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Cyclic Depsipeptides, Ichthyopeptins A and B, from *Microcystis ichthyoblabe*

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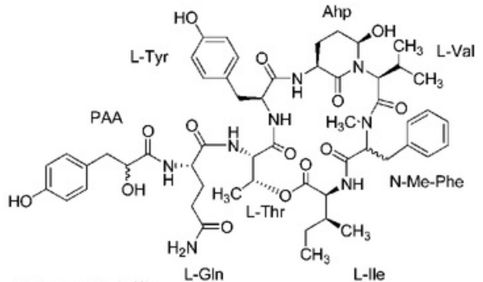
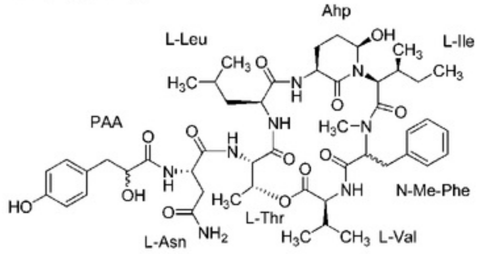
Bioassay-guided isolation of antiviral compounds from the cultured cyanobacterium *Microcystis ichthyoblabe* provided two novel cyclic depsipeptides, ichthyopeptins A (**1**) and B (**2**). Their structures were determined by 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR spectra, ESIMS-MS, and amino acid analysis. The fraction containing both cyclic depsipeptides exhibited antiviral activity against influenza A virus with an IC_{50} value of 12.5 μg/mL.

One of the strategies to combat viral infectious diseases is the search for new metabolites from natural products as antiviral agents. Cyanobacteria have been known to be an enormous resource for compounds with varying bioactivities including antimicrobial, antiviral, and enzyme inhibitory effects. One of the compounds with antiviral potential derived from cyanobacteria is cyanovirin, a protein with 101 amino acids isolated from *Nostoc ellipsosporum*.[1] Several compounds such as cyanopeptolins, micropeptins, oscillapeptins, nostopeptins, agardhipeptins, anabaenopeptins, nodulapeptins, microviridins, and aeruginosins are protease inhibitors. They are widely distributed in species of cyanobacteria such as *Microcystis*, *Oscillatoria*, *Nostoc*, *Nodularia*, and *Anabaena*, and most compounds are cyclic depsipeptides containing the 3-amino-6-hydroxy-2-piperidone (Ahp) unit.[2]

Our previous investigations of several cultured strains of cyanobacteria have shown antiviral effects against influenza A virus, whereas the propagation of respiratory syncytial virus, herpes simplex virus, and adenovirus was not affected.[3−5] Here we report the bioassay-guided fractionation of a methanolic extract of *Microcystis ichthyoblabe* that has resulted in the isolation and identification of two novel cyclic depsipeptides containing the Ahp unit, ichthyopeptins A (**1**) and B (**2**).



Ichthyopeptin A (**1**)



Ichthyopeptin B (**2**)

Results and Discussion

As the strongest activity during screening against influenza A virus was found for the methanolic extract of *M. ichthyoblabe*,[6] this extract was selected for bioassay-guided isolation. Fractionation of the methanolic extract on silica gel using an elution system from 100% EtOAc to 100% MeOH and to 100% H_2O resulted in nine fractions, M-1 to M-9. All fractions were initially tested for cytotoxicity against MDCK cells, but no cytotoxicity was observed. However, antiviral activity was detected in an in vitro system using influenza A virus/MDCK cells and showed the highest activity in fraction M-2, which eluted with 75% EtOAc/MeOH. This fraction was further separated on a silica gel column using a gradient system from 100% EtOAc to 100% MeOH. Of the 11 fractions obtained, three fractions, M2-2, M2-4, and M2-6, showed peaks in the analytical HPLC. Fraction M2-4 was selected for further separation and purification by preparative RP-HPLC with a gradient system of CH_3CN/H_2O, resulting in two pure fractions, M2-4-P1 (t_R 17.93 min, 8.2 mg) and M2-4-P2 (t_R 18.40 min, 7.4 mg), which were obtained as white, amorphous powders after lyophilization.

The combined NMR and MS data of fraction M2-4-P1 allowed unambiguous identification of the novel cyclic depsipeptide ichthyopeptin A (**1**). The positive HRESIMS of **1** showed the $[M + H]^+$ ion at *m/z* 1043.5080, the dehydrated ion $[M - H_2O + H]^+$ at *m/z* 1025.5511, and the mono-sodiated ion $[M + Na]^+$ at *m/z* 1065.4900, which are compatible with a molecular formula of ichthyopeptin A of $C_{53}H_{70}N_8O_{14}$ (calcd for $[M + H]^+$ 1043.5090). Amino acid analysis identified five residues (Gln or Glu, Tyr, Val, Ile, and Thr) in equal ratios in ichthyopeptin A (**1**).

