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Ecotoxicologically relevant cyclic peptides from cyanobacterial bloom (Planktothrix rubescens) – a threat to human and environmental health

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Background. *The information of the overall production of major cyanobacterial cyclic peptides in a water body is essential for risk assessment and for the prediction of future development of the bloom. A procedure that gives a review of both toxic and non-hepatotoxic hydrophilic cyclic peptide production is important to evaluate the ecological conditions in the water environment and to predict the release of dangerous toxic and tumour promoting substances.*

Methods. *The cyclic peptides were identified on the basis of their retention times, characteristic spectra, molecular masses and biological activity. The non-hepatotoxic cyclic peptides were characterised by their inhibition of porcine pancreatic elastase, while cytotoxicity to mammalian cells was tested with the MTT test on B16 cell line.*

Conclusions. *The method presented gives a rapid, simultaneous assessment, preliminary identification and estimation of bioactive cyclic peptides. The synthesis of non-hepatotoxic cyclic peptides can mediate the release various toxic and otherwise biologically active substances that induce systemic genotoxicity in mammals.*

Key words: tumour promoters; microcystin; anabaenopeptin; planktopeptin; toxic cyanobacterial blooms; environmental health

Introduction

Mass occurrence of cyanobacteria decreases the aesthetic value of recreational water bodies and diminishes the applicability of water resources, even for industrial purposes. It poses a serious risk to humans, livestock, wildlife, and consequently to overall environmental health especially during the blooms lysis. As a consequence, the World Health Organization has published provisional guidelines concerning the overall cyanobacterial cell density in environmental waters[1] and, more specifically, the presence of microcystins in drinking water.[2] Health risks arising from cyanobacterial blooms are also unequivocally stated in the new EU directive concerning the management of bathing water quality.[3]

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There are various reasons why the presence of different types of cyclic peptides in the bloom should be monitored. The harmful effects of cyanobacteria cannot be attributed just to hepatotoxins or neurotoxins. It was recently demonstrated that similarly to microcystins also anabaenopeptins and anabaenopeptilides inhibit protein phosphatase activity and that they may be found in tissues of various aquatic animals.[4] There is also the possibility of synergistic interactions between different toxic and "non toxic"cyanobacterial metabolic products released in the water environment. Thus, crude cyanobacterial extracts exert stronger effects on vertebrates and invertebrates than exposure to the purified toxins.[5,6] In the area of human health risk assessment, the genotoxicity of microcystins is probably of major importance.[7,8]

Recent studies have provided evidence that the presence of different cyanobacterial cyclic peptides influence also the physiology of cyanobacteria themselves and may have a strong impact on their blooming capacity.[9-12] Last but not least the presence of cyanobacterial metabolites is in strong negative correlation with phytoplankton biodiversity.[13,14]

Detection of the separated peaks at a number of specific wavelengths, as described in this work, makes possible a simple and rapid qualitative and quantitative assessment of the presence and dominance of specific cyclic peptides. This method has broader application as a tool in ecotoxinological studies and monitoring of cyanobacterial blooms. At the same time the method when used for preparative purposes provides a fast and simple isolation method, especially for the more recent planktopeptins.[15]

Data from isolated cyanobacterial colonies and filaments reveal the enormous potential for synthesis of different types and groups of bioactive peptides. There is great variability, not only in the microcystin group with over 60 known variants[16], but also in the two major groups of "non-toxic" cyclic peptides. These are cyclic depsipeptides with the Ahp moiety, comprising at least 68 variants, and cyclic peptides possessing a ureido linkage such as anabaenopeptins with 29 variants. Representatives of both groups are regularly found in cyanobacterial bloom forming species.[17] In spite of the great metabolic potential and the variety of possible variants, only a few bioactive cyclic peptides dominate in individual natural blooms. This fact is not surprising since we know that representatives of individual groups although slightly diverse (microcystins) show basically the same effects on various phytoplanktons when present in the environment.[11,12] Similarly representatives of the two major groups of "non-toxic" cyclic peptides share a specific activity; the ability to trigger the lytic cycle in lysogen cyanobacterial cells.[18]

