51.9 d	4.32 m	Hty-3,4	
	3	34.9 t	1.91 m, 1.81 m		Hty-6,6′
	4	30.3 t	2.42 m	Hty-2,3,6,6′	Hty-6,6′, NH
	5	131.8 s		Hty-7,7′	
	6, 6′	129.3 d (×2)	6.93 d 8.0 (×2)	Hty-4,8	Hty-3,4,7,7′, Ile-2
	7, 7′	115.2 d (×2)	6.63 d 8.0 (×2)	Hty-6,6′,8,OH	Hty-6,6′,OH
	8	155.5 s		Hty-6,6′,7,7′,OH	
	OH		9.09 s		Hty-7,7′
	NH	114.9 d	7.79 d 8.0	Hty-2,3,4	
Glyceric acid	1	172.1 s		Hty-NH, Ga-3-OH	
	2	73.2 d	3.95 q 5.0, 3.5		Hty-NH
	2-OH		5.63 d 5.0		Hty-NH
	3	64.2 t	3.58 m, 3.51 m		
	3-OH		4.76 m		

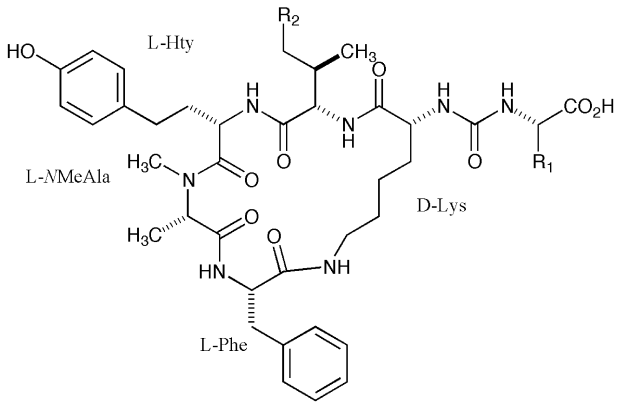
[a] Carried out on a Bruker Avance 400 spectrometer.
[b] Multiplicity and assignment from HMQC experiment.
[c] Determined from HMBC experiment, $^{n}J_{CH}$=8.0 Hz, recycle time 1 s.
[d] By ROESY experiment.

Ahp residue that counts for two amino acids). Analysis of the 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 1) revealed the eight amino acid units and an hydroxy-acid unit, namely isoleucine, *N*Me,*O*Me-tyrosine, *N*,*N*-disubstituted threonine^{I}, amino-hydroxy-piperidone (Ahp), leucine, threonine^{II}, glutamine, homotyrosine and glyceric acid, that build planktopeptin BL1125. The structure of the Ahp is suggested on the basis of comparison of the chemical shifts of the carbon and proton signals with similar moieties in micropeptin SD944,[8] the COSY and HMQC spectra and on the basis of the HMBC correlation between Ahp H-6 and C-2. The ester linkage arises from the carbonyl of isoleucine and the hydroxyl of threonine^{II}. All proton and carbon signals of the latter residues were assigned by the COSY, TOCSY, HMQC and HMBC data (see Table 1). The amino acid sequence of planktopeptin BL1125 was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Ile-*N*Me,*O*Me-Tyr, Ahp-Leu, Leu-Thr^{II}, Thr^{II}-Gln, Gln-Hty and Hty-Ga), the NMe of NMe-Tyr with Thr^{I}-carbonyl and H-2 of Thr^{I} with C-6 of the Ahp residue. The ester bond was assigned by an HMBC correlation between H-3 of threonine^{II} and the carbonyl of isoleucine. The amino acid sequence could also be assembled from the ROESY data (see Table 1). ^{1}H–^{15}N HSQC and HMBC experiments allowed the assignment of the ^{15}N signals of the various amino acids of compound **1** (see Table 1). All of the primary and secondary amide nitrogen signals resonated in the expected region (δ_N 105–120 ppm, relative to ammonia) except for the hydroxy-pyridinone (threonine^{I}) nitrogen signal, which resonates at δ_N 137.5 (s) ppm.[14] Acid hydrolysis of planktopeptin BL1125 and derivatization with Marfey's reagent,[15] followed by HPLC analysis, demonstrated the L-stereochemistry of the isoleucine, *N*Me,*O*Me-tyrosine, leucine, glutamic acid, threonine and homotyrosine residues. Jones oxidation[16] of planktopeptin BL1125, followed by a similar hydrolysis, derivatization and HPLC analysis, determined the *3S*-stereochemistry for the Ahp residue (oxidation and subsequent hydrolysis liberated L-glutamic acid from Ahp). The stereochemistry of C-6 of the Ahp was determined as *R* on the basis of the *J*-values of H-6, <1 Hz, which points to an equatorial orientation of this proton and the chemical shift of the pseudoaxial H-4, δ_H 2.54 brq, which is down-field shifted by the axial hydroxyl.[17] The stereochemistry of the glyceric acid was determined to be D (*R*), by comparison of the retention time of the glyceric acid from the hydrolysate with the retention time of authentic samples of D- and L-glyceric acid on a chiral HPLC column.

