Isolation of New Protein Phosphatase Inhibitors from Two Cyanobacteria Species, *Planktothrix* spp.

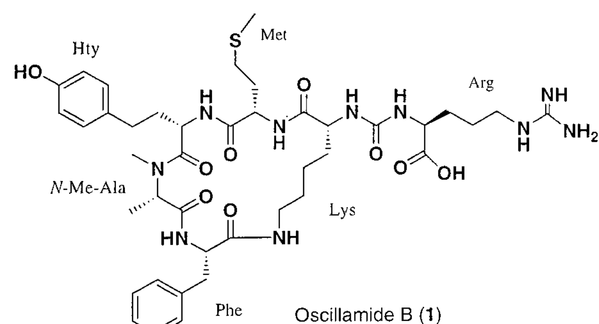
Tomoharu Sano,*,† Takeo Usui,‡ Kazunori Ueda,‡,§ Hiroyuki Osada,‡ and Kunimitsu Kaya†

National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan, RIKEN, 2-1 Hirosawa, Wako, Saitama 305-0198, Japan, and Toyama Prefectural University, 5180 Kurokawa, Kosugi, Imizu, Toyama 939-0398, Japan

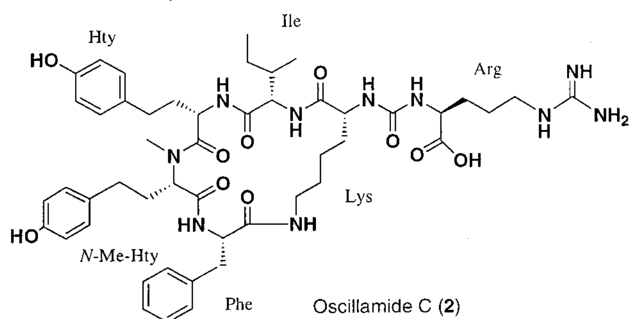
Received November 17, 2000

Two new protein phosphatase inhibitors, oscillamide B (**1**) and C (**2**), were isolated from the cyanobacteria *Planktothrix (Oscillatoria) agardhii* and *P. rubescens*. The structures of the inhibitors were elucidated by analysis of HRFABMS, 1D and 2D NMR spectra, and chemical degradation. These inhibitors are ureido-containing cyclic peptides and inhibited serine/threonine protein phosphatases PP1 and PP2A. The inhibitory activities were closely related to the Arg and *N*-Me-Hty residues in the peptides.

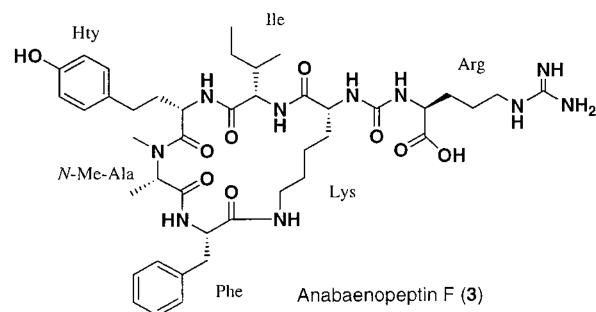
The toxic freshwater cyanobacteria (blue-green algae) *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek (formerly *Oscillatoria agardhii* Gomont) (Oscillatoriales, Oscillatoriaceae) and *Planktothrix rubescens* (DC. ex Gomont) Anagnostidis & Komárek (formerly *Oscillatoria rubescens* DC. ex Gomont) (Oscillatoriales, Oscillatoriaceae) produce many kinds of bioactive cyclic peptides.[1] Microcystins, hepatotoxic cyclic heptapeptides from the cyanobacteria, have been found to inhibit protein phosphatase type 1 and 2A (PP1 and PP2A).[2] Dehydrobutyrine-containing microcystins also hepatotoxic cyclic peptides, and a protein phosphatase-inhibiting cyclic peptide has also been isolated from the filamentous cyanobacteria *Nostoc* and *Planktothrix*.[3−5] Specific inhibitors of protein phosphatases can be utilized to reveal the role of each protein phosphatase that controls the phosphorylation level of proteins. The control of protein phosphorylation level is very important for regulating intracellular events in eukaryotic cells.[6] During our investigation of peptides in *P. agardhii* and *P. rubescens*, we found two novel protein phosphatase-inhibiting cyclic peptides, **1** and **2**. In this paper, we report the structure elucidation of **1** and **2** and their structure−activity relationship as protein phosphatase inhibitors.