Materials and methods

Sampling site

Lake Bled: Latitude N (°) 46.362839, Longitude E (°) 14.098068, height 475m a.s.l. The lake is 2120 m long, 1080 m wide, 30 m deep and ca. 14.000 years old. Currently it contains 31 x 10^6 m^3 water with a retention time of 3 years. The average water temperature is 12° C; in summer it reaches 24° C, and is covered with ice in winter. It is a dimictic oligotrophic - mesotrophic lake. The productivity is normally low and the inflows are rich and permanent. The nutrients in the phase of summer stratification diffuse from the lake bottom and support metalimnetic blooms. Almost every year *Planktothrix rubescens* (DC. ex Gomont) blooms appear in the lake and, under favourable meteorological and climatic conditions, migrate to the surface, frequently covering almost the entire lake.[14]

Species determination

The species was identified according to Starmach[19] as *Oscillatoria rubescens* (DC. ex Gom.). Anagnostidis and Komarek[20] have introduced a new classification system of the order *Oscillatoriales* that takes into consideration up-to-date phenotypic as well as ultrastructural, biochemical, physiological and ecological characteristics. In this work *Oscillatoria rubescens* was redefined as *P. rubescens* comb. n. [basionym *Oscillatoria rubesens* DC. ex Gom. Ann. Sci. Nat. VII Bot., **16**:204, 1892] (family: *Phormidiaceaea*, order: *Oscillatoriales*).[21]

The bloom samples were analysed for plankton species composition and taxonomic determination under an inverted microscope (Nikon Eclipse TE300). Filaments and cells were measured with Lucia (System for Image Processing and Analysis LUCIA 4.6, Laboratory Imaging Ltd.). Cyanobacterial abundance was calculated by measuring the cumulative length of the filaments using the Bürker-Türk haemocytometer. The hypothetical 1 mm *P. rubescens* filament was in average composed of 336 cells. The cell concentration was determined by multiplying the total length in millimetres by the average cell number.

Sampling procedure

The cyanobacterium *P. rubescens* was harvested with a 50 μm plankton net. The samples were kept cool in the dark until brought to the laboratory. They were concentrated in glass cylinders under natural light. In this way cell buoyancy was increased so that the cyanobacterial material that floated towards the surface was collected, while the remaining algal material, together with the zooplankton, sank to the bottom. The bloom was freeze-dried on a Christ Alpha 2-4 freeze dryer (Martin Christ, Germany).

Analytical and preparative HPLC methods

For the extraction and isolation of cyclic peptides we optimized the Harada method.[22,23] Dried cyanobacteria (1000 mg) were extracted three times with 5% aqueous acetic acid (3 x 20 mL) for 30 min while stirring. The mixture was frozen to further disintegrate the filaments and to increase sedimentation. The extracts were centrifuged at 4000 rpm for 10 min. The combined supernatants were applied to preconditioned 500 mg reversed-phase disposable columns (LiChrolut RP-18, Merck). The columns containing the extract were washed with 20 mL of 10% methanol and the cyclic peptides eluted with 2 mL methanol (LiChrosolv, Merck), evaporated to dryness under nitrogen stream and the residues, eluted from the columns dissolved in the buffer for HPLC analysis.

Analytical HPLC method: Samples were analysed by HPLC, using isocratic elution with methanol:0.05 M phosphate buffer 58:42 (v/v) pH 3.0.[13] In order to obtain a better resolution of the peaks of interest, we modified the ratio of the mobile phase to methanol: 0.05 M phosphate buffer 50:50 (v/v) pH 3. The extracts were separated on an analytical Hibar Pre-Packed RT 125-4 LiCrospher 100 RP-18 (5 μm) column (Merck), flow rate 1 mL min^{-1}, using HPLC/PDA (Waters) to visualise cyclic peptides.