The structures and absolute stereochemistry of compounds **2** and **3** were elucidated using similar arguments and methodology. Planktopeptin BL843 (**2**) was isolated as an amorphous white solid. FAB MS measurements for the compound furnished a molecular formula $C_{41}H_{61}N_7O_{12}$.



Planktopeptin BL1125 (**1**) R=A
Planktopeptin BL843 (**2**) R=B
Planktopeptin BL1061 (**3**) R=C



Anabeanopeptin A (**4**) NHR$_1$CHCO = L-Tyr, R$_2$ = H
Anabeanopeptin B (**5**) NHR$_1$CHCO = L-Arg, R$_2$ = H
Anabeanopeptin F (**6**) NHR$_1$CHCO = L-Arg, R$_2$ = CH$_3$

Table 2. NMR data of planktopeptin BL843 (**2**)[a]

Position		δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H–C correlations[c]	NOE correlations[d]
Ile	1	172.7 s		Thr^{II}-3, Ile-2	
	2	55.3 d	4.77 dd 9.5, 5.0	Ile-6	Ile-5
	3	37.7 d	1.83 m	Ile-6,5	
	4	24.6 t	1.01 m, 1.20 m		
	5	11.5 q	0.80 t 7.5	Ile-3	diMeTyr-2
	6	16.2 q	0.84 d 7.0	Ile-2,3	
	NH		7.66 d 9.5		DiMeTyr-NMe, Ahp-6,OH
N,*O*-diMe Tyr	1	169.3 s		Ile-2,NH, diMeTyr-2	
	2	60.8 d	5.01 brd	diMeTyr-2,NMe	diMeTyr-5,5′
	3	33.3 t	2.75 brt 13.0, 3.16 dd 8.0,4.0		diMeTyr-5,5′
	4	129.3 s		diMeTyr-3,6,6′	DiMeTyr-3,NMe, Thr^{I}-4, Ahp-6
	5, 5′	130.6 d (×2)	7.10 d 8.0 (×2)	diMeTyr-6,6′,7	Thr^{I}-4, diMeTyr-Ome
	6, 6′	114.3 d (×2)	6.82 d 8.0 (×2)		
	7	158.5 s		diMeTyr-5,5′,6,6′,OMe	diMeTyr-6,6′
	*O*Me	55.5 q	3.68 s	diMeTyr-7	diMeTyr-5,5′, Ile-NH
	*N*Me	30.1 q	2.70 s		
Thr^{I}	1	170.6 s		Thr^{I}-2, diMeTyr-NMe	
	2	50.9 d	4.34 brd 8.5		Ahp-6, Thr^{I}-4
	3	64.4 d	3.57 m	Thr^{I}-2,4	Ahp-6, Thr^{I}-4
	4	19.2 q	0.19 d 6.0	Thr^{I}-3	diMeTyr-5,5′,6,6′,Thr^{I}-2,Ahp-6
	OH		4.43 d 4.5		
Ahp	2	169.9 s		Ahp-3,6	
	3	48.9 d	4.45m		
	4	22.1 t	2.53 brq 12.0,		Ahp-NH,OH
			1.69 m		Ahp-NH
	5	29.2 t	1.94 m, 1.68 m		Ahp-6, Ahp-6
	6	74.8 d	5.04 brs	Thr^{I}-2	Ahp-4,OH,Ile-NH,Thr^{I}-2,3,4
	OH		6.00 brd 2.4		Ile-NH, Ahp-6
	NH		7.38 d 9.0		Thr^{II}-3, Leu-2,NH,Ahp-4
Leu	1	170.8 s		Ahp-NH, Leu-2	
	2	55.3 d	4.32 m		Ahp-NH, Leu-6
	3	39.0 t	1.41 m, 1.85 m	Leu-5,6, Leu-5,6	
	4	24.4 d	1.54 m		
	5	23.5 q	0.87 d 6.5	Leu-3,4,6	
	6	20.9 q	0.75 d 6.5		Leu-2
	NH		8.41 d 9.0		Thr^{II}-3, Ahp-NH
Thr^{II}	1	169.4 s		Leu-NH, Thr^{II}-2	
	2	55.1 d	4.64 d 9.5	Thr^{II}-1,4	
	3	71.8 d	5.51 brq 6.5		Ahp-NH, Leu-NH
	4	17.9 q	1.21 d 6.5	Thr^{II}-2	
	NH		8.13 d 9.0		Glu-2
Glu-γ-lactam	1	173.7 s		Thr^{II}-NH,2, Glu-3	
	2	55.0 d	4.35 brd 8.0	Glu-4,5	Thr^{II}-NH
	3	25.6 t	1.84 m, 2.27 ddt 9.0		
	4	29.4 t	2.07 m, 2.11 dt 8.0		
	5	177.7 s		Glu-2,4,NH	
	NH		7.84 s		

