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A CYCLIC PEPTIDE, ANABAENOPEPTIN B, FROM THE CYANOBACTERIUM *OSCILLATORIA AGARDHII*

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Key Word Index—*Oscillatoria agardhii*; cyanobacterium; cyclic peptide; anabaenopeptin B.

Abstract—A cyclic peptide, anabaenopeptin B (**1**), was isolated from the cultured cyanobacterium *Oscillatoria agardhii* (NIES-204). The structure of **1** was elucidated by extensive 2D NMR spectroscopy and chemical degradation. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

It is well known that cyanobacteria (blue–green algae) produce novel secondary metabolites such as peptides, macrolides, alkaloids, amides and sulphur compounds [1]. A wide range of biological activities has been reported for cyanobacterial peptides, including cell-differentiation-promoting activity for microcystilide A [2], antifungal activity for laxaphycins A–E [3], tyrosine inhibitory activity for microviridin [4], calcium antagonist activity for scytonemins [5], insecticidal and anticancer activities for majusculamide C [6] and cardioactivity for puwainaphycin C [7].

We have also reported novel serine protease inhibitory peptides such as micropeptins A and B [8], aeruginosin 298-A [9], aeruginosins 98-A and B [10], micropeptin 90 [11], oscillapeptin [12] and microviridins B and C [13] from freshwater cyanobacteria.

Recently, Harada *et al.* reported the isolation of anabaenopeptins A and B as minor components of biologically active peptides from the cyanobacterium *Anabaena flos-aquae* NRC 525-17 [14]. In the course of our continuous screening programme for protease inhibitors from cyanobacteria, we found anabaenopeptin B (**1**) abundantly in the cyanobacterium *Oscillatoria agardhii* (NIES-204). We report here its isolation and structural elucidation.

RESULTS

Compound **1** was isolated as an amorphous powder from the cultured and lyophilized cyanobacterium in a yield of 0.32%: $[\alpha]_D^{20} - 58.9°$ (*c* 0.3, methanol); UV (methanol) λ_{max} 279 nm (log ε 3.22). The molecular formula of **1** was deduced to be $C_{41}H_{60}N_{10}O_9$ from the HRFAB mass spectral (*m/z* 837.4593 $[M+H]^+$ $\Delta -$ 3.0 mmu) and NMR spectral data. Amino acid analysis of the acid hydrolysate of **1** revealed the presence of each one residue of phenylalanine, valine and unknown amino acids. Extensive NMR analyses of **1**, including ^1H–^1H COSY, HMBC, HMQC and HOHAHA spectra, indicated the presence of other structural units, *N*-methylalanine (MeAla), homotyrosine (Hty), arginine and lysine. A fragment ion peak of the negative FAB mass spectrum (*m/z* 661, $[M - Arg - H]^-$) also indicated the presence of arginine. The peptidic nature of **1** was suggested by its ^1H and ^{13}C NMR spectra, showing seven amide protons, six amide carbonyl groups, one ureido carbonyl group, one guanidine group and three non-protonated signals, as shown in Table 1. Compound **1** had six amino acid residues and all of them were readily identified by interpretation of the NMR data, particularly COSY and HMBC spectra. The cyclic pentapeptide moiety of **1** was determined as cyclo-(Phe-MeAla-Hty-Val-Lys) by inter-residual correlations in the HMBC spectrum (Phe NH/MeAla CO, MeAla *N*-Me/Hty CO, Hty NH/Val CO, Val NH/Lys CO, Lys ε-NH/Phe CO) and NOESY spectrum (Phe NH/MeAla α-H, MeAla α-H/Hty α-H, Hty NH/Val α-H, Val NH/Lys α-H and Lys ε-NH/Phe α-H). The remaining Arg residue was attached to Lys through an unusual ureido linkage, which was confirmed by the NOESY correlations (Arg α-NH/Lys α-NH and Arg α-NH/Lys α-H) as well as HMBC cross peaks of Arg α-H/ureido CO (δ_c 157.3) and Lys α-H/ureido CO. It was also established that the free carboxy group was present in the branched Arg group (Fig. 1). The gross structure of **1** based on the NMR data was wholly supported by the FAB mass spectral data.

