CYANOPEPTOLINS, NEW DEPSIPEPTIDES FROM THE CYANOBACTERIUM *Microcystis* sp. PCC 7806

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Four depsipeptides (peptide lactones), called cyanopeptolins A, B, C and D, have been isolated from the cyanobacterium *Microcystis* sp. PCC 7806. They possess identical structures consisting of cyclic L-glutamic acid-γ-aldehyde, L-leucine, *N*-methyl-phenylalanine, L-valine, L-threonine, L-aspartic acid, hexanoic acid and a variable basic amino acid. This variable amino acid can be L-arginine (cyanopeptolin A), L-lysine (cyanopeptolin B), N_ε-methyl-L-lysine (cyanopeptolin C) and N_ε,N_ε-dimethyl-L-lysine (cyanopeptolin D), respectively. The L-glutamic acid-γ-aldehyde and the amino group of L-leucine form an unusual 3-amino-6-hydroxy-2-oxo-1-piperidine system. L-Threonine is connected to L-valine *via* its hydroxy-group forming an ester bonding. The hexanoic acid residue is attached to the *N*-terminal aspartic acid residue which is not a part of the ring structure. The isolation procedure of the four cyanopeptolins as well as structure elucidation are described. Amino acid analysis, GC/MS analysis, FAB-MS and several NMR techniques were used to reveal the structures.

Cyanobacteria (blue green algae) commonly found in fresh and brackish waters are well known to produce a manifold spectrum of secondary metabolites belonging to different substance classes such as alkaloids, phenolic dilactones, polyketides, amides, sulfur compounds, macrolides and peptides[1]. Their most abundant peptide metabolites are cyclic peptide hepatotoxins termed microcystins. They are produced by strains of several waterbloom-forming cyanobacteria mainly from the genus *Microcystis* and are responsible for worldwide animal poisoning. Besides these well investigated toxins little is known about other peptides or peptide-related compounds produced by cyanobacteria. The tricyclic depsipeptide microviridin consisting of 14 L-amino acids has been found in *Microcystis viridis* from a lake in Japan. The depsipeptide acts as a strong inhibitor of thyrosinase[2]. Majusculamide C, a cyclic depsipeptide that inhibits the growth of a number of fungal plant pathogens has been isolated from *Lyngbya majuscula*[3]. The cyanobacterium *Anabaena* sp. BQ-16-1 produces a chlorine-containing cyclic decapeptide named puwainaphycin C which shows cardioactivity[4]. Recently, a fungicidal decapeptide named calophycin has been isolated from *Calothrix fusca*[5], and a cytotoxic bistratamide-related cyclic peptide, called westiellamide, was identified in the terrestrial cyanobacterium *Westiellopsis prolifera*[6]. From *Microcystis* sp. PCC 7806,

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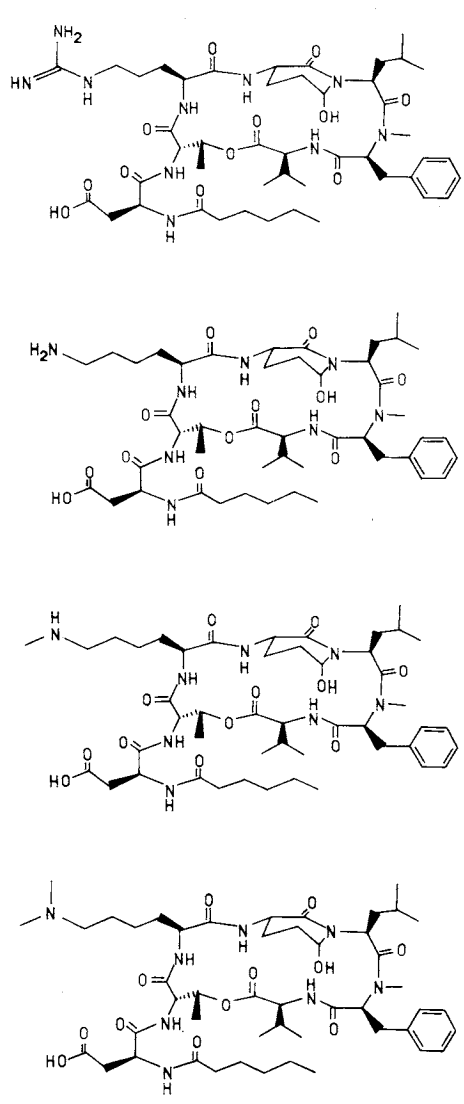
a heterogeneous fraction of peptides was described[7] in addition to microcystins present in this strain[8,9]. This observation led to a detailed study of the peptide pattern of this strain. This paper describes the isolation and structure elucidation of four depsipeptides (peptide lactones) from *Microcystis* sp. PCC 7806 (Fig. 1).

