Oscillapeptin J, a New Grazer Toxin of the Freshwater Cyanobacterium *Planktothrix rubescens*

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Received August 28, 2002

Oscillapeptin J (**1**), a new and highly potent crustacean grazer toxin, was isolated from the axenic cyanobacterium *Planktothrix rubescens*, which frequently forms blooms in freshwater lakes. Chemical and spectroscopic analyses, including high resolving MS and two-dimensional NMR, were used to elucidate the compound's structure as a depsiheptapeptide of the oscillapeptin type. Strict application of a bioassay-guided isolation procedure proved this compound to be one of the major causative agents (besides [D-Asp^3,-(*E*)-Dhb^7]microcystin-RR) of the acute grazer toxicity of *P. rubescens* from Lake Zürich. The LC_{50} value of oscillapeptin J as determined for the freshwater crustacean *Thamnocephalus platyurus* was 15.6 μM.

Cyanobacteria produce a variety of toxins, including hepatotoxins, neurotoxins, cytotoxins, and dermatotoxins.[1] Mass occurrences of cyanobacteria frequently observed in mesotrophic and eutrophic lakes cause severe problems in reservoirs and lakes used as drinking water supplies, recreation areas, and watering places for livestock.[2] The most common cyanobacterium in deep prealpine freshwater lakes in Central Europe is the red filamentous *Planktothrix rubescens*,[3,4] formerly known as *Oscillatoria rubescens* D.C. This cyanobacterium accumulates to very high densities in Lake Zürich (Switzerland) and forms a dense layer at a depth of 10.5−12.5 m in the second part of the year.[5]

P. rubescens and *P. agardhii*, which are closely related species, are efficient producers of microcystins.[6−8] These microcystins are generally believed to be responsible for the majority of the mouse and grazer toxicity observed in these species.

Oscillamides B and C were isolated from *P. agardhii* and *P. rubescens*, respectively, and have exhibited weak inhibitory activity against rabbit skeletal muscle protein phosphatase 1 and human protein phosphatase 2A.[9] Several other bioactive cyclic peptides have been described recently from these sources. Enzyme inhibitor studies have shown that these metabolites are strong inhibitors of serine proteases rather than having properties of acute toxic compounds. Chymotrypsin was inhibited by low concentrations of oscillamide Y, oscillatorin, and microviridins.[10−12] In addition, inhibitory activity against elastase was found for the oscillapeptins.[13,14] Trypsin was inhibited by oscillapeptin F, and anabaenopeptin G was a highly active inhibitor for carboxypeptidase A.[14,15]

When isolating [D-Asp^3,(*E*)-Dhb^7]microcystin-RR from *P. rubescens*, we found a component in the hydrophilic fraction that exhibited strong acute toxicity in a bioassay with *Thamnocephalus platyurus*; this fraction did not show features of a microcystin.[8] This compound was isolated and its molecular structure determined to be a new oscillapeptin. It is the first example of this type of cyclic peptide isolated from *Planktothrix* that exhibits acute grazer toxicity, which was previously known only for the microcystins.

The frozen cyanobacterial biomass was treated with 60% methanol, extracting the majority of the toxin from the cells. After evaporation of the solvent, the residue was dissolved in 60% aqueous methanol to yield a yellow solution. This solution was passed through a particle filter and then subjected to HPLC separations on a C18 reversed-phase column. Five fractions were collected and, after removal of the solvents, subjected to an acute grazer toxicity assay conducted with the crustacean *T. platyurus*. Two baseline-separated toxic fractions were found: one was [D-Asp^3,(*E*)-Dhb^7]microcystin-RR,[8] and the other which eluted much earlier in the diode array detector showed a strong absorption at 200 nm (max), 220 nm (shoulder), and 278 nm (max) rather than the characteristic absorption maximum of microcystins. With electrospray mass spectrometry, the compound exhibited a quasi molecular ion at *m/z* 1093.2 $[M + H]^+$. The toxin was further purified by passage through a C18 cartridge to remove the eluent-derived trifluoroacetic acid (TFA). This was essential because the toxin easily lost a water molecule under acidic conditions and remaining TFA gave false positive results in the bioassays. Because the dehydrated form of the toxin, exhibiting a quasi molecular ion at *m/z* 1075.2 $[M + H]^+$, remained to some extent in the purified toxin, another HPLC separation was conducted with a TFA-free eluent; this latter system easily separated both components, the toxin at $t_R = 5.3$ min and the dehydrated toxin at $t_R = 11.9$ min.