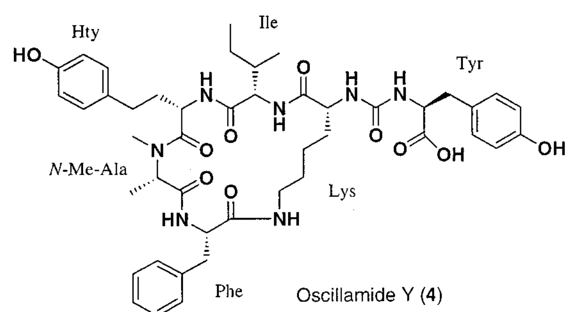
Oscillamide B (**1**)



Oscillamide C (**2**)



Anabaenopeptin F (**3**)



Oscillamide Y (**4**)

Results and Discussion

The extract from *P. agardhii* (CCAP 1459/11A), prepared by soaking with 5% acetic acid aqueous solution, showed inhibitory activity against PP1 and PP2A. The extract was fractionated using Sep-Pak C_{18} cartridges with 20% and 80% aqueous MeOH. The 80% MeOH eluent was chromatographed on reversed-phase HPLC (65% MeOH in 50 mM phosphate buffer, pH 3.0). The active fraction was further purified by normal phase HPTLC to give 21.0 mg (0.2% yield from dry algae) of oscillamide B (**1**). Oscillamide B (**1**) was a colorless, amorphous solid. The molecular formula of **1** was established as $C_{41}H_{60}O_9N_{10}S$ from the positive high-resolution FABMS spectrum ($[M + H]^+$ at *m*/*z* 869.4324, calcd for $C_{41}H_{61}O_9N_{10}S$, 869.4343). The ^1H and ^{13}C NMR spectral data of **1** (Table 1) suggested that it is a peptide. After acid hydrolysis of **1** (6 M HCl, 150 °C, 10 h), homotyrosine (Hty), arginine (Arg), phenylalanine (Phe),

* To whom correspondence should be addressed. Tel: +81-298-50-2449. Fax: +81-298-50-2574. E-mail: sanotomo@nies.go.jp.
† National Institute for Environmental Studies.
‡ RIKEN.
§ Toyama Prefectural University.

10.1021/np0005356 CCC: $20.00 © 2001 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/03/2001

Table 1. ^{1}H and ^{13}C NMR Spectral Data for Oscillamide B (**1**) in CD_3OD

position		^{1}H *J* (Hz)		^{13}C	HMBC (C→H)
Phe	1			174.1	2, 3, Lys-6
	2	4.59	(dd, 3.4, 12.8)	56.8	
	3	3.38	(dd, 3.4, 12.8)	39.1	
		2.82	(t, 12.8)		
	4			139.0	3
	5, 9	7.10	(d, 7.3)	130.1	3
	6, 8	7.24	(t, 7.3)	129.9	
	7	7.15	(t, 7.3)	127.7	
N-Me-Ala	1			172.2	2, 3, Phe-2
	2	4.72	(q, 6.7)	56.5	*N*-Me
	3	1.14	(d, 6.7)	14.2	
	N-Me	1.85	(s)	28.2	
Hty	1			174.1	2, 3, *N*-Me-Ala-2, *N*-Me
	2	4.74	(dd, 5.2, 9.2)	50.2	
	3	2.05	(m)	34.7	
		1.79	(m)		
	4	2.73	(m)	32.1	6, 10
		2.57	(m)		
	5			132.7	4
	6, 10	7.06	(d, 8.2)	130.5	
	7, 9	6.71	(d, 8.2)	116.4	
	8			157.0	
Met	1			175.1	2, 3, Hty-2
	2	4.33	(t, 7.6)	54.8	
	3	2.13	(m)	32.0	
	4	2.68	(m)	31.2	5
	5	2.13	(s)	15.5	4
Lys	1			176.4	2, 3, Met-2
	2	4.12	(dd, 4.3, 10.1)	56.3	
	3	1.90	(m)	32.8	
		1.70	(m)		
	4	1.50	(m)	21.7	
		1.34	(m)		
	5	1.56	(m)	29.4	
	6	3.70	(m)	40.0	
		2.93	(m)		
Arg	1			179.7	2, 3
	2	4.14	(dd, 4.4, 6.0)	55.6	
	3	1.85	(m)	32.0	
		1.67	(m)		
	4	1.63	(m)	26.1	
	5	3.20	(m)	42.1	
	6			158.6	
	CO (ureido)			159.8	Arg-2