Preparative HPLC method: Cyclic peptides were isolated from the combined supernatants under the same conditions as above using a preparative Spherisorb S 10 ODS2 column (Phase Separation Inc., UK) with a flow rate of 10 mL min^{-1}.[13] The HPLC/PDA equipment consisted of a Waters 600 Controller, Waters 616 pump and Waters PDA Detector. Millenium[(32)] software (Ver. 3.0, Waters) was used to run the hardware and to process the data.

Identification and visualisation of cyclic peptides with a photodiode array detector (PDA)

The column eluate was monitored at four different wavelengths (λ_{max}) – 238, 225, 220 and 215 nm – in order to locate and distinguish microcystins from other bioactive cyclic peptides of interest. The wavelengths are characteristic of individual cyclic peptides; microcystins have a characteristic absorption at 238 nm, while representatives of the other two groups have absorption maxima at lower wavelengths. The depsipeptide planktopeptin BL1125 was detected at 225 nm and anabaenopeptins B and F at 220 nm and 215 nm respectively. Both types of non-toxic cyclic peptides have additional characteristic absorption maxima at 278-279 nm.[15] The presence of these absorption maxima confirmed the preliminary identification. From the individual peaks the amounts of the cyclic peptides were calculated by comparison of the integrated peak areas with the values from the calibration curves that were standardised by previously isolated cyclic peptides in pure form.

Molecular mass determination by mass spectrometry (MS)

Molecular masses were determined with a Finnigan LCQ Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, USA) with ESI ion source. Samples, dissolved in pure methanol, were injected directly at a rate of 5 µL min^{-1}. Analysis conditions were: spray voltage 6 kV, sheath gas flow 60 (arbitrary units) and auxiliary gas flow 5 (arbitrary units); tube lens offset 55 V, capillary voltage 40 V and capillary temperature 220 °C. The isolated cyclic peptides were scanned from 150 to 2000 Daltons at positive polarity.

Chlorophyll a determination

Chlorophyll *a* was determined by the method of Vollenweider.[24] Cells were harvested by concentrating samples on glass microfibre Whatman GF/C filters (Whatman Ltd, Maidstone, UK), followed by extraction with hot methanol.

Enzyme assays

Porcine pancreatic elastase (Serva, Germany) activity was assayed spectrophotometrically[25], using N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide ($Suc(Ala)_3$-NA, Sigma, Germany) as the substrate[27] in a total volume of 200 µL. A sample (20 µL of inhibitor) was added to the assay mixture (70 µL) containing 90 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 50 mEU of elastase (10 µl). After incubation for 30 minutes at room temperature (20 °C), 100 µL of 2 mM substrate was added to the assay mixture at 25 °C. The reaction was monitored at 405 nm in microtiter plates, using a GENios microplate reader (Tecan, Austria). The data were analysed using Magellan software (Tecan, Austria).

Values of inhibition constants, K_i, were obtained for inhibition of the elastase catalysed hydrolysis by cyclic peptides. Three different substrate concentrations (0.5 mM, 0.75 mM and 1 mM) were used. The concentrations of inhibitors were: unknown (Unk.): 0.09 – 1.76 µM; AnP B: 0.65 – 64.6 µM; AnP F: 0.68- 68.2 µM; PP BL1125: 0.01 – 5.96 µM. Michaelis constants, K_M, were determined by fitting the Michaelis-Menten equation directly to the data using a Lineweaver-Burk plot[27]. An Easson-Stedman plot yields the apparent inhibition constant, K_i *(app)*, from which K_i was calculated[28] according to

$$K_i = \frac{K_i(app.)}{1 + \frac{(S^\circ)}{K_M}}$$

Table 1. Effectiveness of serial extraction of cyclic peptides from *Planktothrix rubescens* with 5% acetic acid

Extraction number	**AnP B** R.T. 2.9 min		**AnP F** R.T. 3.0 min		**PP BL1125** R.T. 3.3 min		**[D-Asp^3]MC-RR** R.T. 4.7 min	
	mg/ml*	Tot. yield**	mg/ml*	Tot. yield**	mg/ml*	Tot. yield**	mg/ml*	Tot. yield**
I.	1.9	41.2	1.56	43.5	1.0	33.1	9.0	46.4
II.	2.7	57.8	2.0	55.7	2.0	66.2	10.0	51.6
III.	< 0.1	1.0	< 0.1	0.8	< 0.1	0.7	0.4	2.0
Σ =	4.7		3.6		3.0		**19.4**	