The assignment of the NMR spectra of **1** was accomplished using a combination of homonuclear 2D NMR techniques. Spin systems were identified from 2D ^{1}H COSY and TOCSY spectra, starting from the backbone amide protons. Sequence specific assignments were determined from the cross-peaks in 2D ^{1}H ROESY spectra based on short observable distances between H_N, H_α, and H_β of amino acid *i* and H_N of amino acid *i*+1, and from three-bond $^{13}C-^1H$ correlations in 2D HMBC spectra. The full assignments and chemical shift data are presented in Table 1. Using these techniques, spin systems corresponding to Gln, Tyr, Val, Phe, Ile, and Thr were identified in **1**. These were confirmed from the long-range correlations in the 2D HMBC spectrum, which indicated the phenylalanine residue carried an N-methyl group and were compatible with amino acid analysis of the hydrolysate. The same spectra indicated a spin system that showed characteristic ^{1}H and ^{13}C chemical shifts and

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Table 1. ^{1}H and ^{13}C NMR Data of Ichthyopeptin A (**1**) in CD_3CN/D_2O (1:1)

unit	position	δ_H	δ_C	^{1}H correlations to ^{13}C intra-residue; inter-residue	sequential NOEs in ^{1}H ROESY[b]
PAA	1		175.7	2, 3A, 3B; Gln-2	
	2	4.19	73.5		
	3	2.70, 2.90	40.0		Gln-NH[a] (×2)
	4		129.3		
	5, 9	7.01	131.6		
	6, 8	6.67	115.9		
	7		155.5 or 155.7		
Gln	NH	7.71			PAA-3 (×2), Thr-NH[a]
	1		173.2	2, 3; Thr-2, Thr-NH[a]	
	2	4.27	53.0		Thr-NH[a]
	3	1.81	28.3 or 30.3		Thr-NH[a]
	4	1.94, 2.03	31.7		
	5		177.9	3, 4A, 4B	
	NH_2[a]	6.46, 7.14			
Thr	NH[a]	8.09			Gln-2, Gln-3, Gln-NH[a]
	1		171.2	2; Tyr-NH[a]	
	2	4.47	56.1		
	3	5.45	73.5	Ahp-NH[a]	Tyr-NH[a]
	4	1.18	18.9		
Tyr	NH[a]	8.11			Ahp-NH[a], Thr-3
	1		172.9	Ahp-NH[a]	
	2	4.51	56.4		
	3	2.64, 3.32	35.9		
	4		129.6		
	5,8	7.00	130.9		
	6,8	6.65	116.1		
	7		155.7 or 155.5		
Ahp	NH[a]	7.75		Thr-3	Tyr-NH[a]
	1		171.5		
	2	4.53	50.3		Val-4
	3	1.81, 2.54	21.6		
	4	1.81	~30.3		
	5	4.98	75.5		
Val	1		172.2	2; N-CH_3 Phe	
	2	4.29	57.7		Phe-2, Phe-3A, Phe-5,9
	3	1.93	28.2		
	4	0.45	18.1		Ahp-2
	5	−0.19	18.6		Phe-5,9, Phe-6,8
N-Me Phe	1		171.7	3A, 3B; Ile-NH[a]	
	2	4.99	62.5	N-Me	Ile-NH[a], Val-2
	3	2.73, 3.36	34.7		Ile-NH[a] (×2), Val-2, Ile-2
	4		138.0		
	5,9	7.20	130.6		Val-2, Val-5
	6,8	7.25	129.9		Val-5
	7	7.16	127.9		
	N-Me	2.73	31.6		
Ile	NH[a]	7.89			Phe-2, Phe-3
	1		174.8	2; Thr-3	
	2	4.36	57.5		Phe-3A
	3	1.74	37.5		
	4	1.03, 1.26	25.7		
	5	0.73	10.6		
	6	0.80			

[a] From the CD_3CN/H_2O (1:1) spectrum. In all cases the NH always shows a correlation to the directly bonded carbonyl carbon. [b] Each partner is indicated; hence each correlation appears twice in this column.

COSY correlations of the 3-amino-6-hydroxy-2-piperidone (Ahp) system that has been documented in other peptides from similar cyanobacteria.[5−8] Finally, the presence of a further Phe-like system was evident in which the α-H at δ_H 4.19 was attached to a carbon with a shift of δ_C 73.5 indicative of a 2-hydroxy-3-(4 -hydroxyphenyl)acetic acid (PAA) derivative. This was confirmed from mass spectrometric data. A combination of these structural elements was compatible with the molecular mass of 1042.

