Cyanopeptolin 963A, a Chymotrypsin Inhibitor of *Microcystis* PCC 7806

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A new depsipeptide, cyanopeptolin 963 A (**1**), was isolated from an axenic strain of the toxic freshwater cyanobacterium *Microcystis* PCC 7806. The structure of this compound was elucidated by chemical and spectroscopic analyses, including high-resolution ESI-FTICR-MS, 2-D NMR, and GC-MS of the hydrolysate. The major structural difference compared to previously characterized cyanopeptolins of this strain is the replacement of the basic amino acid in position 3 by L-tyrosine. Compound **1** displayed inhibitory activity against chymotrypsin with an IC_{50} value of 0.9 μM.

Cyanobacteria are a rich source of biologically active depsipeptides that exhibit properties of neurotoxins,[1] display acute toxicity to crustacean grazers,[2,3] and contain efficient inhibitors of proteases.[4] The extensive search for new protease inhibitors in recent years was initiated by the finding of micropeptins A and B[5] to be potent inhibitors for plasmin and trypsin. It can be assumed that under natural conditions in aquatic ecosystems these protease inhibitors are directed against digestive proteases of crustacean grazers.[6] The frequently observed high inhibitory activity against trypsin and chymotrypsin, the major digestive proteases of grazers, classifies these inhibitors as important defense molecules. Cyanobacterial biomass containing protease inhibitors is of low nutritional value for the grazers and can severely reduce their growth when ingested.

The freshwater genus *Microcystis* has been extensively screened for depsipeptides, and numerous variants have been described, all of which contain 3-amino-6-hydroxy-2-piperidone (Ahp) as a structural element. Considering Ahp as a derivative of glutamate, hexa-, hepta-, and octadepsipeptides have been found, and chemical structures either partially or fully elucidated. These include the hexadepsipeptides (micropeptin 90,[7] cyanopeptolins S[8] and SS,[2] micropeptins 478-A and B,[9] micropeptin T-20,[10] and micropeptin SF 909[11]), the heptadepsipeptides (aeruginopeptins 228-A and -B,[12] cyanopeptolins A−D,[13] micropeptins A and B,[5] microcystilide A,[1] micropeptin 88-A,[14] micropeptin SF 995,[12] micropeptins SD 944, 979, 999, and 1002,[15] and micropeptins EI 964 and 992[16]), and the octadepsipeptides (aeruginopeptins 95-A and -B,[12] micropeptin 103,[17] and micropeptins 88-B−F[14]). A further characteristic motif of this compound class is the six amino acid-membered macrocyclic lactone ring formed between the *C*-terminus and the hydroxy group of threonine, which is positioned *N*-terminally in the case of the hexadepsipeptides or in position 2 or 3, respectively, of the hepta- and octadepsipeptides. In all known variants of Ahp-containing depsipeptides in *Microcystis* either isoleucine or valine is the *C*-terminal amino acid, all amino acids are in the L-configuration, the amino group of threonine or the *N*-terminal amino acid of the side chain is linked to a short-chain carboxylic acid with or without further functional groups, and the amino acid adjacent to the *N*-terminus is an *N*-methylated aromatic amino acid (Phe, Tyr, ring chlorinated Tyr[10], kynurenine[15]) or *N*-methylated heteroaromatic amino acid (Trp[12,15,17]).

Among the depsipeptides of *Microcystis* PCC 7806, the new variant described herein fits the general features outlined above. Depsipeptides of *Microcystis* of the same basic structure have been labeled cyanopeptolins,[13] micropeptins,[5] aeruginopeptins,[12] and microcystilides.[1] Since the cyanopeptolins were the first variants to be reported with a complete stereochemistry, we use this trivial name for the new compound cyanopeptolin 963A.

Separation of the 60% aqueous methanolic extract of *Microcystis* PCC 7806 on a reversed-phase HPLC column proved the presence of several peptides. Analysis with LC-ESI-MS led to the detection of prominent peaks corresponding to [D-Asp[3]]-microcystin-LR, microcystin-LR, and the cyanopeptolins A and D known from previous studies.[13,18] In addition, we observed a compound that eluted after the cyanopeptolins. The MS/MS mass fragmentation pattern of this compound suggested it to be an as yet unknown variant of the cyanopeptolins. The new compound, cyanopeptolin 963A, exhibited a quasi molecular ion at *m/z* 986.7 $[M + Na]^+$.