Legend:

R.T. – retention time

AnP B – anabaenopeptin B, AnP F – anabaenopeptin F, PP BL – planktopeptin BL1125, [D-Asp^3]MC-RR – [D-Asp^3]microcystin RR

* The quantities for a particular cyclic peptide were read from the related standard curve, based on the peak area.

** Figures are the percentage of the total yield for cyclic peptides.

where (S°) is the substrate concentration. Assays were performed in triplicate.

Cell viability assay (MTT)

The MTT test was used to assess viability, based on the capacity of viable cells to metabolise a tetrazolium colourless salt to the blue formazan in mitochondria.[29] Mammalian B 16 cells (5 x 10^4 cells ml^{-1}) in the exponential growth phase were plated onto 96-microwell plates (200 μl) and chronically exposed to three final concentrations, 1 μM, 10 μM and 100 μM, of individual cyclic peptides – planktopeptin BL1125, anabaenopeptin B and anabaenopeptin F – for 24 hours. After 21 hours the cells were assayed using 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium salt (MTT, Sigma) and incubated for an additional three hours. The medium was removed and the formazan produced was dissolved 200 μl DMSO (Sigma). The optical density (OD) was read at 570 nm, relative to a reference wavelength of 690 nm, with a GENios microplate reader (Tecan, Austria). Cells were grown in a CO_2 incubator at 37^0C in an atmosphere of 5% CO_2, and maintained during the experiment in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (all Sigma). All assays were performed in triplicate.

Results

The *P. rubescens* samples were over 99% monospecific. The species could be determined unequivocally on the basis of the taxonomic characteristics and of the red pigmentation caused by the presence of the accessory pigment phycoerythrin.

Extraction yields

The three fold extraction procedure with acetic acid proved to be appropriate. Different cyclic peptides were extracted in various amounts in the three steps. In the second step the majority of all four major cyclic peptide representatives were extracted in amounts exceeding 50% of the total yield and less than 2% remained available for the third extraction (Table 1). Each 250 ml of concentrated bloom, with chlorophyll *a* content of 52.4 μg mL^{-1} and cyanobacteri-

Table 2. Overall yields of biologically active peptides from *Planktothrix rubescens* bloom using 5 % acetic acid

Cyclic peptide	Extraction yield (% of dry weight)*
	5% CH_3COOH
AnP B	0.35
AnP F	0.27
PP BL1125	0.23
[D-Asp^3]MC-RR	1.5
Σ =	**2.35**

Legend:
Abbreviations as in Table 1.
The percentages of cyclic peptides were read from the related standard curves, based on the peak area.
* Figures are the percentage of the total extraction yields for individual cyclic peptide.

al concentration of 2.8 x 10^8 cells ml^{-1}, gave on average 1.340 mg of lyophilized starting material. Chlorophyll *a* accounted for 0.97% of the dry weight of *P. rubescens*, and cyclic peptides 2.35% (Table 2). The average value for the intracellular content of chlorophyll *a* was 0.18 pg Chl *a* $cell^{-1}$ and, for microcystin RR, which was the major cyclic peptide in the sample, 0.29 pg [D-Asp^3]MC-RR $cell^{-1}$. The average microcystin quota per unit cell volume was 2.6 fg $(\mu m^3)^{-1}$.