The absolute stereochemistry of Phe and Val in **1** was determined to be L-form and that of Lys to be D-form by chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolysate. MeAla and Arg were determined to be L-form by Marfey's method [15]. The absolute configuration of Hty residue remains to be defined.

Table 1. ^{1}H and ^{13}C NMR spectral data for anabaenopeptin B (**1**) in DMSO-d_6

Units	No.	^{13}C (mult.)	^{1}H (mult., J in Hz)	HMBC correlations
Phe	1	170.8 (*s*)		Phe 2, 3, Lys 6, ε-NH
	2	55.0 (*d*)	4.39 (*ddd*, 12.7, 8.8, 3.4)	Phe 3
	3	37.5 (*t*)	2.78 (*dd*, 13.9, 12.7)	Phe 2, 5, 9
			3.32 (*dd*, 13.9, 3.4)	
	4	138.3 (*s*)		Phe 3, 6, 8
	5, 9	128.9 (*d*)	7.06 (*d*, 7.0)	Phe 3, 7
	6, 8	128.3 (*d*)	7.19 (*m*)	
	7	126.1 (*d*)	7.13 (*m*)	Phe 5, 9
	NH		8.67 (*d*, 8.8)	
MeAla	1	169.8 (*s*)		MeAla, 2, 3, Phe NH
	2	54.3 (*d*)	4.78 (*q*, 6.7)	MeAla, 3, *N*-Me
	3	13.8 (*q*)	1.07 (*d*, 6.7)	MeAla 2
	N-Me	27.0 (*q*)	1.78 (*s*)	MeAla 2
Hty	1	170.9 (*s*)		Hty 2, 3, MeAla 2, *N*-Me
	2	48.7 (*d*)	4.73 (*ddd*, 8.2, 5.4, 5.4)	Hty 3, NH
	3	33.2 (*t*)	1.71 (*m*)	Hty 2, 4, NH
			1.88 (*m*)	
	4	30.5 (*d*)	2.42 (*ddd*, 13.7, 10.9, 6.4)	Hty 2, 3, 6, 10
			2.62 (*ddd*, 13.7, 10.9, 4.3)	
	5	131.0 (*d*)		Hty 3, 4, 7, 9
	6, 10	129.0 (*d*)	7.00 (*d*, 8.5)	Hty 4
	7, 9	115.1 (*d*)	6.67 (*d*, 8.5)	
	8	155.6 (*s*)		Hty 6, 7, 9, 10
	NH		8.93 (*d*, 5.4)	
	OH		9.18 (*br s*)	
Val	1	172.6 (*s*)		Val 2, Hty NH
	2	58.1 (*d*)	3.92 (*dd*, 8.8, 7.0)	Val 3, 4, 5
	3	30.0 (*d*)	1.97 (*m*)	Val 2, 4, 5
	4	19.2 (*q*)	0.92 (*d*, 6.6)	Val 2, 3, 5
	5	18.9 (*q*)	1.05 (*d*, 6.7)	Val 2, 3, 4
	NH		7.00 (*d*, 8.5)	
Lys	1	172.2 (*s*)		Lys 2, 3, Val NH
	2	54.7 (*d*)	3.95 (*ddd*, 6.7, 6.7, 4.4)	Lys 3, 4
	3	31.7 (*t*)	1.62 (*m*)	Lys 2, 5
	4	20.3 (*t*)	1.15 (*m*)	Lys 2, 3, 6
			1.32 (*m*)	
	5	28.1 (*t*)	1.45 (*m*)	Lys 6
	6	38.3 (*t*)	2.81 (*m*)	Lys 5
			3.58 (*dddd*, 13.3, 8.7, 8.7, 4.2)	
	α-NH		6.52 (*d*, 7.2)	
	ε-NH		7.14 (*m*)	
Arg	1	174.2 (*s*)		Arg 2, 3
	2	52.0 (*d*)	4.10 (*ddd*, 8.2, 8.2, 5.1)	Arg 3, 4
	3	29.2 (*t*)	1.53 (*m*)	Arg 2, 4, 5
			1.70 (*m*)	
	4	25.0 (*t*)	1.48 (*m*)	Arg 2, 3, 5
	5	40.3 (*t*)	3.12 (*m*)	Arg 3, 4
	6	156.8 (*s*)		Arg 5, δ-NH
	α-NH		6.45 (*d*, 8.2)	
	δ-NH		7.67 (*t*, 5.8)	
	CO(*ureido*)	157.3 (*s*)		Arg 2, Lys 2