Fig. 1. Structures of the cyanopeptolins A (**I**), B (**II**), C (**III**) and D (**IV**).



Materials and Methods

Organism and Cultivation

Microcystis sp. PCC 7806 was obtained from the Pasteur Culture Collection (Paris, France). Cells were grown photoautotrophically at 27°C in 20-liter bottles (Schott, F.R.G.) using BG 11-Medium[10]. Cultures were continuously illuminated with white fluorescent light (30 μ Einstein/m^2) and aerated with 1% CO_2 in air. Cells were harvested after 20 days by centrifugation (12,000 × *g*, 20 minutes) and lyophilized after storage at −20°C.

Extraction and Purification of Cyanopeptolines

The lyophilized cells (up to 25 g dry weight) were extracted twice with 500 ml MeOH for 2 hours at room temperature. After centrifugation the supernatant was concentrated to 50 ml by rotatory evaporation and diluted to a final concentration of 10% MeOH with water. The fraction was applied to ODS silica gel cartridges (Macherey & Nagel, Düren, F.R.G.). After washing with water the bound material was eluted with 30 ml MeOH and concentrated to 3 ml. Gel filtration was carried out on a 1.5 × 60 cm Sephadex LH-20 column (25 ~ 100 mesh, Pharmacia) with MeOH and eluate was collected in 5-ml fractions. The eluate was dried *in vacuo* and redissolved in 10 ml of 30% aqueous MeOH (v/v). Separation of the microcystins was performed by solid phase extraction with a QMA anion exchange cartridge (Millipore) in 30% aqueous MeOH (v/v). The microcystins were bound to the cartridge whereas the cyanopeptolins passed the column. For final separation the fraction containing the cyanopeptolins was concentrated *in vacuo* and applied to a C_{18} Pep-S HPLC column (5 μm, 4 mm × 250 mm, Pharmacia) using a Waters 600 E multisolvent delivery system with a model 991 photodiode array detector (Waters). Elution was performed isocratically at room temperature with 59% A and 41% B as eluent solvent and a flow rate of 1 ml/minute. Eluent A was 0.025 M ammonium acetate, pH 6.0; eluent B 0.125 M ammonium acetate - acetonitrile (1 : 4). Fractions from multiple HPLC runs were collected, desalted with an ODS silica gel cartridge and dried.

Amino Acid and Fatty Acid Analysis, FAB-MS and NMR

Amino acid analysis was performed applying independent methods. Samples (500 μg) were hydrolyzed with 400 μl 6 M HCl at 110°C for 24 hours and dried in a stream of nitrogen at 40°C. The hydrolysate was analyzed on a Biotronic LC 6001 amino acid analyser employing ion exchange chromatography and post

column reaction with ninhydrin. For gas chromatography/mass spectrometry analysis the amino acid mixture was treated with 200 μl 1.4 M HCl - MeOH for 30 minutes at 100°C and subsequently after removal of excess reagent with 200 μl TFAA - CH_2Cl_2 1 : 4 (v/v) for 30 minutes at 100°C in sealed tubes. GC/MS analysis was carried out on a Hewlett Packard 5890 capillary gas chromatograph using a Supelco SP 2380 column (0.25 mm × 50 m) with helium as carrier gas (flow rate: 1.5 ml/minute) under electron impact (70 eV) and chemical ionisation (isobutane) conditions. For configuration analysis of the amino acids a 25 m capillary column coated with octakis (3-*O*-butyryl-2,6-di-O-pentyl)-γ-cyclodextrin (Lipodex E)[11] was used.