Isolation efficiency

Separation on the analytical column, using methanol:phosphate buffer 58:42, resulted in rapid elution of the analytes (Figure 1). However the resolution was better using

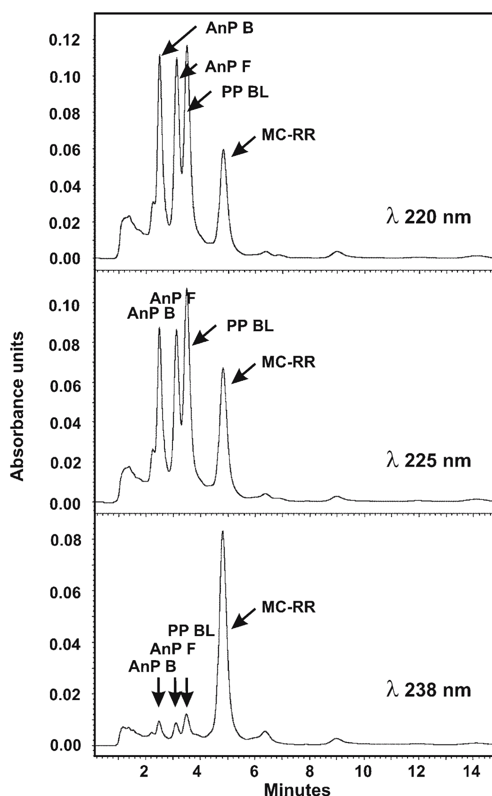

Figure 1. HPLC chromatogram of *Planktothrix rubescens* extract run on an analytical column. The diagrams show the same elution pattern monitored at three different wavelengths. MC-RR is clearly visible at the characteristic λ_{max} of 238 nm, while the other three cyclic peptides are seen only as minor peaks (the lowest panel). PP BL, AnP B and AnP F are better detected at lower wavelengths (upper two panels). Elution was with methanol: 0.05 M phosphate buffer 52:48 (v/v) pH 3. AnP B = anabaenopeptin B; AnP F = anabaenopeptin F; PP BL = planktopeptin BL1125; MC-RR = [D-Asp^3]microcystin RR

a 50:50 ratio of the same mobile phase (Figure 2). Purification on the preparative column gave a larger number of peaks, indicating the presence of five microcystins and one additional protease inhibitor denoted as unknown (Figure 3). All major peaks were clearly separated and gave relatively pure substances as established with MS (Figure 4).

Table 3. The inhibition constants (K_i) for four cyclic peptide inhibitors isolated from *Planktothrix rubescens* for porcine pancreatic elastase, using Suc(Ala)$_3$-NA as substrate are presented. The abbreviations are the same as in Fig. 2. SE represents standard error.

Inhibitor	Elastase K_i (nM) ± SE
Unknown	55.1 ± 1.4
AnP B	1768.0 ± 211.0
AnP F	1400.0 ± 130.0
PP BL 1125	5.5 ± 0.6

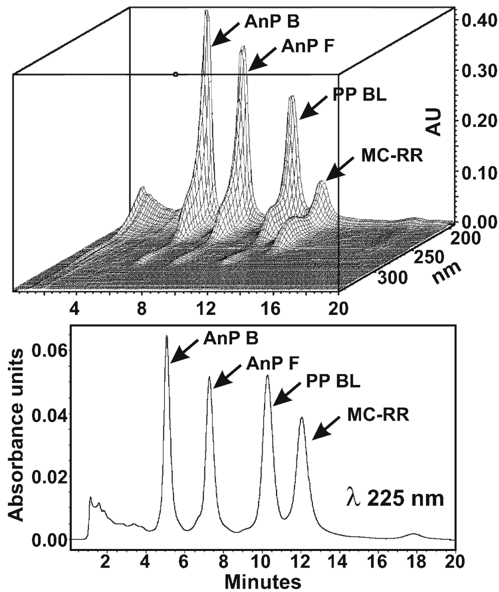

Figure 2. Isocratic elution with methanol: 0.05 M phosphate buffer 50:50 (v/v) pH 3 using the analytical column. The spectra of the four major cyclic peptides demonstrate the effectiveness of separation (upper panel). The same elution monitored at λ 225 nm (lower panel).

