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Hofmannolin, a cyanopeptolin from *Scytonema hofmanni* PCC 7110

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Abstract

Two depsipeptide metabolites, scyptolin A and B, were reported recently from the axenically grown terrestrial cyanobacterium *Scytonema hofmanni* PCC 7110. A related, novel depsipeptide was isolated from this *Scytonema* and designated hofmannolin. The amino acid analysis in context with infrared, mass and $^{1}H/^{13}C$-NMR spectroscopies revealed a cyclic depsipeptide structure of M_r 1073 belonging to the class of cyanopeptolins. Two peculiar features distinguish hofmannolin from other cyanopeptolins: *O*-methylated tyrosine forms the sixth moiety from the amino terminus, and the N-terminus is blocked by 2-hydroxy-3-methyl-valeric acid, a residue that has not yet been reported as a component in other cyanopeptolins. Preliminary assays of peptidase inhibitory and antimicrobial activities suggested negligible bioactivities for hofmannolin.

Keywords: *Scytonema hofmanni*; Cyanobacteria; Cyanopeptolins; Cyclic depsipeptides; Hofmannolin

1. Introduction

Cyanobacteria are a rich source of unusual polypeptide or hybrid polypeptide–polyketide metabolites (Kreitlow et al., 1999), which are synthesized by non-ribosomal polypeptide and/or polyketide synthase complexes as demonstrated for the microcystins or the anabaenopeptilides (Tillett et al., 2000; Rouhiainen et al., 2000). Among the linear polypeptides the cyanophycins have been in the focus of recent biosynthetic studies which identified isoenzymes of the arginine pathway as regulatory components (cf. Oppermann-Sanio and Steinbüchel, 2002). Besides the physiological function as a temporary nitrogen reserve, cyanophycin accumulation has been correlated with salt tolerance in *Scytonema* (Page-Sharp et al., 1998) and other species (Zuther et al., 1998). Numerous other peptide metabolites were identified from cyanobacteria (Burja et al., 2001), and sophisticated bioassays and pharmacological testing revealed various features of potential commercial interest, e.g. antimicrobial activity (Østensvik et al., 1998), specific enzyme inhibition (Radau, 2000) and immunosuppressive (Mundt et al., 1991) or even antitumour activities (Takahiro et al., 1999). This has been exemplified recently with dolastatin and its analogues which reached the stage of human clinical trials for the treatment of cancer (Luesch et al., 2002; Nogle and Gerwick, 2002).

A relatively high proportion of the cyanobacteria accumulate peptide metabolites in the form of cyclic depsipeptides, and some of these compounds also show remarkable bioactivities. Early investigations focused on their toxic potential after livestock lethality and severe human intoxications had incurred (Chorus and Bartram, 1999). Much effort has been spent since on the molecular basis of toxicity, and a large number of toxic metabolites has been characterised. Prominent examples are the microcystins which accumulate in and affect the liver specifically inhibiting protein phosphatases (Harada, 1999). Recently, two new protein phosphatase inhibitors, oscillamid B and C, belonging to the class of anabaenopeptins were isolated from the toxic cyanobacteria *Oscillatoria agardhii* and *Oscillatoria rubescens* (Sano et al., 2001). This search is still in progress and will likely yield many more cytotoxic compounds like

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the obyanamide from *Lyngbya* species (Willams et al., 2002), in addition to alkaloids such as the neurotoxic aphantoxin A or the lipopeptide antillatoxin B (Nogle et al., 2001). A particular group of cyclic depsipeptides is being subsumed as cyanopeptolins (Weckesser et al., 1996) which typically form their ring by *endo*-esterification of a Thr hydroxyl and contain a cyclic glutamaldehyde residue designated Ahp. The cyanopeptolins are furthermore characterised by the *cis*-configuration of the upstream peptide bond joining Ahp to the proximal amino acid. Several cyanopeptolins were reported to be specific inhibitors of serine or cysteine proteases. Two cyanopeptolins, scyptolin A and B, were recently characterized from axenic cultures of *Scytonema hofmanni* PCC 7110 (Matern et al., 2001), and we present in this report a third cyanopeptolin from these cultures including the results from preliminary bioassays.