lysine (Lys), methionine (Met), and *N*-methylalanine (*N*-Me-Ala) were detected by amino acid analysis using Marfey's method[7] and a chiral GC/MS method.[8] From the amino acid analysis, Lys was identified as being in the D-configuration, and all other amino acids are in the L-configuration. From the amino acid composition and the molecular formula, the structure of **1** was expected to be a Met variant of anabaenopeptin F (**3**)[9] at the isoleucine (Ile) position. From extensive analysis of the 2D NMR spectra, such as COSY, HOHAHA, HMQC, and HMBC, these amino acid units were confirmed. The amino acid sequences of **1** were determined from the HMBC spectrum. The cross-peak between H-6 of Lys and the carbonyl carbon of Phe confirmed the connectivity. The connectivity between *N*-Me-Ala and Hty was suggested by the cross-peak between the *N*-Me proton of *N*-Me-Ala and the carbonyl carbon of Hty. The other connectivities were confirmed with the cross-peaks between the α-protons and the carbonyl carbons. These results established the structure of oscillamide B as **1**.

Following the same procedure as used for **1**, oscillamide C (**2**, 7.7 mg, 0.1% yield from dry algae) was isolated as a colorless solid from *P. rubescens* (CCAP 1459/14). The molecular formula of **2** was established as $C_{49}H_{68}O_{10}N_{10}$ on the basis of high-resolution FABMS data ($[M + H]^+$ at *m/z* 957.5198, calcd for $C_{49}H_{69}O_{10}N_{10}$, 957.5198). The ^{1}H and ^{13}C NMR spectral data of **2** (Table 2) suggested that **2** was a peptide. The amino acids Hty, Arg, Phe, Lys, Ile, and *N*-methylhomotyrosine (*N*-Me-Hty) were detected by Marfey's method and chiral GC/MS analysis after acid hydrolysis (6 M HCl, 150 °C, 10 h). The stereochemistry of these amino acids were elucidated. The configuration of Lys was shown to be D, and all other amino acids were identified as having the L-configuration. This amino acid composition and the molecular formula suggested that **2** was the *N*-Me-Hty variant of **3** at the *N*-Me-Ala position. The amino acid sequences of **2** were determined from HMBC and ROESY spectra. From these data, the structure of oscillamide C was confirmed as **2**.