Legend:

R.T. = retention time
AnP B = anabaenopeptin B
AnP F = anabaenopeptin F
MC = undetermined microcystin
PP BL = planktopeptin BL1125
MC-RR = [D-Asp^3]microcystin RR
Unk. = undetermined cyclic peptide

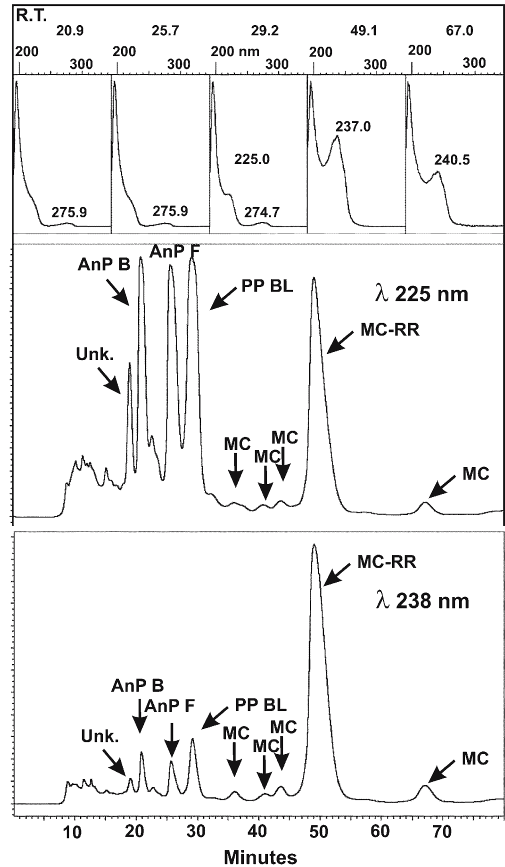

Figure 3. HPLC chromatogram of the *Planktothrix rubescens* extract monitored at 238 nm and 225 nm. The elution pattern was obtained from a preparative column using isocratic elution with methanol: 0.05 M phosphate buffer 50:50 (v/v) pH 3. The upper portion of the Figure shows the spectra of the three major non-toxic cyclic peptides and two microcystins with corresponding retention times. The lower two panels show the same elution chromatogram monitored at two wavelengths; λ 225 nm for planktopeptin and 238 nm for microcystins. Apply legend as in Fig. 2.

Elastase inhibition

All four purified non-toxic cyclic peptides inhibited porcine pancreatic elastase. The unknown inhibitor and the depsipeptide PP BL1125 were the most effective inhibitors, with K_i = 5.5 nM for PP BL1125 (Table 3).

MTT cell proliferation assay

None of the three cyclic peptides tested showed cytotoxic effects even at the highest 100 μM concentration. No effects were observed on the adhesive characteristics of the B16 cells, which remained attached during the course of the experiment.

Discussion

The method makes possible a rapid separation and assessment in one HPLC step

Table 4. The impact of increasing concentrations of anabaenopeptin B anabaenopeptin F and planktopeptin BL1125, (1, 10 and 100 μM) and exposure time (24 hours) on B16 cells as assessed by the MTT assay. The optical density of formazan production was read at 570 nm, relative to a reference of 690 nm. Each value represents triplicate data ± S.E.

Inhibitor	Concentration (μM)			
	Control	1	10	100
	Optical density ± S.E.			
AnP B	1.131 ± 0.056	1.150 ± 0.163	1.108 ± 0.049	0.974 ± 0.134
AnP F	1.131 ± 0.056	1.123 ± 0.072	1.144 ± 0.111	1.198 ± 0.098
PP BL1125	1.003 ± 0.076	1.118 ± 0.124	1.056 ± 0.057	1.148 ± 0.036

of three groups of cyanobacterial cyclic peptides; depsipeptides and cyclic peptides with an ureido linkage and major microcystins. We focused on these three groups because they are related in structure and type of synthesis and are produced in large amounts that exceed even the production of chlorophyll *a*, which is the vital molecule in photoautotrophs (Table 2). The method, when applied for analytical purposes, gave good resolution of the cyclic peptides in symmetrical peaks (Figure 2).