[a] Carried out on a Bruker Avance 400 spectrometer.
[b] Multiplicity and assignment from HMQC experiment.
[c] Determined from HMBC experiment, $^{n}J_{CH}$=8.0 Hz, recycle time 1 s.
[d] By ROESY experiment.

The seven amino acid residues were identified as Ile, *N*Me,*O*Me-Tyr, Thr^{I}, Ahp, Leu, Thr^{II} and Glu-γ-lactam by NMR spectral analysis, all demonstrating the L-stereochemistry.[15] The *3S*,*6R* stereochemistry of the Ahp residue was assigned in a similar way to that of **1**. The proton and carbon NMR chemical shifts of the lacton-ring amino acids of **2** (see Table 2) were found to be similar to the corresponding units in planktopeptin BL1125 (**1**) (see Table 1). A partial amino acids sequence was determined from HMBC correlations between the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Ile-*N*Me,*O*Me-Tyr, Ahp-Leu, Leu-Thr^{II} and Thr^{II}-Glu). An HMBC correlation between the diMeTyr-NMe and the carbonyl of Thr^{I} confirm their connection. The linkage between Ahp and Thr^{I} was established by the HMBC correlations H-2 of Thr^{I} with C-6 of the Ahp residue and NOE correlations between H-6 of Ahp to H-2 and H-3 of threonine^{I}. The lacton-bridge between Thr^{II} and Ile was inferred by the HMBC cross peak between H-3 of threonine^{II} and the carbonyl of isoleucine. The glutamic acid-γ-lactam residue was assigned by a COSY correlation of the glutamic acid α-NH singlet with H-2 of glutamic acid and the HMBC correlation between the C-5 (carbonyl) and α-NH of this residue.

Table 3. NMR data of planktopeptin BL1061 (**3**)[a]