Table 2. ^{1}H and ^{13}C NMR Spectral Data for Oscillamide C (**2**) in CD_3OD

position		^{1}H *J* (Hz)		^{13}C	HMBC (C→H)
Phe	1			174.1	2, 3, Lys-6
	2	4.62	(dd, 3.4, 12.5)	56.8	
	3	3.38	(dd, 3.4, 14.0)	38.9	
		2.87	(dd, 12.8,14.0)		
	4			139.1	3
	5, 9	7.10	(d, 7.3)	130.1	3
	6, 8	7.22	(t, 7.3)	129.7	
	7	7.15	(t, 7.3)	127.6	
N-Me-Hty	1			171.7	2, 3, *N*-Me, Phe-2
	2	4.47	(dd, 5.8, 8.6)	61.1	
	3	2.01	(m)	31.4	
		1.60	(m)		
	4	2.32	(m)	33.0	
		2.21	(m)		
	5			132.8	4
	6, 10	6.92	(d, 8.5)	130.0	4
	7, 9	6.67	(d, 8.5)	116.4	
	8			157.1	
	N-Me	1.89	(s)	28.3	
Hty	1			174.4	2, *N*-Me-Hty-2, *N*-Me
	2	4.67	(dd, 3.7, 10.7)	50.0	
	3	2.07	(m)	34.6	
		1.81	(m)		
	4	2.78	(m)	31.9	
		2.66	(m)		
	5			132.5	4
	6, 10	7.06	(d, 8.6)	130.8	4
	7, 9	6.70	(d, 8.6)	116.5	
	8			157.5	
Ile	1			175.1	2
	2	4.06	(t, 9.2)	60.1	
	3	1.92	(m)	37.5	
	4	1.78	(m)	26.7	
		1.28	(m)		
	5	1.01	(t, 7.3)	12.0	
	6	1.00	(d, 7.0)	16.3	
Lys	1			176.2	2, 3, Ile-2
	2	4.12	(dd, 4.3, 5.5)	56.5	
	3	1.95	(m)	32.5	
		1.75	(m)		
	4	1.55	(m)	21.8	
		1.35	(m)		
	5	1.62	(m)	29.3	
	6	3.70	(m)	40.2	
		3.00	(m)		
Arg	1			179.4	2, 3
	2	4.15	(dd, 6.4, 7.0)	55.5	
	3	1.85	(m)	32.2	
		1.67	(m)		
	4	1.65	(m)	26.0	
	5	3.23	(m)	42.1	
		3.18	(m)		
	6			158.6	5
	CO (ureido)			159.7	Lys-2, Arg-2

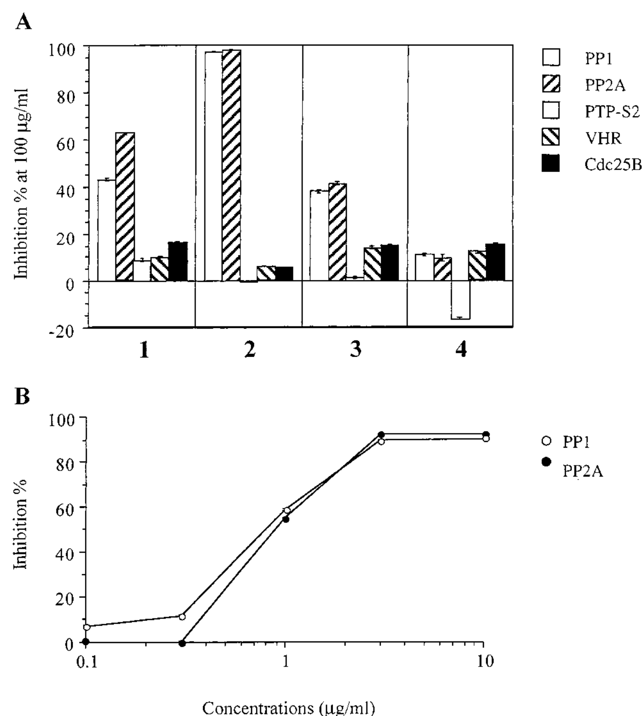


Figure 1. (A) Inhibitory activities of **1**−**4** against protein phosphatases. (B) Inhibition of PP1 and PP2A by **2**.

Table 3. Structures and Protein Phosphatases Inhibition Activities of Peptides **1**−**4**

peptide		Y^2		Z^4		X	inhibition (%) at 100 μg/mL PP1	PP2A
1	Lys	Met	Hty	*N*-Me-Ala	Phe	Arg	43.3	62.8
2	Lys	Ile	Hty	*N*-Me-Hty	Phe	Arg	97.3	98.4
3	Lys	Ile	Hty	*N*-Me-Ala	Phe	Arg	38.1	41.5
4	Lys	Ile	Hty	*N*-Me-Ala	Phe	Tyr	11.2	9.6

Another inhibitor (**3**) was isolated from *P. rubescens* (NIES-610), and its structure was elucidated in the same way as **1** and **2**. From the molecular formula, amino acid composition, and 1D and 2D NMR spectra, **3** was suggested to be identical with anabaenopeptin F.