2. Results

2.1. Isolation of hofmannolin

Lyophilized *Scytonema hofmanni* PCC 7110 that had been grown axenically for 4 weeks under continuous irradiation was extracted with 75% aqueous methanol, and the crude extract was fractionated on Sephadex LH20, Chromabond C_{18}, QMA anion exchange and C_{18}-cartridges followed by RP-HPLC under isocratic conditions in acidic aqueous acetonitrile yielding a single major compound. The purity of this compound, designated hofmannolin, was confirmed by analytical HPLC using a step gradient elution according to Lawton et al. (1994). On average, 6–8 mg of pure hofmannolin were collected from 7–9 g of lyophilized *Scytonema* cell mass.

2.2. Structural analysis

The absorbance of a methanolic solution of hofmannolin with maxima at 223.7 and 277.4 nm pointed to the presence of aromatic residues, and the amino acid analysis revealed Thr, Tyr, Val and Glu in stoichiometric amounts. Taking into account the solubility in methanol, the data suggested a cyclic peptide or a cyanopeptoline. The chirality of these amino acids was not examined experimentally, but their L-configuration can be assumed in accordance with the amino acids identified in scyptolin A or B (Matern et al., 2001) and other cyclic depsipeptides (Jacobi et al., 1995). The banding pattern on IR spectroscopy was fully compatible with this assumption showing NH and OH stretchings as well as aromatic CH stretchings in the range of 3363–3060 cm^{-1}, ester and amide carbonyl stretchings at 1736 and 1666 cm^{-1} besides aliphatic CH stretchings.

MALDI-TOF-MS of hofmannolin in the positive-ion-mode generated three prominent ions at *m/z* 1096.6 (base peak), which is considerably larger than expected for the four amino acids detected by amino acid analysis, at *m/z* 1078.6 (25% rel. intensity) and 1056.6 (17% rel. intensity). The signals can be assigned to the ions resulting from sodium or proton addition and loss of water (*m/z* 1096.6, $[M+Na]$; *m/z* 1078.6, $[MH-H_2O+Na]$; *m/z* 1056.6, $[M-H_2O+H]$). Further ESI-HRMS of hofmannolin which was repeated ten times revealed an experimental mass of 1096.4839 Da (arithmetic means) for the $[M+Na]^+$ ion with a standard deviation of 0.00078 Da, corresponding to a composition of $C_{54}H_{71}NaN_7O_{16}$ (calculated 1096.4855 Da). Fragments inferred from post source decay spectra (Fig. 1) suggested the additional presence of Ahp, *O*-methyl-Tyr, *N*-methyl-Tyr and a hydroxymethylvaleroyl residue. The fragments were compared with a data bank collection of over 2000 cyanobacterial post source decay peptide fragments (Erhard et al., 1997; Fastner et al., 2001), leading to the provisional assignment of a structure for hofmannolin (Fig. 1).

The ^{1}H-NMR of hofmannolin revealed 69 protons resonating in the range from 9.5 to 0.5 ppm and 1–2 broad protons between 10 and 12 ppm (Table 1). HSQC correlations and integration indicated 7 methyl, 8 methylene and 12 methine groups in addition to 12 aromatic protons, leaving 10–11 protons unattached to carbon. The latter protons were assigned to 5 NH groups (doublets in the range 8.5–7.0 ppm), 1 phenolic OH (9.32 ppm) and 2 alcoholic OH groups (5.96 and 5.44 ppm). The broad resonances at 10–12 ppm correspond to phenolic and/or acidic protons. Among the methyl groups, one primary (triplet), four secondary (doublets) and one *N*-methyl group (singlet at 2.73 ppm, resonating at 30.6 ppm on ^{13}C-NMR) and one aromatic methoxy group (singlet at 3.70 ppm, 55.1 ppm on ^{13}C-NMR) were distinguished. All 8 methylene groups are attached to carbons as indicated by their ^{1}H and ^{13}C shifts (Table 1). The ^{13}C correlation of the methine groups revealed that 3 are attached to oxygen and 7 to nitrogen. The 12 aromatic protons, 8 of which resonate in 4 doublets and 4 form a singlet, represent three *p*-disubstituted aromatic rings and are assigned to tyrosine residues.