For fatty acid analysis, the hydrolysate was extracted three times with 1 ml diethyl ether. The diethyl ether phase was dried in a stream of nitrogen and subsequently incubated with a diazomethane solution in diethyl ether for 20 minutes at room temperature. The sample was dried and dissolved in dichloromethane. GC analysis was carried out with a SE 30 capillary column isothermal at 50°C column temperature.

Positive FAB mass spectra were recorded on a VG 70-250 S mass spectrometer (VG Analytical, Manchester, UK) at 8 keV using 3-nitrobenzyl alcohol or thioglycerol as liquid matrix and xenon as a collision gas. All NMR spectra were recorded at 301 K on a Bruker AM 500 spectrometer using a 5 mm inverse probe head and an external temperature control (Haake GmbH, Karlsruhe, F.R.G). ^{1}H NMR spectra of the cyanopeptolins A, B, C and D were measured in DMSO-d_6. All 2D experiments (COSY-DQF[12], TOCSY[13], ROESY[14], inverse proton-carbon COSY[15]) were carried out in phase sensitive mode with time proportional phase increments[16]. A TOCSY spectrum (mixing time 80 milliseconds) of cyanopeptolin D (1.8 mg in 0.5 ml DMSO-d_6) and an inverse proton-carbon COSY of cyanopeptolin A (5.6 mg in 0.5 ml DMSO-d_6), as well as a COSY-DQF and a ROESY from both depsipeptides were measured. The ROESY mixing time was 150 milliseconds using a 30 degree transmitter pulse for the spin lock and the carrier set in the NH-region to prevent TOCSY peaks[17].

Results

Isolation and Purification of the Cyanopeptolins A, B, C and D

The isolation and purification of the cyanopeptolins were carried out according to the scheme given in Fig. 2. The cyanopeptolins, exhibiting no specific UV spectra, were found to coelute in Sephadex LH-20 chromatography with microcystin-LR and 3-demethyl-microcystin-LR which are produced by *Microcystis* sp. PCC 7806 in addition to the cyanopeptolins[8]. In order to identify the part of the eluate which contains the cyanopeptolins, all 5-ml fractions which showed the specific UV spectrum of the microcystins were collected. Due to their negative charge at neutral pH microcystin-LR and 3-demethyl-microcystin-LR were bound to the anion exchange resin. Under these conditions the cyanopeptolins appeared uncharged and did not bind to the resin. Immediate separation of the four cyanopeptolins from one another was not possible because of their similar retention times in reversed phase HPLC (Fig. 3). Therefore time consuming

Fig. 2. Isolation and purification scheme of cyanopeptolins A, B, C and D.