High-resolution electrospray Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) of the sodium adduct $[M + Na]^+$ of oscillapeptin J gave a monoisotopic signal at 1115.43205 amu, corresponding to $[C_{47}H_{68}N_{10}O_{18}SNa]^+$ (theoretical mass = 1115.43260) with a relative mass error of 0.5 ppm. Therefore, uncharged oscillapeptin J has the molecular formula $C_{47}H_{68}N_{10}O_{18}S$. In-source fragmentation led to loss of 18 amu, indicative of hydroxy amino acids, and 80 amu, indicating the presence of sulfate.

Upon acidic hydrolysis of the toxin, derivatization with *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), and separation on a capillary column, peaks were detected that matched the retention times and mass

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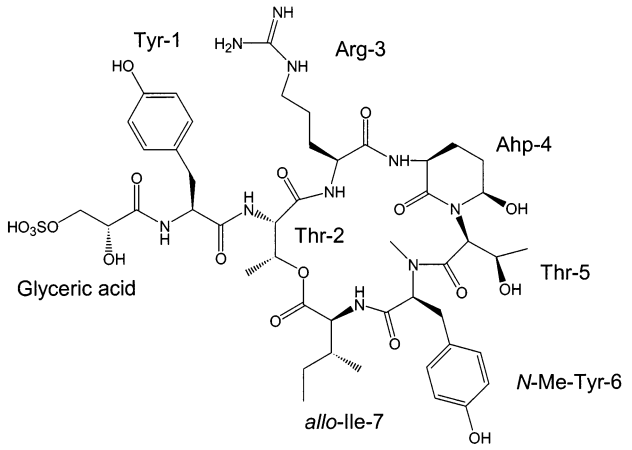
10.1021/np020397f CCC: $25.00
Published on Web 02/21/2003

fragmentation patterns of sulfate (t_R = 7.32 min), *allo*-isoleucine (t_R = 8.49 min), glycerate (t_R = 10.95 min), threonine (t_R = 11.61 min), *N*-methyltyrosine (t_R = 15.16 min), and tyrosine (t_R = 18.9 min). Chiral GC−MS amino acid analysis and quantification by enantiomer labeling of the total hydrolysate detected the presence of L-Thr (1.70), L-*allo*-Ile (ref = 1.00), L-Tyr (0.92), *N*-methyl-L-Tyr (1.05), and L-Arg (0.97) together with (*R*)-glyceric acid (approximately one residue).

The assignment of NMR spin systems to the above-mentioned amino acids and glyceric 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 oscillapeptin J was deduced from the NOESY and HMBC experiments, and lactone ring closure between Thr-2 and L-*allo*-Ile-7 finally was verified by HMBC correlation from the β-proton of Thr-2 to the quaternary carbonyl C atom of L-*allo*-Ile-7. According to a previous publication, the absence of an amido proton, in our case for Thr-5, suggested a N,N-disubstituted derivative, being part of a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety with both amino acids forming a hemiaminal structure.[13] Oxidation of oscillapeptin J with CrO_3[14] followed by acid-catalyzed hydrolysis led to the formation of L-Glu as the major degradation product of Ahp, indicating the L-configuration of the α-position of Ahp.[16] The absolute stereochemistry of (3*S*,6*R*)-L-Ahp was determined by NOESY correlations.



1 Oscillapeptin J

The zwitterionic toxin (**1**) exhibits structural elements that warrant inclusion in the group of oscillapeptins. It is a cyclic depsiheptapeptide which forms a lactone structure between the side chain hydroxy group of threonine (Thr-2) and the carbonyl group of the C-terminal amino acid *allo*-Ile-7. Further characteristic structural elements are Ahp and *N*-methyltyrosine in positions 4 and 6. A common feature of oscillapeptins is the moiety located N-terminally from Thr-2. This moiety is composed of one or two amino acids and (*R*)-glyceric acid, which may be partially methylated or esterified with sulfuric acid at the terminal position.

Up to now seven variants of this molecule have been described in different strains of *O. agardhii*.[13,14,16] Other variants, named cyanopeptolin S, cyanopeptolin SS, and A90720A, were found in a bloom of *Microcystis* and a culture of *Microchaete loktakensis*, respectively.[17,18] The structurally related compounds micropeptin T-20, micropeptin 478-A, micropeptin 90, and cyanopeptolins A−D have been isolated from different strains of the bloom-forming cyanobacterium *Microcystis*.[19−22] Their biological activity has been reported to be serine protease inhibitors rather than toxins. The chymotrypsin IC_{50} values of the oscillapeptins A−E were around 2 μM, while oscillapeptin F showed inhibitory properties against trypsin and plasmin.[14] Oscillapeptin G exhibited inhibitory activity against elastase and low activity against chymotrypsin.[16]