The following *N*-acylated amino acids were identified from the spinsystems in the TOCSY and COSY and in accordance with their H–C correlations in the HSQC and HMBC spectra: Glu, Tyr, Val, *O*-acylated Thr, *N*-methylated Tyr, *N*-alkylated/*O*-methylated Tyr and the Ahp ring system (Radau, 2000). The broad resonances at 10–12 ppm are assigned to the carboxylic proton of Glu^2 and the phenolic proton of Tyr^4 (Fig. 2). The configuration of the Ahp residue was inferred from NOEs and corresponds to the configuration observed in other cyanopeptolins (Martin et al., 1993). The *N*-alkylation of the Thr residue was supported further by the NOE correlation (double-headed arrow in Fig. 2,

sequence								ion	m/z
Tyr	Ahp	omTyr	nmTyr	Val	Thr	Glu	Hmv	M+H	1075
								M-H_2O+H	1057
Tyr	Ahp		nmTyr	Val	Thr	Glu	Hmv	M+H	897
Tyr	Ahp	omTyr	nmTyr	Val	Thr			M-H_2O+2H	814
								M-CO+2H	803
Tyr			nmTyr	Val	Thr	Glu	Hmv	M+H	784
								M-CO	756
Tyr	Ahp			Val	Thr			M-CO+H	625
			nmTyr	Val	Thr	Glu	Hmv	M+H	621
	Ahp	omTyr						M-H_2O+H	273
		omTyr	nmTyr					M	354
								M-CO	326
	Ahp	omTyr	nmTyr	Val	Thr	Glu	Hmv	M-H_2O+H	950
						Glu	Hmv	M	245
				Val	Thr			M+H	200
			nmTyr					immonium ion	150
Tyr								side chain	107
						Glu		immonium ion	102
				Val				immonium ion	72

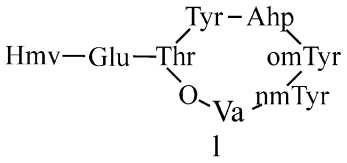

Fig. 1. PSD mass fragments and proposed composition of hofmannolin. Hmv, 2-hydroxy-3-methylvaleric acid; Ahp, 3-amino-6-hydroxy-2-oxo-1-piperidine; omTyr, *O*-methyl-Tyr; nmTyr, *N*-methyl-Tyr.

Table 1). Finally, the spin system of a 2-hydroxy-3-methyl-valeroyl (Hmv) residue was established. This Hmv moiety is attached to the N-terminus of the amino acid sequence, which could be deduced from sequential proton NOEs like α-$N_{i,\ i+1}$, β-$N_{i,\ i+1}$, δ-$\beta_{i,\ i+1}$ or α–$\alpha_{i,\ i+1}$ (Table 1). The α–α NOE between Tyr^6 and $mTyr^7$ indicates a *cis*-amide bond between these two amino acids. All other amide bonds show *trans*-configuration, and the sequential NOEs (Table 1) are pointed out by double-headed arrows in Fig. 2. No NOE correlation is observed across the ester bridge between Val^8 and Thr^3, while the HMBC correlations (dotted arrows, Fig. 2) supported the Thr^3–Val^8 ester linkage. The NMR data of hofmannolin are fully compatible with the composition of $C_{54}H_{71}N_7O_{16}$ derived from HRMS and amounting to a nominal M_r of 1073. Due to the marginal difference of 1.6 mmu between the experimental and theoretical mass units the molecular formula of hofmannolin can be unequivocally assigned (Fig. 2).

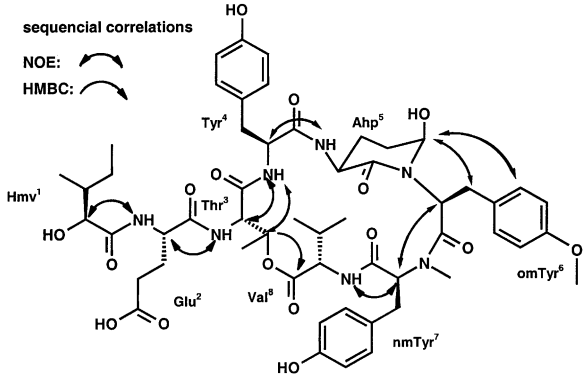

Fig. 2. Structure of hofmannolin. Hmv, 2-hydroxy-3-methylvaleric acid; Ahp, 3-amino-6-hydroxy-2-oxo-1-piperidine; omTyr, *O*-methyl-Tyr; nmTyr, *N*-methyl-Tyr.