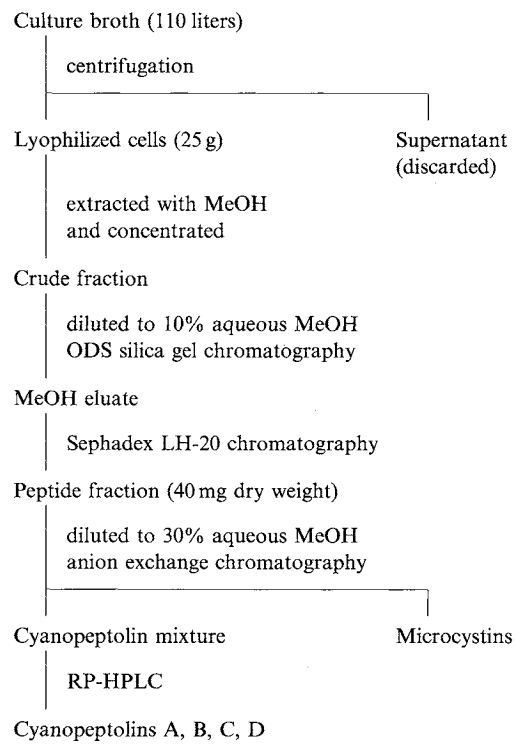
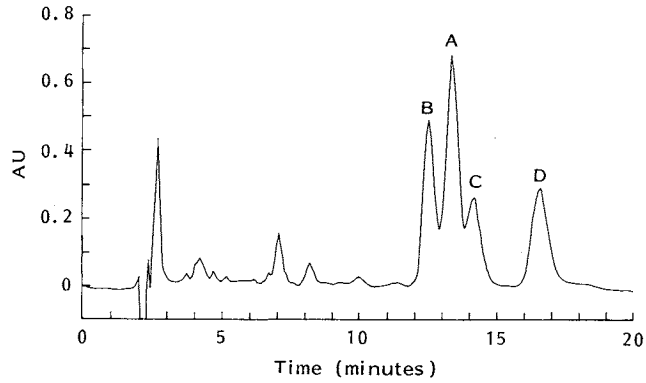


Fig. 3. HPLC profile of cyanopeptolins A, B, C and D monitored at 214 nm.



rechromatography of the four fractions obtained was necessary. Thus, the yield of cyanopeptolins could be only roughly estimated to be approximately 10~20% of that of microcystins (1~2% of cell dry weight). Several other reversed phase-HPLC eluent systems tested did not improve separation.

Structural Analysis

Amino acid and fatty acid analysis of the four cyanopeptolins yielded identical constituents except for their basic amino acids (Table 1). All amino acids were detected in nearly equimolar amounts. Hexanoic acid was identified as its methyl ester by capillary gas chromatography using the authentic standard. Partial hydrolysis of the cyanopeptolins and subsequent GC/MS analysis showed that hexanoic acid was bound to the amino group of aspartic acid.

Table 1. Amino acids and fatty acids in the hydrolysates of cyanopeptolins A, B, C and D (for dehydro-proline see Discussion section).

Amino acid	Cyanopeptolin			
	A	B	C	D
L-Arginine	+[a]	−[b]	−	−
L-Lysine	−	+	−	−
N_ε-Methyl-L-lysine	−	−	+	−
N_ε,N_ε-Dimethyl-L-lysine	−	−	−	+
L-Leucine	+	+	+	+
N-Methyl-L-phenylalanine	+	+	+	+
L-Valine	+	+	+	+
L-Threonine	+	+	+	+
L-Aspartic acid	+	+	+	+
Hexanoic acid	+	+	+	+

[a] +, Present.
[b] −, Absent.

$(M+H)^+$ signals at *m/z* 957.8 (cyanopeptolin A), 929.8 (cyanopeptolin B), 943.8 (cyanopeptolin C) and 957.8 (cyanopeptolin D) indicated relative molecular masses of 956, 928, 942 and 956, respectively. The differences in molecular masses indicated by FAB-MS are in accordance with the differences of the molecular masses of the respective basic amino acids (Table 1).

The 1D and 2D spectra of cyanopeptolins A and D show high similarity. Table 2 lists the ^1H chemical shifts of all assigned protons in the order of the amino acid sequence of the depsipeptides (for numbering of amino acids see Fig. 4). However, the basic amino acid in position 1 causes local shift differences. The protons 1-α and 6-NH give broad signals. It is conceivable that the chemical shift of 6-NH correlates with the basicity of the amino acid-1 side chain. A weak interaction of these functional groups seems possible, as we found Thr-NH to be in slow exchange in both cyanopeptolins measured (A and D), as judged by the broaden NH-resonance. Full spin systems of lysine in cyanopeptolin D (arginine in cyanopeptolin A), valine, aspartic acid and *N*-substituted systems of leucine and *N*-methyl-phenylalanine were detected in the COSY spectrum. In the spin systems of threonine the OH-group was absent but the

Table 2. ^{1}H Chemical shifts of cyanopeptolins A and D, determined in DMSO (2.5 ppm) at 301 K.