Position		δ_C/δ_N, mult.[b]	δ_H, mult., J (Hz)	LR H-C correlations[c]	NOE correlations[d]
Ile	1	172.7 s		Thr^{II}-3, Ile-2	
	2	55.5 d	4.73 brd 5.0		Ile-5
	3	24.4 d	0.98 m		Ahp-OH
	4	24.6 t	1.19 m	Ile-6	Ahp-OH
	5	11.5 q	0.78 t 7.5		Ile-2
	6	21.9 q	0.82 d 8.0	Ile-4	
	NH	111.8 d	7.62 d 9.0		diMeTyr-2
N,*O*-diMeTyr	1	169.2 s		Ile-2,NH, diMeTyr-2	
	2	60.8 d	4.96 m		diMeTyr-6,6′,Ile-NH
	3	33.3 t	2.75 m, 3.15 m	diMeTyr-2, diMeTyr-2	
	4	129.0 s		diMeTyr-6,6′	
	5, 5′	130.6 d (×2)	7.08 d 8.0 (×2)	diMeTyr-6,6′,OMe	diMeTyr-2,NMe, Thr^{I}-2,4
	6, 6′	114.3 d (×2)	6.80 d 8.0 (×2)	diMeTyr-4,5,5′	Thr^{I}-3,4
	7	158.4 s		diMeTyr-6,6′,5,5′	
	OMe	55.3 q	3.64 s	diMeTyr-5,5′	diMeTyr-5,5′
	NMe	30.1 q	2.67 s		
Thr^{I}	1	170.6 s		Thr^{I}-2, diMeTyr-NMe	
	2	55.5 d	4.30 br	Thr^{I}-4	diMeTyr-5,5′
	3	64.5 d	3.53 br		diMeTyr-6,6′
	4	19.2 q	0.16 d 6.0	Thr^{I}-2	diMeTyr-6,6′,5,5′
	OH		4.41 m		
Ahp	2	169.9 s		Ahp-6,3	
	3	49.0 d	4.42m	Ahp-4	
	4	22.1 t	2.50 m, 1.65 m		Ahp-OH, Ahp-OH
	5	29.4 t	1.90 m, 1.61 m		Ahp-6, Ahp-6
	6	74.8 d	5.01 brs	Thr^{I}-2	Ahp-5
	OH		5.96 s		Ile-3,4, Ahp-4
	NH	117.6 d	7.35 d 9.0		Thr^{II}-3, Leu^{I}-2
Leu^{I}	1	170.8 s		Ahp-NH, Leu^{I}-2	
	2	50.9 d	4.28 m		Ahp-NH, Leu^{I}-6,NH
	3	40.0 t	1.34 brdd, 1.80 m		
	4	24.3 d	1.48 m		
	5	23.5 q	0.84 d 7.5	Leu^{I}-3	
	6	21.3 q	0.73 d 6.0	Leu^{I}-3	Leu^{I}-2
	NH	118.3 d	8.36 d 8.5		Thr^{II}-3, Leu^{I}-2
Thr^{II}	1	169.3 s		Leu^{I}-2,NH, Thr^{II}-2	
	2	55.1 d	4.59 d 9.0		
	3	71.9 d	5.45 brq 6.5		Thr^{II}-NH, Ahp-NH, Leu^{I}-NH
	4	17.8 q	1.16 d 6.5		
	NH	107.3 d	8.00 d 9.0		Thr^{II}-3, Gln-2
Gln	1	172.2 s		Thr^{II}-NH,2, Gln-2	
	2	52.1 d	4.40 m		Thr^{I}-NH
	3	28.1 t	1.85 m		
	4	31.6 t	1.68 m		
			2.06 m		Gln-NH_2
	5	174.0 s		Gln-4,NH_2	
	NH_2	108.2 t	7.19 s, 6.70 s		Gln-4, Gln-4
	NH	117.2 d	8.06 d 7.5		Leu^{II}-2
Leu^{II}	1	171.9 s		Gln-2,NH, Leu^{II}-2	
	2	50.7 d	4.33 brd 8.5	Ga- 3	Gln-NH, Leu^{II}-5
	3	41.4 t	1.47 brt 7.0		
	4	37.9 d	1.77 m	Ga-3-OH	
	5	23.3 q	0.84 d 8.0		Leu^{II}-2
	6	16.3 q	0.82 d 8.0		
	NH	116.3 d	7.66 d 8.5		Ga-2-OH,2,3
Glyceric acid	1	172.0 s		Leu^{II}-2,NH	
	2	73.1 d	3.88 brs	Ga-2-OH	Leu^{II}-NH
	2-OH		5.59 brs		Leu^{II}-NH
	3	64.2 t	3.53 br	Ga-2-OH,3-OH,Leu^{II}-2	Leu^{II}-NH
			3.45 brq	Ga-2-OH,3-OH,Leu^{II}-2	Leu^{II}-NH
	3-OH		4.73 brd 4.0		

[a] Carried out on a Bruker Avance 400 spectrometer.
[b] Multiplicity and assignment from HMQC experiment.
[c] Determined from HMBC experiment, $^{n}J_{CH}$=8.0 Hz, recycle time 1 s.
[d] By ROESY experiment.