2.3. Bioassays

Hofmannolin was examined for eventual bioactivities in assays monitoring the inhibition of serine- and cysteine-type proteases or the antibiotic efficiency as had been done before with the scyptolins (Matern et al., 2001). Under the conditions employed (up to 25 μg hofmannolin per ml in the assay), the activities of elastase, plasmin, thrombin, trypsin or of papain were not inhibited to a significant extent. Since the search for novel antibiotics is a permanent problem in human therapy and some cyanobacterial cyclic depsipeptides had been shown to possess antibiotic activity (Skulberg, 2000), the effect of hofmannolin on the growth of representative Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Corynebacterium diphteriae*, *Bacillus subtilis* or the aquatic *Micrococcus luteus*) as well as of *Candida albicans* was examined in agar diffusion tests. However, in all of these tests the growth rate of cells was hardly affected by hofmannolin.

3. Discussion

Cyanobacteria produce numerous depsipeptides, among which the cyanopeptolins form a structurally distinct group of metabolites. These peptides partially cyclize to a lactone by reaction of the C-terminal carboxyl with the hydroxyl group of an internal Thr residue and contain an unusual Ahp residue corresponding to a cyclized Glu-semialdehyde (Weckesser et al., 1996). Their N-terminus is typically blocked by aromatic acids, e.g. 4-hydroxyphenyllactic acid, medium chain-length aliphatic acids (hexanoic, octanoic acids), or more

Table 1
NMR data of hofmannolin

Amino acid	Pos.	δ^{13}C mult.	δ^{1}H mult., *J* (Hz)	LR H–C correlations[a]	NOE-correlations[a]
Hofmannolin					
Hmv[1]	1	173.8 *s*		Glu[2]-NH(weak)	
	2	75.3 *d*	3.75 *s broad*		Glu[2]-NH
	3	38.5 *d*	1.70 *m*		
	4	24.0 *t*	1.40/1.14 *2*x *m*		
	5	12.0 *q*	0.81 *t 7.5*		
	6	15.8 *q*	0.97 *d* 7.0		
	OH		5.44 *s broad*		
Glu[2]	1	172.1 *s*		Thr[3]-2, Glu[2]-3	
	2	52.5 *d*	4.37 *m*		Thr[3]-NH
	3	28.5 *t*	1.78/1.85 *2*x *m*		
	4	31.5 *t*	2.08 *m*		
	5	174.2		Glu[2]-3,4 (weak)	
	5-OH		9-11 *broad*		
	NH		7.99 *d*, 8.0	Hmv[1]-1	Hmv[1]-2, Thr[3]-NH
Thr[3]	1	168.8 *s*		Thr[3]-2, Tyr[4]-NH	
	2	54.5 *d*	4.54 *d,* 9.5		Tyr[4]-NH
	3	72.3 *d*	5.36 *q,* 6.6		Tyr[4]-NH
	4	17.8 *q*	1.08 *d*, 6.6		
	NH		7.70 *broad*		Glu[2]-2, Glu[2]-NH
Tyr[4]	1	170.0 *s*		Ahp[5]-NH	
	2	53.9 *d*	4.32 *m*		Ahp[5]-NH
	3	35.3 *t*	3.13/2.45 *2*x *m*		
	1′	128.0 *s*			
	2′/6′	129.8 *d*	6.87 *d*, 8.3		
	3′/5′	115.4 *d*	6.55 *d*, 8.3		
	4′	156.5 *s*			
	NH		8.45 *d*, 9.0	Thr[3]-1	Thr[3]-2,3, Ahp[5]-NH
	OH		9-11 *broad*		
Ahp[5]	1	170.2 *s*		omTyr[6]-2 (weak)	
	2	49.0 *d*	3.69 *m*		
	3	21.8 *t*	2.41/1.62 *2*x *m*		
	4	29.6 *t*	1.58/1.69 *2*x *m*		
	5	73.9 *d*	5.03 *s*		omTyr[6]-2′/6′
	NH		7.07 *d*, 9.0	Tyr[4]-1	Tyr[4]-NH,1, Thr[3]-3
	5-OH		5.96 *d*, 1.7		Val[8]-NH,5
Tyr[6]	1	170.8 *s*		nmTyr[7]-NCH_3	
	2	50.7 *d*	4.67 *m*		nmTyr[7]-2,2′/6′
	3	34.7 *t*	2.77/1.71 *2*x *m*		Ahp[5]-5
	1′	128.8 *s*			
	2′/6′	130.6 *d*	6.74 *s*		Ahp[5]-5
	3′/5′	113.4 *d*	6.74 *s*		Tyr[6]-OCH_3
	4′	157.9 *s*		omTyr[6]-OCH_3	
	OCH_3	55.1 *q*	3.70 *s*	omTyr[6]-4′	omTyr[6]-3′/5′
mTyr[7]	1	169.6 *s*		Val[8]-NH, Tyr[7]-2	
	2	61.1 *d*	4.89 *d*, 11.4		omTyr[6]-2, Val[8]-NH
	3	33.2 *t*	3.04/2.69 *2*x *m*		
	1′	127.7 *s*			
	2′/6′	130.6 *d*	6.97 *d*, 8.3		omTyr[6]-2
	3′/5′	115.6 *d*	6.76 *d*, 8.3		
	4′	156.5 *s*			
	OH		9.32 *s*		
	NCH_3	30.6 *q*	2.73 *s*	omTyr[6]-1	Val[8]-4,5
Val[8]	1	172.2 *s*		Thr[3]-3, Val[8]-2	
	2	55.9 *d*	4.68 *m*		
	3	31.2 *d*	2.00 *m*		
	4	19.5 *q*	0.82 *d*, 7.0		nmTyr[7]- NCH_3
	5	17.4 *q*	0.68 *d*, 7.0		nmTyr[7]-NCH_3,Ahp[5]-OH
	NH		7.35 *d*, 9.6	mTyr[7]-1	nmTyr[7]-2, Ahp[5]-OH