Cyanopeptolin compound	NH(N–CH_3)	α-H	β-H	γ-H	δ-H	Other
Cyanopeptolin A						
L-Arginine [1]	8.47	4.29	1.98/1.55	1.50/1.26	2.93/2.98	ε-NH: 10.40; z-NH/NH_2: 6.95
3-Amino-6-hydroxy-2-oxo-1-piperidine ring system [2]	7.19	4.38	2.53/1.70	1.70/1.70	4.87	δ-OH: 6.03
L-Leucine [3]		4.56	1.55/0.27	0.95	0.66/0.42	
N-Methyl-phenylalanine [4]	(2.75)	5.03	3.24/2.83			arom: 2,6-H: 7.14; 3,5-H: 7.26; 4-H: 7.22
L-Valine [5]	7.50	4.70	2.04	0.88/0.74		
L-Threonine [6]	7.20	4.64	5.39	1.16		
L-Aspartic acid [7]	8.04	4.53	2.54/2.10			
Hexanoic acid:						2-CH_2: 2.11; 3-CH_2: 1.52; 4-CH_2/5-CH_2: 1.18/1.28; 6-CH_3: 0.86
Cyanopeptolin D						
N_ε,N_ε-Dimethyl-L-lysine [1]	8.43	4.20	2.02/1.52	1.20/1.30	1.30/1.43	ε-N(CH_3)$_2$: 2.32
3-Amino-6-hydroxy-2-oxo-1-piperidine ring system [2]	7.24	4.38	2.53/1.70	1.70/1.70	4.87	δ-OH: 6.01
L-Leucine [3]		4.56	1.55/0.27	0.95	0.66/0.42	
N-Methyl-phenylalanine [4]	(2.75)	5.03	3.24/2.83			identical to cyanopeptolin A
L-Valine [5]	7.51	4.74	2.08	0.88/0.74		
L-Threonine [6]*	7.70	4.59	5.40	1.19		
L-Aspartic acid [7]	8.10	4.62	2.48/2.48			
Hexanoic acid:						identical to cyanopeptolin A

[ ] Position in depsipeptide sequence (see Fig. 4).
( ) Protons of the *N*-methyl group.
The abbreviation "arom" stands for aromatic protons.

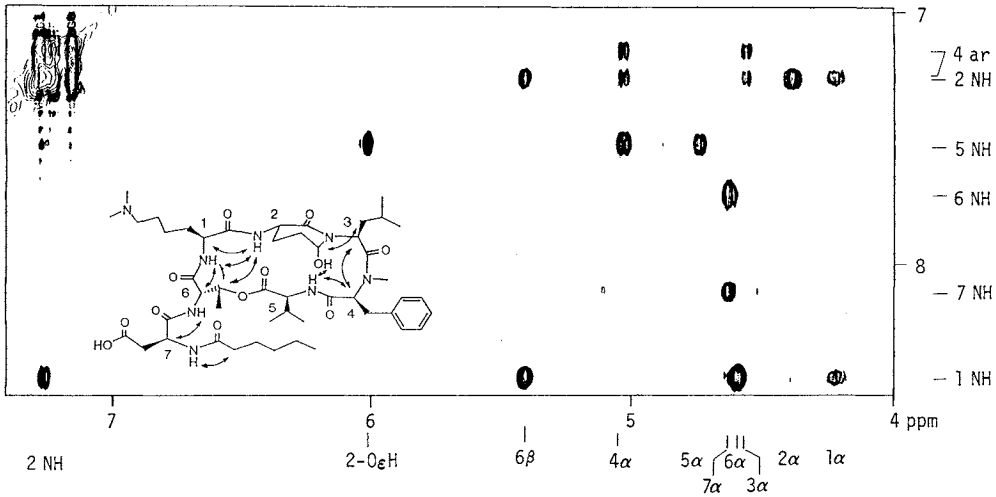
shift of the β-proton (5.40 ppm) indicated a substitution of the OH-proton by the acyl group of L-valine.