High-resolution electrospray Fourier transform ion cyclotron mass spectrometry (ESI-FT-ICR-MS) of the sodium adduct of cyanopeptolin 963A gave a monoisotopic signal at 986.48418 amu, corresponding to $[C_{49}H_{69}N_7O_{13}Na]^+$ (theoretical mass = 986.48456, relative mass error Δ_m = 0.38 ppm). Therefore, uncharged cyanopeptolin 963A has the molecular formula $C_{49}H_{69}N_7O_{13}$. Chiral GC-MS amino acid analysis and quantitation by enantiomer labeling of the hydrolysate gave the following relative molar concentrations: L-Leu (1.00 ref), L-Val (0.96), L-Asp (1.42), L-Thr (0.96), *N*-Me-L-Phe (0.83), and L-Tyr (1.16). The assignment of NMR spin systems to the above-mentioned amino acids and hexanoic acid was done from the COSY and TOCSY experiments. The assignment of ^{13}C signals was derived from HSQC and HMBC spectra. The peptide sequence of cyanopeptolin 963A was deduced from the NOESY and HMBC experiments, and the lactone ring closure between Thr-2 and Val-7 was verified by HMBC correlation from the β-proton of Thr-2 to the quaternary carbonyl C atom of Val-7. According to a previous publication,[19] the absence of an amido proton, in this case for Leu-5, suggested an *N,N*-disubstituted derivative, being part of a 3-amino-6-

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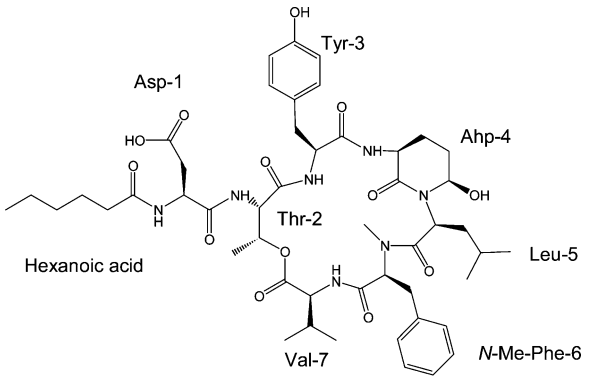
hydroxy-2-piperidone (Ahp) moiety with both amino acids forming a hemiaminal structure. After CrO_3 oxidation[20] followed by hydrolysis and derivatization, L-glutamic acid was detected by GC-MS, along with the above-mentioned amino acids (with the exception of tyrosine). Reduction of cyanopeptolin 963A with $NaBH_4$[20] yielded pentahomoserine (5-hydroxy-2-aminopentanoic acid). Lack of an authentic standard, however, precluded the determination of its configuration. These results are consistent with the presence of Ahp, the 3-position of which has the L-(*S*) configuration.[21] The absolute stereochemistry of Ahp (3*S*,6*R*) was determined on the basis of chemical degradation and NOESY correlations.[22]

A common feature of many cyanopeptolins is the moiety located *N*-terminally from Thr-2. This moiety was composed of one amino acid (L-Asp) and *n*-hexanoic acid. *n*-Hexanoic acid was detected as the sole carboxylic acid by GC-MS of the TMS derivatives from the ethyl acetate extract of the hydrolysate, and its presence was confirmed by 1-D NMR (DEPT 135) and 2-D NMR experiments. The complete set of ^1H and ^{13}C chemical shifts is summarized in the Experimental Section.



1 Cyanopeptolin 963A

Cyanopeptolin 963A was found to be a specific inhibitor of chymotrypsin. The IC_{50} value determined was 0.9 μM and thus is in the range of several other Ahp-containing depsipeptides. The strongest chymotrypsin inhibitor so far described[10] is micropeptin T-20, with an IC_{50} of 2.5 nM, while the majority of micropeptins exhibit IC_{50} values around 2−4 μM.[11,14,15,20] Trypsin and cathepsin B were not inhibited at a 15 μM concentration of cyanopeptolin 963A.