The inhibitory activities of these cyclic peptides (**1**−**3**) and oscillamide Y (**4**)[10] against protein phosphatases were tested. The peptides showed inhibitory activities against protein serine/threonine phosphatase PP1 and PP2A at 100 μg/mL (Figure 1), except for peptide **4**, but did not inhibit protein tyrosine phosphatase (PTP-S2) or dual-specificity phosphatase (VHR and Cdc25B) activity even at 100 μg/mL (Figure 1). The most effective inhibitor of the peptides tested was peptide **2**, which had IC_{50} values of 0.90 and 1.33 μM against PP1 and PP2A, respectively. The inhibitory activity values (%) of **2** against PP1 and PP2A at 100 μg/mL were significantly different from those of **1** by the Student's t-test ($\alpha = 0.001$) after arcsine transformation.

The general structure of the tested peptides was shown to be cyclo[(**X**-α-NH-CO-α-NH)-Lys[1]-**Y**[2]-Hty[3]-**Z**[4]-Phe[5]-ε-NH-(Lys[1])]. In the case of peptide **1**, **X**, $\mathbf{Y}^2$, and $\mathbf{Z}^4$ were Arg, Met, and *N*-Me-Ala, respectively (Table 3). Also, in peptide **2**, **X**, $\mathbf{Y}^2$, and $\mathbf{Z}^4$ were Arg, Ile, and *N*-Me-Hty, and **X**, $\mathbf{Y}^2$, and $\mathbf{Z}^4$ of peptide **3** were Arg, Ile, and *N*-Me-Ala, respectively. In peptide **4**, **X**, $\mathbf{Y}^2$, and $\mathbf{Z}^4$ were shown to be Tyr, Ile, and *N*-Me-Ala, respectively. When **X** was Arg as in peptides **1**, **2**, and **3**, considerable inhibitory activities against PP1 and PP2A were observed, but in the case of Tyr, no inhibition of the enzymes was observed. In peptides **1**−**4** the *N*-methyl amino acid unit was located at **Z**.[4] When $\mathbf{Z}^4$ was *N*-Me-Hty as in peptide **2**, the inhibitory activities against PP1 and PP2A were much greater than when $\mathbf{Z}^4$ is *N*-Me-Ala. The amino acid unit at $\mathbf{Y}^2$ did not significantly affect the inhibitory activities. From these findings, the structure−activity relationship suggests that *N*-Me-Hty at $\mathbf{Z}^4$ and a ureido-linked Arg at **X** in the peptides are closely related to the inhibition of PP1 and PP2A activities, but *N*-Me-Ala at $\mathbf{Z}^4$ and ureido-linked Tyr at **X** are not. A similar structure−activity relationship was observed in the case of inhibition of carboxypeptidase A by anabaenopeptins.[11] The positive charge of the guanidyl group of Arg and the hydrophobic residue of *N*-Me-Hty in the peptides may play an important role in the inhibition of PP1 and PP2A. The reversible phosphorylation of proteins is recognized to be a major mechanism for the control of intracellular events in eukaryotic cells.[6] Protein phosphatases act as key enzymes in the control of the phosphorylation level of proteins. Therefore, these protein phosphatase inhibitors can be utilized for biological research.

Experimental Section

General Procedures. NMR spectra were recorded on a JEOL JNM A-500 spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C) in CD_3OD. ^1H and ^{13}C chemical shifts are referenced to the solvent peaks (^1H, 3.30 ppm, and ^{13}C, 49.5 ppm in CD_3OD). Low- and high-resolution FABMS spectra were performed with a JEOL JMS-700 spectrometer using glycerol as the matrix. Specific rotations were obtained on an Atago POLAX-D polarimeter.

Chemicals. D- and L-homotyrosine were gifts from Dr. Mark Bradley at University of Southampton, U.K. All other solvents and reagents were purchased from commercial sources.