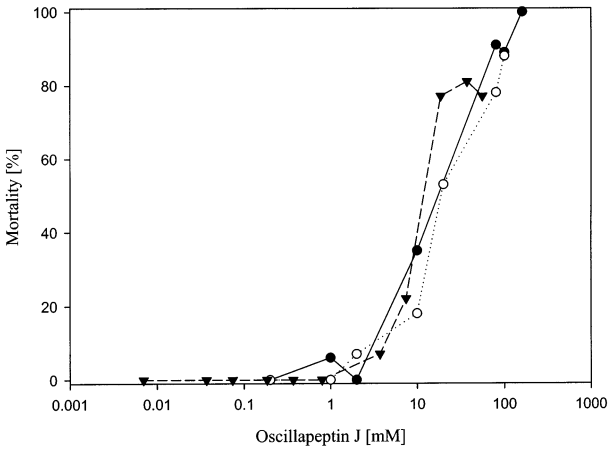


Figure 1. Dose−response curves for the mortality [%] of *Thamnocephalus platyurus* (24 h acute toxicity) exposed to different concentrations of oscillapeptin J in three independent experiments. Each curve is the mean of three replicates.

Toxicity assays of extracts from microcystin-containing cyanobacteria have demonstrated that microcystins account for only a part of the total toxicity observed. Aqueous extracts of *Microcystis flos-aquae* PCC 7806 and *P. agardhii* NIVA 34 that were free of microcystins were determined to be toxic, but the responsible compound could not be elucidated.[23,24] Oscillapeptin J, a constituent of the aqueous extract of *P. rubescens*, showed toxic properties (IC_{50} 15.6 μm) when tested against the crustacean grazer *T. platyurus*. Although oscillapeptin J is slightly less toxic than [D-Asp^3,(*E*)-Dhb^7]microcystin-RR, which accounts for the major grazer toxicity of *P. rubescens*, the acute toxicity is strong enough to contribute significantly to the total grazer toxicity of this cyanobacterium.

Experimental Section

Instrumental Analyses. UV-spectra were recorded on a Cary III photometer (Varian) and high-resolution FTICR-MS spectra on an APEX II ESI-FTICR mass spectrometer equipped with a 4.7 T magnet (Bruker-Daltonics, Bremen, Germany). The two-dimensional 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 probehead. Mass spectra were obtained on a LC-MS (LCQ Duo mass spectrometer, Finnigan Thermoquest) equipped with an electrospray source. The composition of the derivatized hydrolysate was determined using GC (HP 5890A Agilent, Waldbronn, Germany with FID detection) and GC−MS (Fison Instruments, GC 8000 Top, MD 800 or 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 Conditions and Harvest. *P. rubescens*, an axenic isolate from Lake Zürich, was grown under weak continuous light in 300 mL Erlenmeyer flasks as described recently.[8] The biomass was concentrated on a plankton net (25 μm mesh size), removed with a spatula, and frozen at −20 °C. The yield was ca. 3 g wet biomass per 1500 mL of culture.

Extraction and Isolation. Portions of the frozen cyanobacterial biomass (3 g wet weight) were extracted with 60% MeOH (80 mL) for 3 h in the dark, and the extract was

Table 1. ^{1}H and ^{13}C NMR Data for Oscillepeptin J in DMSO-D_6

position		δ_H	δ_C
glyceric acid	1		170.32
	2	4.02	70.78
	3	3.66	68.28
		3.97	
	2-OH	–[a]	
	3-OH	–	
Tyr-1	1		171.43
	2	4.68	53.14
	3	2.95	36.43
		2.84	
	4		126.90
	5,9	6.97	130.06
	6,8	6.61	114.63
	7		155.42
	NH	7.68	
	OH	9.18	
Thr-2	1		168.88
	2	5.49	71.95
	3	4.64	54.75
	4	1.20	17.54
	NH	8.35	
Arg-3	1		169.70
	2	4.36	51.55
	3	1.39	27.36
		2.10	
	4	1.46	24.91
		1.46	
	5	3.11	40.21
		3.11	
	6		156.16
	6-NH	7.46	
	NH_2	7.24; 6.74	
	NH	8.53	
Ahp-4	1		169.38
	3	4.46	48.58
	4	1.73	21.73
		2.52	
	5	1.67	28.88
		1.94	
	6	5.06	74.36
	NH	7.52	
	OH	5.97	
Thr-5	1		170.21
	2	4.39	54.94
	3	3.58	64.19
	4	0.24	18.87
	OH	–	
N-Me-Tyr-6	1		169.25
	2	4.99	60.57
	3	2.68	32.96
		3.16	
	4		126.92
	5,9	6.97	130.06
	6,8	6.64	114.98
	7		155.93
	Me	2.69	29.78
	OH	9.18	
allo-Ile-7	1		172.30
	2	4.96	53.89
	3	1.87	36.73
	4	1.11	25.98
		1.28	
	5	0.91	11.39
	6	0.70	14.34
	NH	7.54	