Careful inspection of the NOE correlations in the 2D ROESY spectrum afforded the partial sequence NH-Tyr-Ahp-Val-(N-Me-Phe)-Ile-CO (Table 1). Long-range correlations in the HMBC spectrum indicated the sequence NH-Gln-Thr-Tyr and showed that Ile was attached to the β-hydroxyl group of Thr to form a cyclic lactone system. Finally the PAA was found to be the acyl residue attached to the peptidic NH of Gln through the observation of a correlation of Hα of Gln with the CO of PAA. These data afforded the cyclic peptide structure shown in the Figure 1. Furthermore, a detailed analysis of the ESIMS/MS (Table 2) confirmed the sequence assignment. At this stage it was necessary to carefully look at the long-range correlations in both CD_3CN/H_2O (1:1) and CD_3CN/D_2O (1:1) to establish the mode of attachment of the PAA residue to the Gln residue. From the observation of long-range correlations to the two carbonyl carbons at δ 173.2 and 177.9, the mode of attachment of the PAA residue to the Gln residue was established as shown in **1**. Enantioselective GC-MS analysis indicated the five regular amino acids (Gln, Tyr, Val, Ile, and Thr) had the L-configuration. Appropriate reference material was not available for the three remaining moieties, although their configurations can be inferred from the absolute stereochemistries reported in the literature for N-Me-Phe[8,9] and Ahp and PAA[8,10] in related depsipeptides.

A similar approach afforded the structure of the major component of fraction M2-4-P2, ichthyopeptin B (**2**). The positive ion HRESIMS of **2** identified the $[M + H]^+$ ion at *m/z* 979.5160,

Table 2. MS−MS Fragmentation of Ichthyopeptin A (**1**)

m/z	fragment[a]
134.11	F
195.13	X-V(-H_2O)
293.22	Y*-Q(-H_2O)
356.23	X-V-F(-H_2O)
376.19	Y*-Q-T(-2H_2O)
487.34	X-V-F-L
519.31	Y-X-V-F(-H_2O)
539.26	Y*-Q-T-Y(-2H_2O)
650.41	Y*-X-V-F-L
669	Y*-Q-T-L-F
670	Y*-Q-T(-Y)-L
715.44	Y*-Q-T-Y-X-V(-4H_2O)
733.45	Y*-Q-T-Y-X-V(-3H_2O)
751	Y*-Q-T-Y-X-V(-2H_2O)
831.46	Y*-Q-T(-Y)-L-F
894.49	Y*-Q-T-Y-X-V-F(-3H_2O)
912.50	Y*-Q-T-Y-X-V-F(-2H_2O)
1025.58	$[M + H - H_2O]^+$
1043.59	$[M + H]^+$

[a] For convenience the following abbreviations are used for the residues: Y* = PAA, Q = Gln, T = Thr, Y = Tyr, X = Ahp, V = Val, F = N-Me Phe, I = Ile, N = Asn, L = Leu.

$[M - H_2O + H]^+$ at *m/z* 1067.4955, and $[M + Na]^+$ at *m/z* 1001.4960, which are compatible with the molecular formula $C_{49}H_{70}N_8O_{13}$. Five amino acids, Asn or Asp, Leu, Ile, Val, and Thr, were identified from the amino acid analysis of **2** and were confirmed from the NMR data (Table 3). The latter also indicated the presence of spin systems belonging to N-Me-Phe, Ahp, and PAA moieties, suggesting a further cyclic depsipeptide related to **1**. The absolute stereochemistries of **2** have not been determined but can be inferred from those found for **1**. NOE and inter-residue long-range C−H correlations (Table 3) identified two sequences, Ile-NMePhe-Val and Asn-Thr-Leu. The absence of an amidic proton for the Thr residue suggested this residue was attached to Ahp. As in **1** the low-field shift of H-3 of Thr indicated acylation at this position through binding the free carboxylic acid group of Val or PAA. Only the former is compatible with the attachment of the PAA moiety on Asn as shown for **2**. This was confirmed from the detailed interpretation of the MS−MS fragmentation data shown in Table 4.