Table 4. Inhibitory activity of protease inhibitors isolated from *Planktothrix* spp.[a]

Name	Position of amino acids[b]									Enzyme inhibited/IC50[c]		
	1	2	3	4	5	6	7	8	9	Elastase	Chymo-trypsine	Trypsin/plasmin
1	Ile	diMeTyr	Thr	Ahp	Leu	Thr	Gln	Hty	Ga	0.11	0.9	
2	Ile	diMeTyr	Thr	Ahp	Leu	Thr	Gll			1.4	11.8	
3	Ile	diMeTyr	Thr	Ahp	Leu	Thr	Gln	Leu	Ga	0.04	2.2	
Oscillapeptin A[d]	Ile	diMeTyr	Ile	Ahp	Hty	Thr	Hty	Mgs		0.3	2.2	
Oscillapeptin B[d]	Ile	diMeTyr	Ile	Ahp	MeHty	Thr	Hty	Mgs		0.05	2.1	
Oscillapeptin C[d]	Ile	NMeTyr	Ile	Ahp	HcAla	Thr	Hty	Mga			3.0	
Oscillapeptin D[d]	Ile	NMeTyr	Ile	Ahp	HcAla	Thr	Hty	Mgs		30.0	3.0	
Oscillapeptin E[d]	Ile	NMeTyr	Ile	Ahp	Hty	Thr	Hty	Mgs		3.0	3.0	
Oscillapeptin F[d]	Ile	NMeTyr	Ile	Ahp	Lys	Thr	Hty	Mgs				0.2/0.03
Oscillapeptin G[e]	*Allo*- Ile	diMeTyr	Thr	Ahp	Leu	Thr	Gln	Hty	Ga	1.12	11.4	
Oscillapeptilide 97-A[e]	Ile	diMeTyr	Phe	Ahp	Leu	Thr	Gln	Pro	Ac	0.73	12.9	
Oscillapeptilide 97-B[e]	Ile	NMeTyr	Phe	Ahp	Leu	Thr	Gln	Pro	Ac	0.41	10.7	

[a] *Oscillatoria*.
[b] Numbering starts from the carboxyl end of the linear peptide, diMeTyr=*N*,*O*-diMeTyr, MeHty=8-methyl homotyrosine, HcAla=3-(4′-hydroxy-2′-cyclohexenyl)alanine, Gll=glutamic acid γ-lactam, Mgs=2-methoxy-3-sulfoglyceric acid.
[c] μg/mL.
[d] Taken from Ref. 9b.
[e] Taken from Ref. 9c.

Planktopeptin BL1061 (**3**) was isolated as an amorphous white solid. Mass spectral analysis (HR FAB MS) of the molecular cluster ion of **3** showed a sodiated ion (*m*/*z* 1084.5542) consistent with the molecular formula $C_{50}H_{79}N_9NaO_{16}$. The proton and carbon NMR spectra of planktopeptin BL1061 (**3**) differed slightly from that of planktopeptin BL1125 (**1**). The aromatic signals of Hty were absent in the spectra of **3**. Instead, two doublet methyl groups appeared in the aliphatic region of these spectra. Detailed examination of the various 1D and 2D NMR spectra (see Table 3) determined eight amino acid residues Ile, *N*Me,*O*Me-Tyr, Thr^{I}, Ahp, Leu^{I}, Thr^{II}, Gln, Leu^{II} and a glyceric acid residue. The assignment of the amino acid sequence of planktopeptin BL1061 (**3**) could be established either by HMBC or ROESY correlations (see Table 3). Marfey's analysis[15] of the derivatized hydrolysate of **3** established an L-stereochemistry for all of the amino acids except, Ahp. The *3S*,*6R*-stereochemistry of the 3-amino-6-hydroxy pyridinone moiety was determined as for **1** and **2**. The absolute stereochemistry of the glyceric acid was determined on a chiral HPLC column as for **1**.

The inhibitory activity of **1**–**3** was determined for three enzymes, the serine proteases elastase, chymotrypsin and trypsin. Planktopeptin BL1125 (**1**), planktopeptin BL843 (**2**) and planktopeptin BL1061 (**3**) inhibited elastase activity with IC_{50} values of 96 nM, 1.7 μM and 40 nM, respectively, and chymotrypsin with IC_{50} values of 0.8 μM, 14.0 μM and 2.1 μM, respectively, but not trypsin at a concentration of 50 μg/mL. *Planktothrix* spp. have, thus far, furnished the most potent Ahp-containing chymotrypsin and elastase inhibitors. Comparison of the inhibitory activity of **1**–**3** with the activity of inhibitors from other *Planktothrix* spp. (see Table 4), reveals that **1** and **3** are the most potent inhibitors of chymotrypsin and elastase from this group of protease inhibitors. Examination of the structure activity relationship of these cyclic depsipeptides reveals that lipophilic amino acids, such as Leu, Hty and HcAla, at position 5, select for inhibition of chymotrypsin related serine-proteases, while basic amino acids such as Lys and Arg select for trypsin related serine-proteases. Furthermore, the flexible side chain composition of compounds **1** and **3** show some marginal selectivity for elastase and chymotrypsin. Compound **1**, with Hty at position 8, is the most potent chymotrypsin inhibitor of this group of compounds while **3** is the most potent inhibitor of elastase.