Problems with overlapping in the spin system of the 3-amino-6-hydroxy-2-oxo-1-piperidine ring system were resolved with the TOCSY. The ring structure mentioned was finally classified by its carbon shifts obtained from cyanopeptolin A: C-α 49.2, C-β 22.1, C-γ 30.1, C-δ 73.9 ppm (relative DMSO = 40 ppm). The 9 carbonyl groups in the shift range of 169 to 176 ppm were not assigned.

A small and a large coupling constant from the 2-α to 2-β and 2-β′ and two small coupling constants between 2-δ and the two 2-γ protons pointed to a twisted chair conformation of the 3-amino-6-hydroxy-2-oxo-1-piperidine ring system where 2-α is an axial and 2-δ is an equatorial proton. Structural NOEs and coupling constants of this ring system indicated an *S*-configuration in α-position and *R*-configuration in δ position.

The sequential and structural NOEs observed in the ROESY spectra were identical in cyanopeptolin A and D. The connectivities of the residues were obtained by sequential NOEs. The NOE between 3-α and 4-α indicated a *cis*-amide bond. The condensed residues 2 and 3 showed NOEs from 2-δ to 3-β′ and 3-γ. Due to the ester bonding, no NOE is seen between the protons of the backbone of Val-5 and Thr-6. A part of the ROESY spectrum is given and a selection of sequential and structural NOEs is marked in Fig. 4.

Fig. 4. ROESY: α-NH region of cyanopeptolin D.



A selection of NOE cross peaks from the α region in ω2 to the NH region in ω1. (4 ar: aromatic protons of *N*-methyl-phenylalanine; 2′-H/6′: 7.14 ppm; 3′-H/5′: 7.26 ppm). *Insertion*: structure of cyanopeptolin D, a selection of sequential and structural proton-proton NOEs are indicated with arrows. The numbers account to the sequence of the amino acid constituents.

Discussion

The cyanobacterium *Microcystis* sp. PCC 7806 was found to produce a group of four new depsipeptides termed here cyanopeptolins A, B, C and D. They are not related to the microcystins simultaneously produced by this microorganism. The four cyanopeptolins are structurally identical except for their basic amino acids in position 1.

The cyanopeptolins are peptide lactones which possess a new ring structure formally built up by condensation of the aldehyde function of reduced L-glutamic acid with the amino group of L-leucine. In addition to the NMR studies, we have confirmed the 3-amino-6-hydroxy-2-oxo-1-piperidine ring structure by hydrolysis of the cyanopeptolins and subsequent oxidation with $Na_2Cr_2O_7$ yielding L-glutamic acid (without oxidation, dehydro-proline is formed as a minor compound by elimination of water). To our knowledge this ring structure has not been described in any naturally occurring molecule until now (source: Chemical Abstracts). *N*-Methylation, cyclic structure and exchange of related amino acids in one position—properties reminding to peptides and depsipeptides such as cyclosporins, bacitracins and actinomycins[18]—suggest non-ribosomal biosynthesis of cyanopeptolins.

A significant antibacterial effect of the cyanopeptolins has not been observed so far. Their possible other biological activities are subject of further studies. The appearance of different secondary metabolites with peptide character synthesized by a single cyanobacterial strain gives rise to the assumption that cyanobacteria are a rich and fairly unexploited source of pharmacologically interesting compounds.

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