Extraction of cyclic peptides

Five percent acetic acid aqueous extraction, in combination with solid phase extraction, was used for microcystin isolation, as introduced by Harada and co-workers.[22,23] It is effective for extracting almost all the peptides produced by cyanobacteria[30], and does not extract many of the pigments that often make purification difficult; it enhances pellet formation and gives reasonable recovery.[32] The extraction procedure has proved to be selective for the cyclic peptides, since the major peaks corresponded to the three groups of cyanobacterial non-ribosomal cyclic products of interest (Figure 1 and Table 2).

HPLC chromatography

Use of a PDA detector at a number of wavelengths characteristic of the different groups of cyclic peptides enabled the latter to be more readily identified and quantitatively estimated. Separation using the preparative column was highly effective and no further purification of cyclic peptides was needed (Figures 3, 4).

Identification of cyclic peptides

The identity of individual cyclic peptide was confirmed with the molecular weight information obtained from mass spectrometry (Figure 4). The "non-toxic" peptides were additionally identified by biological assay using elastase inhibition (Table 3). PP BL1125 exhibited the lowest inhibition constant, and the putative absence of cytotoxicity to mammalian cell lines suggested that this cyclic peptide is a potentially useful tool for studying the role of elastase in pathophysiological processes, such as inflammation and cancer. Planktopeptin and the anabaenopeptins were confirmed to be non-cytolytic, using the MTT cell proliferation assay (Table 4).

Ecological implications of cyclic peptide production

Cyanobacterial blooms are almost always mixtures of different cyanobacterial species and other phytoplankton organisms. Algae are progressively excluded from eutrophic water, and dominated by cyanobacteria.[14] Surface blooms and scums exhibit the lowest diversity which correlates higher total microcystin concentrations.[13]