Culture Conditions. *Planktothrix agardhii and P. rubescens* (CCAP 1459/11A and CCAP 1459/14) were kindly provided by Dr. John G. Day of CCAP, Scotland. *P. rubescens* (NIES-610 = CCAP 1459/22 = NIVA CYA 18) was obtained from National Institute for Environmental Studies Microbial Culture Collection. *Planktothrix* were grown in 10 L culture bottles with CT medium [$Ca(NO_3)_2 \cdot 4H_2O$ (15 mg), KNO_3 (10 mg), β-Na_2 glycerophosphate (5 mg), $MgSO_4 \cdot 7H_2O$ (4 mg), vitamin B_{12} (0.01 μg), biotin (0.01 μg), thiamin HCl (1 μg), $FeCl_3 \cdot 6H_2O$ (0.06 mg), $MnCl_2 \cdot 4H_2O$ (0.01 mg), $ZnSO_4 \cdot 7H_2O$ (7 μg), $CoCl_2 \cdot 6H_2O$ (1.2 μg), $Na_2MoO_4 \cdot 2H_2O$ (0.75 μg), $Na_2EDTA \cdot 2H_2O$ (0.3 mg), TAPS (*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) (40 mg), distilled water 100 mL, pH 8.2].[12] The cells were grown isothermally at 20 °C (light intensity, below 250 μmol photon m^{-2} s^{-1}; aeration rate, 1.5 L min^{-1}) for 3 weeks. The cultured cells were harvested by continuous flow centrifugation at 10 000 rpm. The harvested cells were lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. The freeze-dried algae were suspended in 5% aqueous acetic acid solution. The suspensions were centrifuged (3500*g*, 10 min), and the supernatants were passed through Sep-Pak C_{18} cartridges (Waters). The cartridges were washed with 20% aqueous MeOH and eluted with 80% aqueous MeOH. The eluate was evaporated *in vacuo*. The residue was dissolved in 20% aqueous MeOH and separated by HPLC (column, Mightysil RP-18 20 × 250 mm (Kanto Chemical Co., Inc.); eluent, 65% MeOH in 50 mM phosphate buffer, pH 3.0; flow rate, 9 mL/min). The active fractions were further purified with HPTLC (Si gel 60 F254 (Merck), thickness, 0.25 mm; developing solvent, $CHCl_3$−MeOH−H_2O (6:4:1)). Oscillamide B (**1**, t_R 9.3 min, R_f 0.7) and oscillamide C (**2**, t_R 8.3 min, R_f 0.8) were obtained from CCAP 1459/11A and CCAP 1459/14, respectively. Compound **3** (anabaenopeptin F) was isolated from *P. rubescens* (NIES-610) in the same way as **1** and **2**. Compound **4** (oscillamide Y) was isolated previously.[10]

Protein Phosphatase Inhibition Assay. Protein phosphatase type-1 (PP1 from rabbit skeletal muscle) and protein phosphatase type-2A (PP2A from human red blood cells) were purchased from Upstate Biotechnology (NY). *In vitro* protein phosphatase assays were carried out in duplicate at 30 °C for 10 min using 0.05 unit/50 μL of protein phosphatase and ^{32}P-labeled myelin basic protein (New England Biolabs), which was prepared from the catalytic subunit of cAMP-dependent kinase and γ-[^{32}P]ATP. The reaction was terminated by addition to each sample of 200 μL of 20% trichloroacetic acid and chilling on ice. After centrifugation, the clear supernatant was collected and the amount of the radioactivity released as ^{32}Pi was determined using a liquid scintillation counter (Packard TRICURVE 2000). Control experiments were performed by addition of MeOH.