DISCUSSION

Compound **1** was isolated from *O. agardhii* (NIES-204) in a high yield and its structure was elucidated unambiguously by the extensive NMR and FAB mass spectral data. Anabaenopeptins A and B were first isolated as a minor group of bioactive compounds from the cyanobacterium *A. flos-aquae* NRC 525-17, and these compounds produced concentration-dependent relaxations in rat aortic preparations with endothelium precontracted with 0.1 mM norepinephrine [14]. In the present study, **1** was isolated from a 60% methanol fraction of *O. agardhii*, which showed potent serine protease inhibitory activity. Purified **1**, however, had no activity.

It is interesting that *O. agardhii* and *A. flos-aquae* produce the same compound, **1**, as a major and minor peptide, respectively. It is a unique cyclic peptide having an unusual homotyrosine residue and a ureido bond. In this connection, keramamide A [16] and

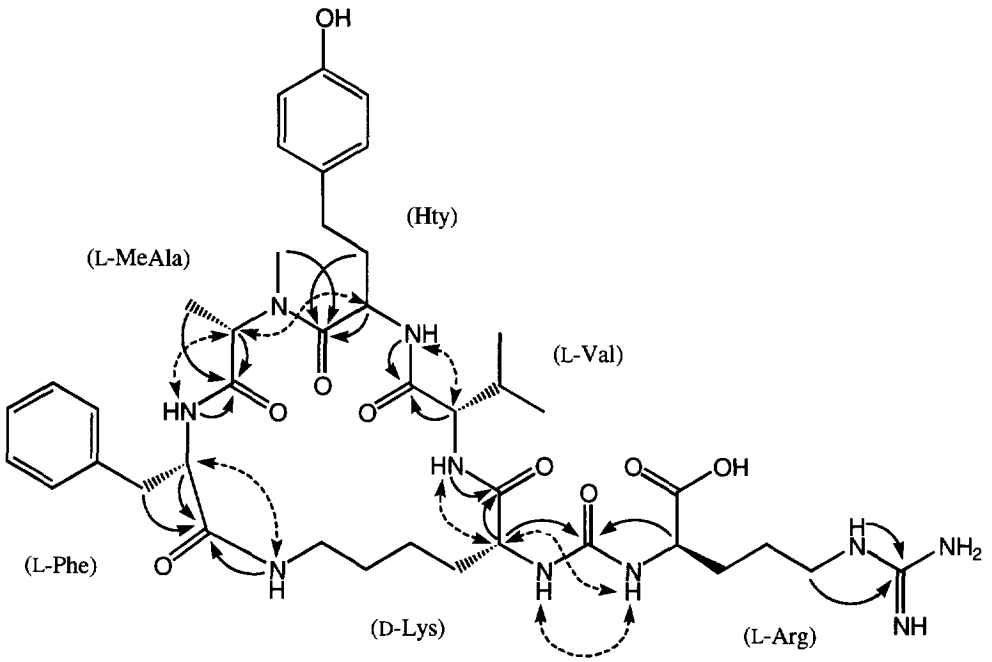

Fig. 1. Structure of anabaenopeptin B (**1**). Arrows represent the selected HMBC (→) and NOESY (←--→) correlations.

konbamide [17], closely related to **1** in structure, were isolated from the Okinawan marine sponge *Theonella* sp., and the authors speculated that these unique peptides might be produced by symbiotic microorganisms such as microalgae, bacteria or fungi. The fact that *Oscillatoria* and *Anabaena* produce **1**, similar to keramamide A and konbamide in structure, suggests that the true producer of those compounds might be cyanobacteria.