Hmv = 2-hydroxy-3-methyl-valerianic acid. omTyr = *N*-methyl-tyrosine.
[a] HMBC and NOE correlations within the spinsystem of an amino acid are not listed.

hydrophilic amino and other acids such as disulfated glyceric acid. Recently, the first cyclic depsipeptides, scyptolin A and B, were reported from *Scytonema* (Matern et al., 2001), and hofmannolin represents the third example. The general composition of hofmannolin resembles that of the scyptolins, although several amino acid substitutions are apparent. In particular, hofmannolin contains Tyr^4 and *O*-methyl-Tyr^6 in the lactone-ring (Fig. 2) as compared to Leu^5 and Thr^7 in the scyptolins, which likely affects the lipophilic character of the ring portion. Furthermore, the side chain of hofmannolin is composed of *N*-acylated Glu rather than *N*-butyryl-Ala-Thr in scyptolin A or $(N\text{-butyryl-Ala})_2$-Thr in scyptolin B, and due to the acidic residue hofmannolin could be separated from the scyptolins through fractionation on anion exchange cartridges (Matern et al., 2001). 2-Hydroxy-3-methylvaleric acid which has not yet been reported as an *N*-acyl-moiety of a cyanopeptolin contributes with Glu to the hydrophilicity of the side chain in hofmannolin relative to that of the side chains of the scyptolins. Overall, the data predict a stronger amphiphilic character for hofmannolin as compared to the scyptolins.

Various physiological functions have been ascribed to the depsipeptide metabolites in cyanobacteria, ranging from defence (Rohrlack et al., 2001) and metal chelation (Humble et al., 1997) to the inhibition of protein phosphatases (Boland et al., 1993; Rapala, 1998) or of peptidases supposedly involved in cell cycle regulation (for review see Mann, 2000). Unfortunately, the assays employed did not reveal a pronounced bioactivity of hofmannolin, but the lack of serine protease inhibition might be explained in light of very recent findings on scyptolin A. The scyptolins were shown to inhibit porcine pancreatic elastase activity in vitro at low concentrations (Matern et al., 2001), and the elastase-scyptolin A-complex was crystallized and analyzed by X-ray diffraction (Matern et al., manuscript in preparation). Accordingly, the Leu^5-Ahp^6 partial ring motif is essential for elastase inhibition as had been proposed earlier for the inhibition by nostopeptins (Okino et al., 1997), and this motif is replaced in hofmannolin by Tyr^4-Ahp^5 (Fig. 2). Furthermore, the hydrophobic side chain of scyptolin A is essential for tight binding to elastase. Thus, the Glu^2 and Tyr^4 residues (Fig. 2) likely interfere with binding of hofmannolin to elastase or other proteases.