1. Experimental

1.1. Instrumentation

High resolution MS were recorded on a Fisons VG AutoSpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. Optical rotation measurements were obtained on a Jasco P-1010 polarimeter. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^{1}H and 125.76 MHz for ^{13}C and a Bruker Avance 400 spectrometer at 400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C and 40.549 MHz for ^{15}N. ^{1}H, ^{13}C, DEPT, gCOSY, gTOCSY, gROESY, gHMQC and gHMBC 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 and Merck–Hitachi HPLC system (model L-4200 UV–VIS detector and model L-6200A Intelligent pump).

1.2. Biological material

Planktothrix rubescens strain of cyanobacteria was collected from the Bled lake in Slovenia in the summer of 1999. The cells were freeze-dried and kept at −20°C until extracted.

1.3. Isolation procedure

The freeze-dried cells (180 g) were extracted with 7:3 MeOH/H_2O. The crude extract (20 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 5 (40:60 MeOH/H2O) was subjected to a reversed-phase HPLC (YMC C-18 5 μm, 250×20 mm, DAD at 238 nm, 55:10:35 0.1% TFA in water/methanol/acetonitrile, flow rate 5.0 mL/min). Compound **4** was eluted from the column with a retention time of 29.3 min. In another experiment, fraction 6 (50:50 MeOH/H_2O) was subjected to a reversed-phase HPLC (YMC C-18 5 μm, 250×20 mm, DAD at 238 nm, 55:20:25 water/methanol/acetonitrile, flow rate 5.0 mL/min). Compounds **5**, **6** and **1** were eluted from the column with a retention times of 21.1, 25.7 and 42.4 min, respectively. Subsequently, fraction 7 (60:40 MeOH/H_2O) was subjected to a reversed-phase HPLC (YMC C-18 5 μm, 250×20 mm, DAD at 238 nm, 65:35 water/acetonitrile, flow rate 5.0 mL/min). Compounds **6**, **1**, **2** and **3** were eluted from the column with a retention times of 15.2, 25.0, 34.6 and 36.8 min, respectively. The overall quantities and yields based on the dry weight of the bacteria of compounds **1**, **2**, **3**, **4**, **5** and **6** are 380 mg (0.2%), 9.5 mg (0.005%), 15.6 mg (0.009%), 10.7 mg (0.006%), 252 mg (0.14%) and 322 mg (0.19%), respectively.

1.3.1. Planktopeptin BL1125 (1). Colorless oil; $[\alpha]_D^{20}=-60$ (*c* 0.06, MeOH); UV (MeOH) λ_{max} (ε) 226 (18,000), 278 (3,000) nm; for ^1H, ^{13}C and ^{15}N NMR data see Table 1; HRFABMS *m/z* 1148.5520 [MNa^+] (calcd for $C_{54}H_{79}N_9O_{17}Na$, 1148.5491).

1.3.2. Planktopeptin BL843 (2). Colorless oil; $[\alpha]_D^{20}=-55$ (*c* 0.006, MeOH); UV (MeOH) λ_{max} (ε) 224 (9,500), 278 (1,600) nm; for ^1H, ^{13}C and ^{15}N NMR data see Table 2; HRFABMS *m/z* 866.4275 [MNa^+] (calcd for $C_{41}H_{61}N_7O_{12}Na$, 866.4276).

1.3.3. Planktopeptin BL1061 (3). Colorless oil; $[\alpha]_D^{20}=-52$ (*c* 0.01, MeOH); UV (MeOH) λ_{max} (ε) 225 (8,700), 277 (1,400) nm; for ^1H, ^{13}C and ^{15}N NMR data see Table 3; HRFABMS *m/z* 1084.5472 [MNa^+] (calcd for $C_{50}H_{79}N_9O_{16}Na$, 1084.5542).