EXPERIMENTAL

General instrumentation. NMR spectra were recorded on a Bruker AM600 NMR spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C using DMSO as solvent at 27°. FAB-MS were measured by using polyethyleneglycol sulphate or glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyser. Chiral GC experiments were performed on a Shimadzu GC-9A gas chromatograph fitted with an Alltech Chirasil-Val capillary column (25 m × 0.25 mm) with a FID. The oven temp. was increased from 80 to 200° at a rate of 4° min^{-1}. HPLC was performed on a Shimadzu LC-6A liquid chromatograph with an ODS L-column (10 × 250 mm, Chemicals Inspection and Testing Institute). UV spectra were measured on a Hitachi 330 spectrometer. Optical rotations were determined with a Jasco DIP-140 digital polarimeter.

Culture conditions. Oscillatoria agardhii (NIES-204, Cyanophyceae) was obtained from the NIES collection (Microbial Culture Collection, National Institute for Environmental Studies, Japan) and cultured in 10 l glass bottles containing CB medium [18] with aeration (filtered air, 0.3 l min^{-1}) at 25° under illumination of 250 $\mu E\ m^{-2}\ s$ on a 12 hr:12 hr light–dark cycle. Cells were harvested after 10–14 days incubation by continuous centrifugation at 10 000 MV min^{-1}. Harvested cells were lyophilized and kept at −20° until extraction.

Extraction and isolation. Freeze-dried cells (138 g from 400 l of culture) were extracted ×3 with 80% MeOH and concd to give a crude extract. This extract was partitioned between Et_2O and H_2O. The H_2O-soluble fr. was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was subjected to ODS flash CC and eluted with aq. MeOH and CH_2Cl_2. The 60% MeOH fr. was purified by HPLC on the ODS column with 35% MeCN containing 0.05% TFA to yield 440 mg of **1**.

Amino acid analysis. Compound **1** (100 μg) was dissolved in 6 M HCl (500 μl) and sealed in a reaction vial. The vial was heated at 110° for 16 hr. The soln was evapd in a stream of dry N_2 with heating and redissolved in 0.1 M HCl for amino acid analysis.

Chiral GC analysis of amino acids. The hydrolysate of **1** was added with a soln of 10% HCl in iso-PrOH to a reaction vial and heated at 100° for 30 min. The solvent was removed in a stream of dry N_2. $(CF_3CO)_2O$ (300 μl) in CH_2Cl_2 (300 μl) was added to the residue, the vial was capped, and the soln heated at 100° for 5 min and evapd in a stream of dry N_2. The residue was dissolved in CH_2Cl_2 (500 μl) and immediately analysed by chiral GC.

Derivatization of amino acids and HPLC analysis. Compound **1** (100 μg) was dissolved in 6 M HCl (500 μl) and heated at 110° for 16 hr. After removal of HCl in a stream of dry N_2, the residue was treated with a 10% Me_2CO soln of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) in 1 M $NaHCO_3$ at

80–90° for 3 min followed by neutralization with 50 μl 2 M HCl. The reaction mixt. was dissolved in 50% MeCN and subjected to reverse-phase HPLC: column, cosmosil MS (Nacalai Tesque Co., 4.6 × 250 mm), gradient elution from H_2O–TFA (100:0.1) to MeCN–H_2O–TFA (50:50:0.1) in 60 min, UV detection (340 nm).

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