4. Experimental

4.1. General

Amino acid analysis was carried out as described before (Matern et al., 2001) using a AccQ Tag kit (Waters, Eschborn, Germany). Polarimetry of hofmannolin (1.75 mg/ml in methanol) was carried out in a Perkin-Elmer 241 Polarimeter (Überlingen, Germany) at 589 nm and 20 °C. The ultraviolet spectrum was recorded from 195 to 350 nm (at 0.1 g/l in methanol) on a Perkin-Elmer Lambda 19 spectrometer. Transmission infrared spectroscopy of lyophilyzed hofmannolin was conducted from 4000 to 680 cm^{-1} in a FT-IR-Microscope i-Series coupled with a Spectrum 2000 FT-IR Spectrometer (Perkin-Elmer, Überlingen, Germany).

For MALDI-TOF-MS, the peptide was dissolved in 10 μl water/ethanol/acetonitrile (1/1/1). All samples were analyzed in a saturated α-cyano-4-hydroxycinnamic acid matrix solubilized in 50% acetonitrile, 0.3% TFA, and a mixture of 1 μl matrix and 1 μl sample was prepared directly on the plate. The samples were analyzed using a MALDI Voyager Elite time of flight mass spectrometer from PerSeptive BioSystems (Framingham, MS, USA), containing a nitrogen laser giving a 337 nm output. The ions were accelerated with a voltage of 20 kV. Measurements were performed in the delayed extraction mode, allowing the determination of monoisotopic mass values. A low mass gate of 400 improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was used in the positive ion detection and reflector mode. Post source decay (PSD) measurements were performed with the same probes on the template as used for peptide mass determination. The operating voltages of the reflectron were reduced stepwise to record 12 spectral segments sequentially.

For ESI-MS analysis, the samples were loaded onto an Inertsil ODS-3, C_{18}, 5 μm, 50×0.8 mm column equipped with a C_{18}, 2×0.8 mm μ-guard column (LC Packings) using a FAMOS μ-sampling workstation (LC Packings, Amsterdam, The Netherlands). Chromatography was carried out using an HP1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) running at 100 μl/min. The flow was split by an Acurate μ-flow processor (LC Packings) installed between the HPLC pump and the injector of the FAMOS workstation to yield a flow of 25 μl/min on the reversed-phase column. The HPLC solvents were: A, 0.05% TFA/water; B, 96% acetonitrile/0.05% TFA/water. Linear gradients from 10 to 80% B were run over 12 min, with an isocratic step of 3 min at 95% at the end of the gradient. The column effluent passed through a U-Z view 30 nl flow cell (LC Packings) with the detector set at 214 nm, and from there was then directed through a fused silica capillary (340 μm o.d.×50 μm i.d.) into the electrospray source of an LCQ ion trap mass spectrometer (Finnigan Corp., San Jose, CA, USA) run in the full scan mode for mass determination with the scan range set between 200 and 2000 amu. The source was operated at 4.5 kV with the heated capillary set at 220 °C and sheath nitrogen gas flow rate at 80. The ion time was set at 500 ms and the target number of ions at 5×10^7. Three microscans/spectrum were performed. The electron

multiplier was set at −1000 V and spectra were collected in the positive-ion mode.

The NMR spectra were recorded on a BRUKER DMX 500 (Faellanden, Switzerland) spectrometer using a selective probe for the 1D-^{13}C spectrum and a triple inverse probe for the 1D-^{1}H and 2D spectra. The sample concentration was <2 mg/0.5 ml DMSO-d_6. ^{1}H and ^{13}C shifts are referenced to DMSO-d_6 = 2.49 ppm and 39.9 ppm respectively. The following NMR experiments were carried out: 1D-^{1}H, 1D-^{13}C, ^{1}H–^{1}H-COSY, ^{1}H–^{1}H-ROESY, ^{1}H–^{1}H-TOCSY, ^{1}H–^{13}C-COSY (HSQC), and the amino acid sequence was deduced primarily by HMBC correlations.