3-Amino-6-hydroxy-2-piperidone (Ahp)-containing cyclic depsipeptides are widely distributed in cyanobacteria.[7] Recently, Yamaki et al.[8] published the structure of micropeptins 88-N and 88-Y from *Microcystis aeruginosa* NIES-88, which have a cyclic moiety in common with the present structure of ichthyopeptin A (**1**). The difference is the nature of the chain attached to the amide nitrogen of the Thr moiety. Both ichthyopeptins A (**1**) and B (**2**) possess the Ahp unit, which is also found in those micropeptins. In addition, the sequence Tyr-Ahp-Val-NMePhe-Ile-Thr found in the micropeptins is also present in ichthyopeptin A (**1**). However, there are differences between the micropeptins and ichthyopeptins, as N-butyl-Leu or N-acetyl-Tyr in the micropeptins is substituted by the PAA unit in the ichthyopeptins. The position of Val and Ile in **1** is the same as in both micropeptins; in **2** the position of these amino acids is interchanged.

Most of the described Ahp-containing compounds exhibit serine proteases inhibitory activity. However, the fraction containing **1** and **2** did not show any trypsin inhibitory activity. This is compatible with the data of Yamaki et al.,[8] who have shown that the residue linked to Ahp is responsible for regulating the trypsin inhibitory activity. If the residue is Tyr or Phe, HcAla, Leu, HTyr, Glu, or HSer, then the compound does not possess trypsin inhibitor activity but may be able to inhibit other serine proteases such as chymotrypsin. Clearly the Tyr and Leu residues linked to the Ahp unit in ichthyopeptins A (**1**) and B (**2**) fall into the non-trypsin inhibitory category.

However, the fraction containing both cyclic depsipeptides exhibited a strong antiviral activity against influenza A virus with an IC_{50} value of 12.5 μg mL^{-1}, essentially at the same level as amantadine, with 15 μg mL^{-1}. To date, antiviral activities reported for cyanobacteria are based on sulfated polysaccharides,[11] indolocarbazoles,[12] chlorine-containing alkaloids,[13] and proteins such as cyanovirin-N[14]. This protein, consisting of 101 amino acids, is a fusion inhibitor of HIV with virucidal activity by multivalent interactions with high mannose oligosaccharides comprising the HIV glycoprotein envelope and is a potential candidate for preventing HIV transmission.[14] To our knowledge, there are no reports on the antiviral activity of Ahp-containing cyclic peptides. However, since the processing of virus proteins is an essential step in the life cycle of influenza virus in order to generate infective virus particles, our results indirectly suggest that the mode of action of the ichthyopeptins may be based on a protease inhibition.

Experimental Section

General Experimental Procedures. All 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, ROESY, HMQC, and HMBC) NMR spectra were recorded at 300 K on a Bruker AVANCE DMX-600 NMR spectrometer locked to the major deuterium resonance of the various solvent systems used, which included CD_3CN/H_2O (1:1) and CD_3CN/D_2O (1:1). Measurements were carried out with mixing times period of 100 ms for TOCSY and 500 ms for ROESY. Chemical shifts were referenced to the residual proton resonance of the acetonitrile signal at δ 1.93 (^1H) and δ 1.28 (^{13}C). Mass spectra were recorded on a Micromass Q-Tof 2 mass spectrometer. Amino acid analysis was performed on an Applied Biosystems ABI-420-A amino acid analyzer.

Column chromatography was carried out on silica gel (Si 60, 0.040−0.063 mm, Merck, Germany). Fractions were monitored by TLC (Si 60 GF 254 nm, Merck, Germany) with *n*-PrOH/EtOAc/H_2O (7:2:1) or EtOAc/MeOH/H_2O (100:13.5:10) as mobile phases. Detection was done under UV light at 254 nm or by spraying with anisaldehyde/sulfuric acid reagent and heating. Analytical and preparative HPLC were performed on a component system (Kontron Instruments, Italy), consisting of pumps 422 and 422 S, auto sampler 360, and diode array detector DAD 440. A Synergi POLAR-RP column, 4.6 × 250 mm, 4 μm, 80 Å (Phenomenex, USA), and a gradient of deionized H_2O (Clear UV plus SG, Water Preparation and Recycling GmbH, Germany) and MeCN (gradient grade, ROTH, Germany) from 100% H_2O to 100% MeCN in 30 min was used for analytical HPLC with a flow rate of 1.0 mL min^{-1}. Preparative HPLC was performed on an equivalent POLAR-RP column (10 × 250 mm) with a flow rate of 3 mL min^{-1}. HPLC runs were recorded using the Geminyx HPLC data system 1.91 SST version 1.6. All chemicals were used as received, and solvents were distilled prior to use except for HPLC.