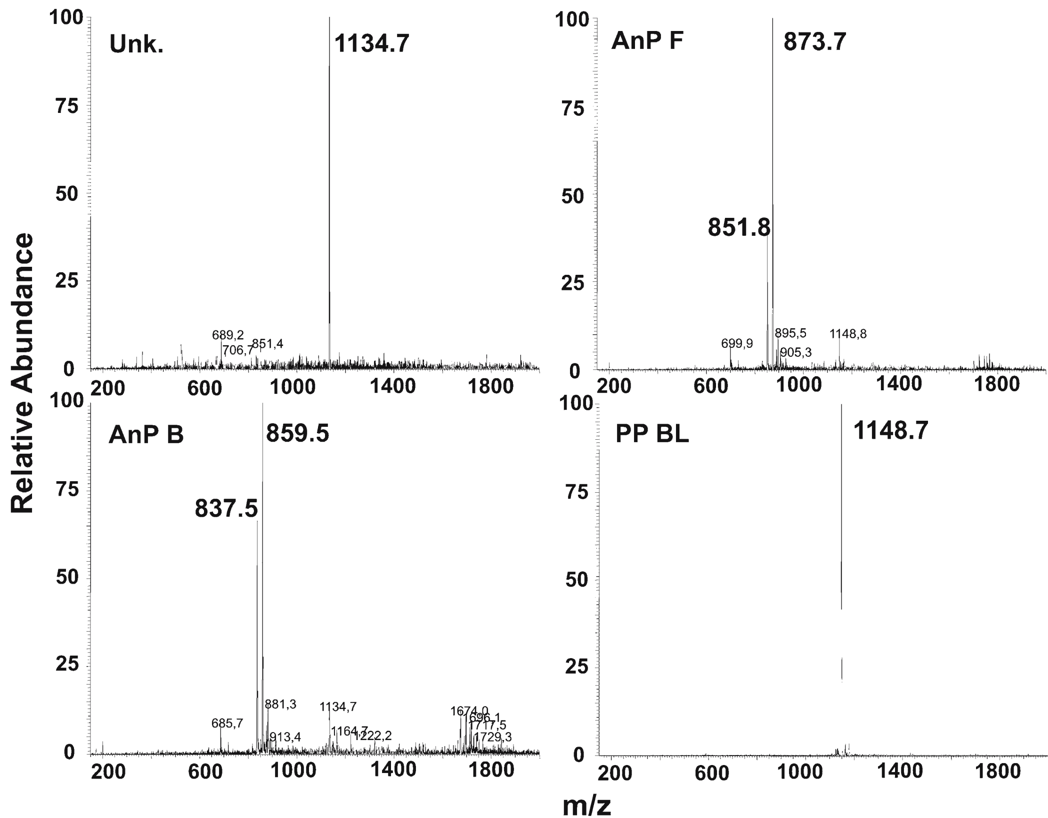

Figure 4. The MS spectra of four different cyclic peptides isolated from *Planktothrix rubescens*; unknown (Unk.), An F (upper figs.), AnP B and PP BL1125 (lower figs.). Note that, on MS spectra, the Unk. value 1134.7 refers to the ion $[M+H]^+$, AnP B value 859.5 refers to the ion $[M+Na]^+$, AnP F value 873.7 refers to the ion $[M+Na]^+$ and PP BL1125 value 1148.7 refers to the ion $[M+Na]^+$. Abbreviations as in Fig. 2.

However, even in monospecific blooms, the production of bioactive cyclic peptides is highly variable. Bloom forming species belonging to the coccoid genera, such as *Microcystis*, and to filamentous genera, such as *Planktothrix*, include microcystin-producing and non-microcystin-producing strains.[32,33] Additionally, those genotypes that contain *mcy* genes can, in the case of the toxic cyanobacterium *Microcystis* spp.[34] and of the toxic cyanobacterium *Planktothrix* spp.[36], be either active or inactive. The determination of the actual cyclic peptide presence is therefore the only reliable measure for their production.

In general, only particular cyanobacterial species prevail under specific environmental conditions. For example, the filamentous cyanobacterium *Planktothrix agardhii* is primarily distributed in eutrophic polymictic shallow lakes, frequently blooming during late summer[36], whereas *P. rubescens* occurs in oligotrophic to mesotrophic deep dimictic lakes, blooming throughout the year, and often stratifies in the metalimnic layer.[37] Although the two species occupy different types of water body their natural blooms show very similar and stable cyclopeptide production. The main microcystin is almost always MC-RR, with its

de-methylated variant, together with anabaenopeptins B and F and micropeptin representatives.[15,38,39,40,41,42] The microcystin composition in field populations of a single species changes little over time, as long as dominance of the particular species persists.[40] However there is also evidence that the predominating anabolism of defined bioactive cyclic peptides is not linked exclusively to cyanobacterial species and genera, but that their synthesis can be affected by geographical trends.[42]

The production of microcystins in *P. rubescens* blooms in Slovenia is high, which correlates well with the most recent data on toxin content from other locations.[44] The co-occurring non-toxic peptides, as well as the microcystin variants, were similar regardless of geographical provenance*e.g.*.[42] Thus, in the case of *Planktothrix rubescens*, the anabolism of cyclic peptides is linked primarily to the species and depends less on ecological factors.

Planktopeptins are new micropeptin-type serine protease inhibitors that are the most abundant of the "non-toxic" cyclic depsipeptides produced in *P. rubescens* blooms in Slovenia. Planktopeptin BL1125 is also the most potent Ahp-containing chymotrypsin and elastase inhibitor discovered so far[15], with an inhibition constant in the nanomolar range.

It seems that all cyanobacterial cyclic peptides possess strong biological activities ranging between lethal effects on mammals[45] to the influence on morphology, physiology on algae and cyanobacteria[11] and gene expression in cyanobacteria themselves.[12] Evidently we have to reconsider the term non toxic cyclic peptides, since beside their strong biological activity as protease inhibitors they may be the main cause of cyanobacterial bloom collapse. Recently it was demonstrated that representatives of both groups of "non-toxic" cyclic peptides, anabaenopeptins and planktopeptins, are able to trigger the lytic cycle of temperate cyanophages inducing rapid lysis of cyanobacterial cells. The monitoring of these metabolic and/or ultrastructural disturbers is important, since they can be a valuable element for the prediction of cyanobacterial bloom lysis and the consequent release of toxic genotoxic and tumour promoting substances.[19,46]

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