Recent genetic studies on depsipeptide syntheses in cyanobacteria have proven the nonribosomal synthesis of these compounds. Peptide synthetase genes were characterized for anabaenopeptilides[23] in *Anabaena* and nostocyclopeptide[24] and nostopeptolide A[25] in *Nostoc*. The modular structure of the peptide synthetases[26] facilitates the exchange of the amino acid constituents within the peptides and the formation of a large number of variants that exhibit different enzyme target specifities and effectivities. It can be assumed that the primary targets of the protease inhibitors found in cyanobacteria are the digestive proteases of grazers and that the driving force for the development of depsipeptide variants is caused by the grazing pressure in the natural ecosystem.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Cary III spectrophotometer (Varian), and high-resolution FTICR-MS spectra were measured on an APEX II ESI-FTICR mass spectrometer equipped with a 4.7 T magnet (Bruker-Daltonics, Bremen, Germany). The 2-D NMR spectra (COSY, TOCSY, NOESY, HSQC, HMBC) were measured on an AMX 600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm Z-Grad triple resonance probe head. HPLC-ESI-MS-derived mass spectra were obtained on a LC-MS (LCQ Duo mass spectrometer, Finnigan Thermoquest) equipped with an electrospray source (ESI). The composition of the derivatized hydrolysate was determined by GC-MS (Agilent MSD 5973/6890, Agilent, Waldbronn, Germany). HPLC separations were performed on a Shimadzu 10AVP system equipped with a diode array detector and autosampler.

Culture of *Microcystis*. *Microcystis* PCC 7806 was cultivated at 25 °C under sterile conditions in 5 L glass tower-type reactors. The reactors had an inner diameter of 7.2 cm and were aerated through a central glass tube with CO_2-enriched air (600 mL min^{-1}, 0.15 vol % CO_2) and stirred with a magnetic stirrer to prevent settling of the cyanobacteria. The reactors were continuously illuminated by a fluorescent tube that was positioned at a distance of 35 cm, providing 15 μmol quanta m^{-2} s^{-1}. A mineral medium optimized for growth of *Microcystis* was used.[27] The yield was 1.0 g L^{-1} dry mass, corresponding to 13 g L^{-1} wet biomass.

Isolation of Cyanopeptolin 963A. In a typical experiment, 2 g of lyophilized biomass was extracted with 200 mL of 60% MeOH followed by a further 100 mL of 60% MeOH for 2 h at ambient temperature. After centrifugation, the supernatants were combined and the solvent was removed in a rotary evaporator at 45 °C. The residue was taken up in 20 mL of 60% MeOH and applied to four C18 cartridges (Varian, 12 mL size, equilibrated with 10% aqueous MeOH). Each cartridge was rinsed with 60 mL of 10% MeOH and the compound eluted with 7 mL of 60% MeOH and 15 mL of 80% MeOH. After evaporation of the solvent in a rotary evaporator, the extract was dissolved in 5 mL of 60% MeOH and subjected to about 50 HPLC runs. A semipreparative reversed-phase column was used (Grom-Sil 120 ODS-4HE, 5 μm particle size, 250 × 10 mm, Grom Analytik, Rottenburg-Hailfingen, Germany). Separations were performed with a linear solvent gradient starting with aqueous ACN (30−100% ACN in 20 min, 3 mL min^{-1} flow rate) under acidic conditions (0.05% TFA). The microcystins eluted at 11−12 min, the cyanopeptolins A and D at 12−13.5 min, and cyanopeptolin 963A at 14.5−16 min. When a narrow peak cut was applied, the yield of cyanopeptolin 963A was 0.7 mg per 2 g biomass.

Amino Acid Analysis. The composition and the configuration of the amino acids of the inhibitor were determined after hydrolysis in 6 N HCl at 110 °C for 21 h. The dry hydrolysate was derivatized to the *N*-(*O*-)tfa/ethyl esters (the tfa/methyl esters were used to determine the configuration of *N*-Me-Phe) and analyzed by chiral GC-MS on a 20 m × 0.25 mm Lipodex E/PS255 (30:70) capillary column. Quantitation of amino acids was performed by the method of enantiomer labeling,[28] for which an *N*-Me-Phe standard was synthesized according to published procedures.[29] CrO_3 oxidation of cyanopeptolin 963A was carried out according to a previously described method.[20] For the determination of hexanoic acid, the hydrolysate was saturated with NaCl and extracted with ethyl acetate. The extract was dried over anhydrous Na_2SO_4, derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), and analyzed by GC-MS on a DB 5 capillary column (J&W, Folsom, CA).

Molar Extinction Coefficient. The determination of the molar extinction coefficient was based on a mass determination calculated from the quantitative analysis of L-valine of the hydrolysate. l-U-$^{13}C_5$-valine (Cambridge Isotope Laboratories Inc. MA, 99% purity) served as internal standard. The *tert*-butyldimethylsilyl derivatives of the amino acids were quantitatively analyzed by GC-EIMS in the SIM mode using the fragment ions *m*/*z* 186 and 260 for Val and *m*/*z* 190 and 264 for the ^{13}C-labeled Val.