Culture Conditions. The cyanobacterium *Microcystis ichthyoblabe* strain BM Mi/13 was isolated from a sample of water collected from the Passader See, Schleswig-Holstein, Germany, and established as laboratory culture by Dr. Barbara Meyer (Max Planck Institute for Limnology, Plön). The strain is maintained in the culture collection of the Institute of Pharmacy, EMAU Greifswald, as a stock culture. The cyanobacterium was cultured in a glass column containing 40 L[15] of MBL medium.[16] The growth was routinely monitored by measuring the optical density at 730 nm using a spectrometer (Uvicon 930, Kontron Instruments, Italy). After 28 days the cells were harvested by centrifugation at 6500 rpm in a continuous flow centrifuge (Stratos, Heraeus Instruments, Germany), lyophilized, and kept at −20 °C until used. The yield of lyophilized biomass was 0.2 g L^{-1}.

Extraction and Isolation. The lyophilized cells (5 g) of *M. ichthyoblabe* were successively extracted three times with 250 mL of *n*-hexane followed by MeOH under stirring for 1 h, respectively. After separation by centrifugation at 4500 rpm at 4 °C for 10 min the methanolic supernatants were pooled and evaporated to provide a crude extract of about 0.5 g. The crude methanolic extract (500 mg) was separated on Si gel [open column, 3 × 40 cm, flow rate 2 mL min^{-1} realized by a VAC V-500 vacuum pump (Büchi, Switzerland)] using a stepwise gradient of EtOAc/MeOH/H_2O, each 250 mL [100% EtOAc (M-1), 75% EtOAc in MeOH (M-2), 50% EtOAc in MeOH (M-3), 25% EtOAc in MeOH (M-4), 100% MeOH (M-5), 75% MeOH in H_2O

Table 3. ^1H and ^{13}C NMR Data of Ichthyopeptin B (**2**) in CD_3CN/H_2O (1:1)

unit	position	δ_H	δ_C	^1H correlations to ^{13}C intra-residue; inter-residue	sequential NOEs in ^1H ROESY^b
PAA	1		a		
	2	4.20	73.8		
	3	2.68, 2.98	40.2		
	4		129.8		
	5, 9	7.05	131.4		
	6, 8	6.68	116.0		
	7		156.1		
Asn	NH	8.05			
	1		173.0	2; Thr-2	
	2	4.72	50.5		Thr-NH
	3	2.70	37.3		
	4				
	NH_2	6.51, 7.24			
Thr	NH^a	7.95			Asn-2
	1		171.6	2	
	2	4.63	56.5		Leu-NH
	3	5.50	73.7	Ahp-NH	Leu-NH
	4	1.27	18.7		
Leu	NH	8.03			Thr-2, Thr-3
	1		a		
	2	4.30	53.6		
	3	1.53, 1.80	39.9		
	4	1.51	25.4		
	5	0.86	23.4		
	6	0.75	21.0		
Ahp	NH	7.72		Thr-3	
	1		a		
	2	4.50	50.2		
	3	1.80, 2.55	21.9		
	4	1.80	30.4		
	5	4.99	75.7		
Ile	1		172.5	2; N-CH_3 Phe	
	2	4.35	56.2		Phe-2
	3	1.74	33.8		
	4	1.04, 0.58	24.5		
	5	0.82	14.3		
	6	−0.25	14.6		
N-Me Phe	1		a		
	2	5.08	62.6		Ile-2, Val-NH
	3	2.72, 3.38	34.7		Val-NH, Val-4
	4				
	5,9	7.20	130.5		Val-2
	6,8	7.23	129.8		
	7	7.15	127.8		
	N-Me	2.74			
Val	NH	7.91			Phe-2, Phe-3
	1		a		
	2	4.30	59.0		Phe-5,9
	3	1.95	31.2		
	4	0.81	19.5		Phe-3
	5	0.75	19.0		

^{a} Cannot be unambiguously assigned. ^{b}Each partner is indicated; hence each correlation appears twice in this column.

(M-6), 50% MeOH in H_2O (M-7), 25% MeOH in H_2O (M-8), and 100% H_2O (M-9)]. Fraction M-2 (yield 28 mg of yellow oil) exhibited the highest anti-influenza activity. A portion of 60 mg of M-2 was further separated on Si gel [open column, 1.2 × 30 cm, flow rate 0.2 mL min^{-1}] with a step gradient from 100% EtOAc to 100% MeOH (steps of 10%, each 30 mL), and 11 fractions were collected according to the bands detected on TLC. All fractions were analyzed by HPLC with a linear gradient from 2% MeCN in H_2O to 100% MeCN in 25 min, flow rate 1 mL min^{-1}. Only fraction M-2-4 [eluted with EtOAc/MeOH (90:10) from Si gel, yield 2.9 mg of a yellowish oil] showed two detectable peaks, which were collected by preparative HPLC (46 runs, 500 µg/injection, flow rate 3 mL min^{-1}) with the same H_2O/MeCN gradient. The pure compounds ichthyopeptins A (**1**) and B (**2**) eluted at t_R = 17.93 min and t_R = 18.40 min, respectively. The collected peaks of 46 HPLC runs were combined, and after removing the solvent under reduced pressure 8.2 mg of ichthyopeptin A (**1**) and 7.4 mg of ichthyopeptin B (**2**) were obtained as white, amorphous solids.