[a] (–) assignment ambiguous.

separated by centrifugation from the residue. The supernatant was evaporated in a vacuum rotary evaporator at 40 °C. The residue was dissolved in 60% MeOH (1 mL), filtrated through 0.2 μm PTFE filter (Semadeni, Switzerland), and fractionated by reversed-phase HPLC (C18 Grom-Sil 120 ODS-4 HE, 4.6 × 250 mm, Stagroma, Germany; flow rate 1 mL min^{-1}; solvent A: UV-treated H_2O and 0.05% TFA; solvent B: acetonitrile and 0.05% TFA). The following gradient was applied: solvent B from 30% to 35% in 10 min, 35% to 70% in 30 min, 70% to 100% in 2 min, isocratic 8 min. The peak eluting at 5–6 min, which showed high toxicity against *T. platyurus*, was collected in about 20 HPLC runs and applied to a C18 cartridge (Varian; equilibrated with 10% aqueous MeOH). The cartridge was flushed with Nanopure water (2 L) and the toxin eluted with 50% MeOH (25 mL) and then with 80% MeOH (55 mL). The eluates were combined and evaporated in a rotary evaporator. The yield was about 0.5 mg highly purified oscillapeptin J.

Oscillapeptin J (1): amorphous powder; UV (MeOH) λ_{max} 278 nm (ϵ 6200); for ^{1}H and ^{13}C NMR, see Table 1; HR-ESI-FTICR-MS was measured by direct infusion of a methanolic solution containing NaCl with internal calibration using polypropyleneglycol 1020.

Analysis of the Hydrolysate. The acids and amino acids of the toxin were determined with GC–MS after hydrolysis in 6 M HCl at 110 °C for 48 h. The derivatization of the hydrolysate was performed with MTBSTFA (Fluka, Switzerland) in tetrahydrofuran and TFA (20:25:0.05). Analyses were conducted on a capillary column (30 m DB-1301, 0.32 mm i.d., 0.25 μm film thickness) under the following separation conditions: 1 min at 120 °C, 120 to 250 °C at a rate of 10 °C min^{-1}. The configuration of the amino acids was determined by GC–MS of their *N*-(*O*-)trifluoroacetyl/ethyl ester derivatives on both Chirasil Val and Lipodex E/PS255 (30:70) capillaries. Quantification of amino acids was performed by the method of enantiomer labeling on the Chirasil Val capillary column with FID detection.[25] The configuration of glyceric acid was determined by GC of its *n*-propylamide/O-TMS derivative on a Chirasil Val capillary column.[26] CrO_3 oxidation was performed with oscillapeptin J according to a previously described method.[14]

Determination of the Molar Absorption Coefficient. The molar absorption coefficient was determined by quantitative analysis of *allo*-Ile after acidic hydrolysis. L-U-$^{13}C_6$ isoleucine (Cambridge Isotope Laboratories, Inc., MA) was added as an internal standard before hydrolysis. After derivatization with MTBSTFA and GC-EIMS, the integrals of the fragment ions at *m*/*z* 200 and 205, *m*/*z* 274 and 279, and *m*/*z* 344 and 350 were used for quantitative analysis. This value was correlated to the UV absorption of oscillapeptin J measured before hydrolysis.

Biological Assay. The highly purified oscillapeptin J was tested in a 24 h acute toxicity assay with *T. platyurus* (Thamnotoxkit F; G. Persoone, State University of Ghent, Belgium) in a multiwell plate using instar II–III larvae hatched from cysts. The toxin was tested in seven to 10 different concentrations; each concentration was tested in three parallels in three independent replicates. The nonlinear regression analysis and determination of the LC_{50} value was calculated with Sigma Plot 2002 for Windows Version 8.0 (SPSS Inc.).

Acknowledgment. The authors thank A. E. Walsby (School of Biological Sciences, University of Bristol) for the culture of *Planktothrix rubescens*, and the National Science Foundation, Bern, for financial support. R.D.S. acknowledges an Emmy-Noether Fellowship for young investigators of the Deutsche Forschungsgemeinschaft (DFG SU 239/2-1).

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NP020397F