Inhibition of Proteases. The procedures to measure the inhibition of serine proteases were essentially the same as described recently by Banker and Carmeli.[11] Solutions (2 mM) of benzoyl-L-Arg-*p*-nitroanilide (Bachem) and *N*-succinyl-Gly-

Gly-Phe-*p*-nitroanilide (Sigma) in a Tris/HCl buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.6) were used as substrates (2 mM) for both trypsin from porcine pancreas (Fluka) and α-chymotrypsin (7.7 U mL^{-1}) from bovine pancreas (Sigma). The absorbance was measured for 30 min at 37 °C and 405 nm in 200 μL well microtiter plates on a Spectra Max 190 (Molecular Devices) absorbance reader.

The determination of the activity of cathepsin B (7.5 mU mL^{-1}) was performed according to Greenspan and co-workers.[30] Cbz-Ala-Arg-Arg-4-methoxy-β-naphthylamide acetate (Sigma) was used as the substrate for cathepsin B from bovine spleen (Sigma). The fluorescence was measured on a Spectra Max Gemini XS (Molecular Devices) fluorescence reader at λ_{Ex} 345 nm and λ_{Em} 425 nm.

Cyanopeptolin 963A (1): white amorphous solid; UV (MeOH) λ 225 (ϵ 16 000 sh), λ_{max} 277 (ϵ 2 500) nm; ^{1}H NMR (600 MHz, DMSO-d_6) hexanoic acid: δ 2.12 (2H, H-2), 1.54 (2H, H-3), 1.25 (4H, H-4, H-5), 0.85 (3H, H-6); Asp-1: 4.61 (1H, H-α), 2.68, 2.50 (2H, H-β), 12.18 (1H, O*H*), 8.17 (1H, N*H*); Thr-2: 4.54 (1H, H-α), 5.44 (1H, H-β), 1.33 (3H, H-γ), 7.52 (1H, N*H*); Tyr-3: 4.41 (1H, H-α), 3.21, 2.60 (2H, H-β), 6.94 (2H, H-2, H-5), 6.60 (2H, H-3, H-6), 9.06 (1H, O*H*), 8.48 (1H, N*H*); Ahp-4: 4.37 (1H, H-3), 1.74, 2.56 (2H, H-4), 1.72 (2H, H-5), 4.90 (1H, H-6), 6.01 (1H, O*H*), 7.31 (1H, N*H*); Leu-5: 4.58 (1H, H-α), 0.31, 1.52 (2H, H-β), 0.93 (1H, H-γ), 0.66, 0.44 (6H, H-δ); *N*-Me-Phe-6: 5.01 (1H, H-α), 2.82, 3.21 (2H, H-β), 7.13 (2H, H-2, H-5), 7.25 (2H, H-3, H-6), 7.20 (1H, H-4), 2.72 (3H, N-C*H*$_3$); Val-7: 4.63 (1H, H-α), 2.02 (1H, H-β), 0.85, 0.73 (6H, H-γ), 7.52 (1H, N*H*); ^{13}C NMR (600 MHz, DMSO-d_6) hexanoic acid: δ 171.7 (C-1), 34.8 (C-2), 24.5 (C-3), 30.5 (C-4), 21.5 (C-5), 13.5 (C-6); Asp-1: 168.1 (*C*ONH), 49.1 (C-α), 35.2 (C-β), 170.9 (C-γ); Thr-2: 167.7 (*C*ONH), 54.0 (C-α), 71.7 (C-β), 17.3 (C-γ); Tyr-3: 168.9 (*C*ONH), 53.6 (C-α), 34.8 (C-β), 127.9 (C-1), 129.2 (C-2, C-5), 114.5 (C-3, C-6), 155.2 (C-4); Ahp-4: (C-2*), 48.9 (C-3), 21.5 (C-4), 29.4 (C-5), 73.1 (C-6); Leu-5: 169.7 (*C*ONH), 47.3 (C-α), 38.0 (C-β), 23.2 (C-γ), 23.4, 22.0 (C-δ); *N*-Me-Phe-6: 170.4 (*C*ONH), 60.2 (C-α), 33.8 (C-β), 137.4 (C-1), 128.7 (C-2, C-5), 128.6 (C-3, C-6), 126.0 (C-4), 30.1 (N-*C*H$_3$); Val-7: 170.7 (*C*ONH), 55.7 (C-α), 30.4 (C-β), 18.8, 17.1 (C-γ); HR-ESI-FTICR-MS *m*/*z* $[M + Na]^+$ 986.48418 (calcd for $[C_{49}H_{69}N_7O_{13}Na]^+$ 986.48456, relative mass error $\Delta_m = 0.38$ ppm). (*) assignment ambiguous.

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