Enantioselective Analysis of Amino Acids. Peptide was hydrolyzed using 6 N HCl at 100 °C for 16 h, conditions that result in the conversion of Gln to Glu. After drying, the resulting free amino acids were derivatized with 4 N HCl/propan-2-ol (1h, 110 °C), and after removal of reagents, the amino acid isopropyl esters were then acylated by pentafluoropropionic acid anhydride in CH_2Cl_2 (150 °C, 12 min). Excess reagents were again removed and the amino acid derivatives analyzed on a Chirasilval column (50 m) connected to a GCQ ion trap mass spectrometer. The constituent amino acids were identified by their characteristic mass spectra, and their enantiomerity was determined by comparison to standard D,L amino acids.

Antiviral Assay. MDCK cells, 4 × 10^4 in 200 µL of MEM with 5% FCS (Invitrogen GmbH, Germany), were seeded per well in 96-well tissue culture plates (Falcon, USA) and incubated in a humidified 5% CO_2 atmosphere for 24 h. Confluent monolayers were preincubated with 100 µL of medium containing the extract or the fractions in nontoxic concentrations (100, 50, 25, 12.5 µg mL^{-1}) for 30 min. Cells were infected with 30 $TCID_{50}$ of influenza virus A/WSN/33/London (H1N1) and incubated for 72 h. Antiviral effects were determined by the dye uptake assay using neutral red.[17] Cell controls without extract and virus controls were included. Antiviral activity was calculated as percentage of protection from virus-induced cell destruction in relation to infected cells without test compounds and the mock-infected control. The 50% inhibitory concentration (IC_{50}) was determined from graphic plotting, percent protection against extract concentrations.

Table 4. MS−MS Fragmentation of Ichthyopeptin B (**2**)

m/z	fragment[a]
134.11	F
209.15	X-L(-H_2O)
279.20	Y*-N(-H_2O)
362.17	Y*-N−T(-2H_2O)
370.25	X-L-F(-H_2O)
475.27	Y*-N-T-L(-2H_2O)
483.34	L-X-I-F(-H_2O)
493.28	X-I-F-V
622.35	L-X-I-F-V
640.35	Y*-N-T-V-F
683.46	Y*-N-T-L-X-I(-3H_2O)
701	Y*-N-T-L-X-I(-2H_2O)
753.44	Y*-N-T(-L)-V-F
844.49	Y*-N-T-L-X-I-F(-3H_2O)
862.50	Y*-N-T-L-X-I-F(-2H_2O)
961.57	$[M + H - H_2O]^+$
979.58	$[M + H]^+$

[a] For convenience the following abbreviations are used for the residues: Y* = PAA, Q = Gln, T = Thr, Y = Tyr, X = Ahp, V = Val, F = N-Me Phe, I = Ile, N = Asn, L = Leu.

Protease Inhibition Assay. Protease inhibitory effects were tested using trypsin and BAPNA (α-*N*-benzoyl-DL-arginine-*p*-nitroanilide) as substrate for colorimetric analysis at 405 nm.[18]

Ichthyopeptin A (1): white, amorphous powder; UV$_{\lambda max}$ (CH_3CN/H_2O) 205 and 276 nm; ^1H and ^{13}C NMR in CD_3CN/D_2O (1:1), see Table 1; MS−MS fragmentation, see Table 2; HRESIMS *m/z* 1043.5080 $[M + H]^+$, *m/z* 1025.4950 $[M - H_2O + H]^+$, and *m/z* 1065.4900 $[M + Na]^+$ (calcd for $C_{53}H_{70}N_8O_{14}$, 1043.5090, 1025.4984, and 1065.4909, respectively).

Ichthyopeptin B (2): white, amorphous powder; UV$_{\lambda max}$ (CH_3CN/H_2O) 205 and 276 nm; ^1H and ^{13}C NMR in CD_3CN/D_2O (1:1), see Table 3; MS−MS fragmentation, see Table 4; HRESIMS *m/z* 979.5160 $[M + H]^+$, *m/z* 961.5040 $[M - H_2O + H]^+$, and *m/z* 1001.4960 $[M + Na]^+$ (calcd for $C_{49}H_{70}N_8O_{13}$, 979.5141, 961.5035, and 1001.4960, respectively).