Tyrosine phosphatase PTP-S2 and dual-specificity phosphatase VHR[13] were overexpressed in *Escherichia coli* JM109, and phosphatase assays were performed in triplicate according to our previous report.[14] The catalytic subunit of human Cdc25B was also expressed in BL21 (DE3) using pET21b (Novagen) and purified according to the methods of Fauman *et al.*[15] Phosphatase assays were performed at 37 °C using 200 μM 3-*O*-methylfluorescein phosphate as a substrate in 0.7 mL of reaction solution (100 mM Tris-HCl, 40 mM NaCl, 1 mM dithiothreitol, 20% glycerol, pH8.2). After incubation for 20 min, the reaction was terminated by adding 0.55 mL of the stop solution (6 M guanidine-HCl, 1 M 2-(*N*-morpholino)-ethanesulfonic acid, pH 5.5), and the increase in the absorbance at 477 nm was measured. Control experiments were performed by addition of MeOH.

Oscillamide B (1): colorless solid (21.0 mg, 0.2% dry wt); $[\alpha]^{25}_{D}$ −83° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 279 nm (3.5); ^{1}H and ^{13}C NMR, see Table 1; FABMS (pos. glycerol) *m/z* 869 [M + H]$^{+}$; HRFABMS (pos., glycerol) *m/z* 869.4324 [M + H]$^{+}$ (calcd for $C_{41}H_{61}O_{9}N_{10}S$, 869.4343).

Oscillamide C (2): colorless solid (7.7 mg, 0.1% dry wt); $[\alpha]^{25}_{D}$ −111° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 279 nm (3.5); ^{1}H and ^{13}C NMR, see Table 2; FABMS (pos. glycerol) *m/z* 957 [M + H]$^{+}$; HRFABMS (pos., glycerol) *m/z* 957.5198 [M + H]$^{+}$ (calcd for $C_{49}H_{69}O_{10}N_{10}$, 957.5198).

Anabaenopeptin F (3): colorless solid (25.2 mg, 0.03% dry wt); $[\alpha]^{25}_{D}$ −60° (*c* 0.08, MeOH) (lit. −41.4°, *c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 279 nm (3.2); FABMS (pos. glycerol) *m/z* 851 [M + H]$^{+}$; HRFABMS (pos., glycerol) *m/z* 851.4818 [M + H]$^{+}$ (calcd for $C_{42}H_{63}O_{9}N_{10}$, 851.4779).

Acknowledgment. The authors thank Drs. H. Ito and S. Serizawa (NIES) for MS analyses.

References and Notes

(1) Moore, R. E. *J. Ind. Microbiol.* **1996**, *16*, 134−143.
(2) Yoshizawa, S.; Matsushima, R.; Watanabe, M. F.; Harada, K.; Ichihara, A.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1990**, *116*, 609−614.
(3) Sano, T.; Kaya, K. *Tetrahedron* **1998**, *54*, 463−470.
(4) Beattie, K. A.; Kaya, K.; Sano, T.; Codd, G. A. *Phytochemistry* **1998**, *47*, 1289−1292.
(5) Kaya, K.; Sano, T.; Beattie, K. A.; Codd, G. A. *Tetrahedron Lett.* **1996**, *37*, 6725−6728.
(6) Cohen, P. *Annu. Rev. Biochem.* **1989**, *58*, 453−508.
(7) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(8) Sano, T.; Kaya, K. *J. Nat. Prod.* **1996**, *59*, 90−92.
(9) Shin, H. J.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 139−141.
(10) Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, *36*, 5933−5936.
(11) Itou, Y.; Suzuki, S.; Ishida, K.; Murakami, M. *Bioorg. Medic. Chem. Lett.* **1999**, *9*, 1243−1246.
(12) Watanabe, M. M.; Hiroki, M. Eds. *NIES−Collection. List of Strains*; National Institute for Environmental Studies: Tsukuba, Japan, 1997; p 31.
(13) Ishibashi, T.; Bottaro, D. P.; Chan, A.; Miki, T.; Aaronson, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 12170−12174
(14) Usui, T.; Marriott, G.; Inagaki, M.; Swarup, G.; Osada, H. *J. Biochem.* **1999**, *125*, 960−965.
(15) Fauman, E. B.; Cogswell, J. P.; Lovejoy, B.; Rocque, W. J.; Holmes, W.; Montana, V. G.; Piwnica-Worms, H.; Rink, M. J.; Saper, M. A. *Cell* **1998**, *93*, 617−625.

NP0005356