4.2. Organism and culture conditions

Scytonema hofmanni PCC 7110 was obtained from the Pasteur Culture Collection (Institut Pasteur, Paris, France). The cells were cultivated photoautotrophically under stationary conditions at 28 °C in BG-11 growth medium (Rippka et al., 1979) as described previously (Matern et al., 2001).

4.3. Extraction and purification

The lyophilized cell material was extracted with 75% aqueous methanol, and the extract was fractionated over Sephadex LH20 (Amersham Biosciences, Freiburg, Germany), Chromabond C_{18} (Macherey & Nagel, Düren, Germany) and QMA anion cartridges (1 g, Waters, Eschborn, Germany) (Matern et al., 2001). The bound material was eluted from the cartridges with 0.05 mM ammonium hydrogencarbonate in 30% aqueous methanol, diluted to 10% methanol and applied to a C_{18}-cartridge (1 g, Macherey & Nagel, Düren, Germany). The material eluted with approx. 8 ml of 90% aqueous methanol were applied to a SP 250/21 Nucleosil 100-7 C_{18}-HPLC-column (Macherey & Nagel, Düren, Germany), and final separation was accomplished by isocratic elution in water/0.125% TFA-acetonitrile 60/40 at 40 °C and at a flow rate of 10 ml/min using a Waters 600 multisolvent delivery system in line with a model 991 photodiode array detector (Waters, Eschborn, Germany). The fractions containing hofmannolin were pooled and dried in a speed vac (maxi dry lyo, Heto-Holten GmbH, Wettenberg, Germany) at 35 °C for 12 h. For analytical purposes, the RP-HPLC was run in a gradient according to Lawton et al. (1994). Briefly, this protocol employs a three-step gradient made up of bidistilled water/ acetonitrile/0.5% TFA in acetonitrile in ratios of (a) 70:20:10, (b) 65:25:10, (c) 30:60:10 and (d) 0:90:10 at a flow rate of 1 ml/min, and the elution starts with a linear gradient from (a) to (b) in 10 min followed successively by linear gradients of (b) to (c) in 30 min and (c) to (d) in 2 min. Pure hofmannolin was collected as a colourless amorphous powder.

4.3.1. Hofmannolin

Optical rotation $[\alpha]_D^{20}$ +2.29° (MeOH).; Ultraviolet absorbance λ_{max} (methanol) nm (log ε): 223.66 (3.37), 277.41 (3.47); IR (film): ν_{max}^{film} cm^{-1}: 3363, 3297 (NH and OH), 3060 (aromatic CH), 2966, 2936, 2878 (aliphatic CH), 1736 (ester C=O), 1666 (amide C=O). For PSD mass fragments and NMR data see Fig. 1 and Table 1.

4.4. Bioactivity assays

Pure hofmannolin was employed in a range of concentrations up to 0.02 mg/ml in multiple assays aiming at the inhibition of elastase, plasmin, thrombin, trypsin and papain, respectively. The kinetics of peptidolysis was monitored at 405 nm with various substrate peptides labeled as *p*-nitroanilides recording the release of *p*-nitroaniline (Matern et al., 2001).

Plate diffusion tests on BST Medium (Difco Laboratories, Heidelberg, Germany) were used to determine the antibiotic potential of hofmannolin to *B. subtilis*, *E. coli*, *C. diphteriae*, *P. aeruginosa*, *M. luteus* and *C. albicans*. The liquid inoculum (10^4–10^5 cells) in peptone-bouillon was streaked on the plates, and after a short period of incubation the plates were dried in an incubator at 37 °C for 20 min. Hofmannolin was dissolved in 5% aqueous methanol (2 mg/ml), and 100 µl of this solution was placed in a well of the preincubated agar plate, while a second well in the same plate was filled with the solvent only. The plates were kept for 10 min at room temperature, then placed in an incubator at 36 °C over night and examined for growth inhibition around the wells.

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