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References and Notes

(1) Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Burgess, J. G.; Wright, P. C. *Tetrahedron* **2001**, *57*, 9347−9377.
(2) Radau, G. *Pharmazie* **2000**, *55*, 555−560.
(3) Wegner, U.; Mundt, S.; Kliefoth, C.; Meyer, B.; Lindequist, U.; Mentel, R. *Hyg. Med.* **2000**, *25*, 75−78.
(4) Mundt, S.; Nowotny, A.; Mentel, R.; Lesnau, A.; Lindequist, U. *Pharm. Phamacol. Lett.* **1997**, *7*, 161−163.
(5) Nowotny, A.; Mentel, R.; Wegner, U.; Mundt, S.; Lindequist, U. *Phytother. Res.* **1997**, *11*, 93−96.
(6) Zainuddin, E. N.; Mundt, S.; Wegner, U.; Mentel, R. *Med. Microbiol. Immunol.* **2002**, *191*, 181−182.
(7) Radau, G. *Curr. Enzyme Inhib.* **2005**, *1*, 295−307.
(8) Yamaki, H.; Sitachitta, N.; Sano, T.; Kaya, K. *J. Nat. Prod.* **2005**, *68*, 14−18.
(9) Ploutno, A.; Shoshan, M.; Carmeli, S. *J. Nat. Prod.* **2002**, *65*, 973−978.
(10) Harada, K.; Mayumi, T.; Shimada, T.; Suzuki, M. *Tetrahedron Lett.* **1993**, *34*, 6091−6094.
(11) Hayashi, T.; Hayashi, K.; Maeda, M.; Kojima, I. *J. Nat. Prod.* **1996**, *59*, 83−87.
(12) Knübel, G.; Larsen, L. K.; Moore, R. E.; Levine, I. A.; Patterson, G. M. L. *J. Antibiot.* **1990**, *43*, 1236−1239.
(13) Larsen, L. K.; Moore, R. E.; Patterson, G. M. *J. Nat. Prod.* **1994**, *57*, 419−421.
(14) Tsai, C. C.; Emau, P.; Jiang, Y.; Agy, M. B.; Shattock, R. J.; Schmidt, A.; Morton W. R.; Gustafson, K. R.; Boyd, M. R. *AIDS Res. Hum. Retroviruses* **2004**, *20*, 11−18.
(15) Mundt, S.; Kreitlow, S.; Nowotny, A.; Effmert, U. *Int. J. Hyg. Environ. Health* **2001**, *203*, 327−334.
(16) Lorenzen, C. J.; Guillard, R. R. L. *J. Phycol.* **1972**, *8*, 10−14-
(17) Mothana, R. A. A.; Mentel, R.; Reiss, C.; Lindequist, U. *Phytotherapy* **2006**, *20*, 298−302.
(18) Pilgrim, H.; Haasmann, S.; Schröder, K. *Zentralbl. Mikrobiol.* **1992**, *147*, 400−402.

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6	www.jbc.org Internet Source	%1

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8	bmcstructbiol.biomedcentral.com Internet Source	%1
9	Alexei Ploutno, Maria Shoshan, Shmuel Carmeli. " Three Novel Protease Inhibitors from a Natural Bloom of the Cyanobacterium ", Journal of Natural Products, 2002 Publication	%1
10	Preisitsch, Michael, Kirsten Harmrolfs, Hang TL Pham, Stefan E Heiden, Anna Füssel, Christoph Wiesner, Alexander Pretsch, Monika Swiatecka-Hagenbruch, Timo HJ Niedermeyer, Rolf Müller, and Sabine Mundt. "Anti-MRSA-acting carbamidocyclophanes H–L from the Vietnamese cyanobacterium Nostoc sp. CAVN2", The Journal of Antibiotics, 2014. Publication	%1
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	Journal of the American Chemical Society, 2013 Publication	
12	dmd.aspetjournals.org Internet Source	<%1
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15	www.chemotaxis.usn.hu Internet Source	<%1
16	marine.rutgers.edu Internet Source	<%1
17	www.mdpi.com Internet Source	<%1
18	Tomoharu Sano, Jiawan He, Yongding Liu, Kunimitsu Kaya. "Isolation of bioactive compounds in cyanobacteria from Chinese fresh water. 1. Trypsin inhibitor", Phycological Research, 2006 Publication	<%1