1.3.4. Anabaenopeptin A (4). Colorless oil; $[\alpha]_D^{20}=-40$ (*c* 0.007, MeOH), lit.,[10d] $[\alpha]_D=-61$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (ε) 212 (18,000), 279 (3,000) nm; FABMS *m/z* 844 [MH^+].

1.3.5. Anabaenopeptin B (5). Colorless oil; $[\alpha]_D^{20}=-44$ (*c* 0.03, MeOH), lit.,[10d] $[\alpha]_D=-62$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (ε) 222 (7,200), 279 (1,200) nm; FABMS *m/z* 837 [MH^+].

1.3.6. Anabaenopeptin F (6). Colorless oil; $[\alpha]_D^{20}=-39$ (*c* 0.04, MeOH), lit.,[10a] $[\alpha]_D^{20}=-41$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (ε) 215 (11,900), 279 (1,800) nm; FABMS *m/z* 851 [MH^+].

1.4. Determination of the absolute configuration of the amino acids

0.5 mg portions of compounds **1**–**3** were dissolved in 6N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110°C overnight. In another experiment, 0.25 mg portions of compounds **1**–**3** were first oxidized with Jone's reagent (2 drops) 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 110°C overnight. In order to determine the absolute stereochemistry of L-NMe,OMe-Tyr, compounds **1**–**3** were dissolved in 1.5N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110°C for 7 h. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (100 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 similarly derivatized standard AA by HPLC analysis: Purospher STAR RP-18e 5 μm, 4.6×250 mm, flow rate 1.00 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-Thr, 27.58 min; D-Thr, 33.24 min; L-Glu, 33.16 min; D-Glu, 36.21 min; L-Ile, 47.21 min; D-Ile, 54.01 min; L-Leu, 47.77 min; D-Leu, 54.26 min; L-*NMe*,*OMe*-Tyr, 49.04 min, L-Hty, 44.0 min. HPLC analysis of derivatized hydrolysates of **1** established L-Ile, L-*NMe*,*OMe*-Tyr, L-Thr, L-Leu, L-Glu and L-Hty; that of **2** established L-Ile, L-*NMe*,*OMe*-Tyr, L-Thr, L-Leu and L-Glu; that of **3** established L-Ile, L-*NMe*,*OMe*-Tyr, L-Thr, L-Leu and L-Glu. HPLC analysis of the FDAA derivatives of oxidized **1**–**3** hydrolysates established L-Glu for all three compounds and thus confirmed the L configuration of the Ahp units in these compounds.

1.5. Determination of the absolute configuration of glyceric acid

The dried peptide hydrolysate (see above) was resuspended in water and injected to a chiral HPLC column: Astec Chirobiotic T, 5 μm, 4.6×250 mm, flow rate 1.00 mL/min, UV detection at 270 nm, isocratic 100:0.1:0.1 (v/v/v) MeOH/acetic acid/triethylamine. The determination of the absolute configuration of the glyceric acid was confirmed by spiking the hydrolysate with authentic samples of D- and L-glyceric acids. L-Glyceric acid eluted from the column with a retention time of 2.4 min while D-glyceric acid had a retention time of 2.7 min. The glyceric acid derivatives in **1** and **3** were determined as, D (*R*).

1.6. Protease inhibition assays

Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Trypsin was dissolved in a 50 mM Tris–HCl/100 mM NaCl/1 mM $CaCl_2$ pH 7.5 buffer 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 were used as substrate solutions. 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 measured every 5 s for a period of 30 min (405 nm, 37°C). The procedure was repeated with different concentrations of the test solution.

Elastase was purchased from Sigma Chemical Co. and dissolved in a 0.2 M Tris–HCl pH 8.0 buffer to prepare a 0.075 mg/mL solution. A 2 mM solution of *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide in the same buffer was used as a substrate solution. The test samples were dissolved in DMSO. A series of dilutions (10 μL portions) were preincubated with a 150 μL of buffer solution and 10 μL enzyme solution at 30°C for 20 min. Then, a 30 μL substrate solution was added to begin the reaction. The absorbance of the microtiter plate wells was measured every 5 s for the period of 20 min (405 nm, 30°C).

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