19	patents.google.com Internet Source	<%1
20	Dwivedi, Deepti, Rolf Jansen, Gabriella Molinari, Manfred Nimtz, Bhavdish N Johri, and Victor Wray. "Antimycobacterial Serratamolides and Diacyl Peptoglucosamine Derivatives from *Serratia* sp.", Journal of Natural Products, 2008. Publication	<%1
21	Okino, Tatsufumi, Sun Qi, Hisashi Matsuda, Masahiro Murakami, and Katsumi Yamaguchi. "Nostopeptins A and B, Elastase Inhibitors from the Cyanobacterium *Nostoc minutum*", Journal of Natural Products, 1997. Publication	<%1
22	Wolfram Gronwald, Dietmar Schomburg, Michael P. F. Harder, Hubert Mayer, Jens Paulsen, Edgar Wingender, Victor Wray. "Structure of Recombinant Human Parathyroid Hormone in Solution Using Multidimensional NMR Spectroscopy", Biological Chemistry Hoppe-Seyler, 1996 Publication	<%1
23	Zainuddin E., Mundt S., Wegner U., Mentel R.. "Cyanobacteria a potential source of antiviral substances against influenza virus", Medical Microbiology and Immunology, 2002 Publication	<%1

24	limnology.ch Internet Source	<%1
25	dadospdf.com Internet Source	<%1
26	Muftah A. M. Shushni. "Balticols A-F, New Naphthalenone Derivatives with Antiviral Activity, from an Ascomycetous Fungus", Chemistry & Biodiversity, 02/2009 Publication	<%1
27	Mundt, S.. "Biochemical and pharmacological investigations of selected cyanobacteria", International Journal of Hygiene and Environmental Health, 2001 Publication	<%1
28	www.proteinscience.org Internet Source	<%1
29	www.j3.jstage.jst.go.jp Internet Source	<%1
30	mdpi.com Internet Source	<%1
31	TORSTEN FEDERAU. "Solution structure of the cytoplasmic domain of the human immunodeficiency virus type 1 encoded virus protein U (Vpu)", International journal of peptide & protein research, 01/12/2009 Publication	<%1

32	Stefanie Klein. "Adaptation of *Pseudomonas aeruginosa* to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol", Molecular Microbiology, 02/2009 Publication	<%1
33	Sabine Mundt, Susann Kreitlow, Andrea Nowotny, Uta Effmert. "Biochemical and pharmacological investigations of selected cyanobacteria", International Journal of Hygiene and Environmental Health, 2001 Publication	<%1
34	Susan Matthew, Cliff Ross, James R. Rocca, Valerie J. Paul, Hendrik Luesch. " Lyngbyastatin 4, a Dolastatin 13 Analogue with Elastase and Chymotrypsin Inhibitory Activity from the Marine Cyanobacterium ", Journal of Natural Products, 2007 Publication	<%1
35	Submitted to University of Hong Kong Student Paper	<%1
36	ec.asm.org Internet Source	<%1
37	wgbis.ces.iisc.ernet.in Internet Source	<%1

Kiyonaga Fujii, Kaarina Sivonen, Emiko

38	Naganawa, Ken-ichi Harada. "Non-Toxic Peptides from Toxic Cyanobacteria, Oscillatoria agardhii", Tetrahedron, 2000 Publication	<%1
39	sundoc.bibliothek.uni-halle.de Internet Source	<%1
40	He, Z.D.. "Rourinoside and rouremin, antimalarial constituents from Rourea minor", Phytochemistry, 200607 Publication	<%1
41	Marina Lifshits, Shmuel Carmeli. " Metabolites of Bloom Material from Lake Kinneret, Israel ", Journal of Natural Products, 2012 Publication	<%1
42	Yamaki, Hiroshi, Namthip Sitachitta, Tomoharu Sano, and Kunimitsu Kaya. "Two New Chymotrypsin Inhibitors Isolated from the Cyanobacterium *Microcystis aeruginosa* NIES-88", Journal of Natural Products, 2005. Publication	<%1
43	George R. Pettit, John C. Knight, Delbert L. Herald, Robin K. Pettit et al. " Antineoplastic Agents. 570. Isolation and Structure Elucidation of Bacillistatins 1 and 2 from a Marine ", Journal of Natural Products, 2009 Publication	<%1
	Alberto Plaza, Carole A. Bewley. " Largamides	

44	A−H, Unusual Cyclic Peptides from the Marine Cyanobacterium sp. ", The Journal of Organic Chemistry, 2006 Publication	<%1
45	Michael Preisitsch, Timo H. J. Niedermeyer, Stefan E. Heiden, Inga Neidhardt et al. " Cylindrofridins A–C, Linear Cylindrocyclophane-Related Alkylresorcinols from the Cyanobacterium ", Journal of Natural Products, 2015 Publication	<%1

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