Microcyclamide, a Cytotoxic Cyclic Hexapeptide from the Cyanobacterium *Microcystis aeruginosa*

Keishi Ishida, Hidenori Nakagawa, and Masahiro Murakami*

Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Received April 12, 2000

Microcyclamide (**1**), a cytotoxic cyclic hexapeptide, was isolated from the cultured cyanobacterium *Microcystis aeruginosa* (NIES-298). Its structure was elucidated to be **1** on the basis of two-dimensional $^1H-^1H$, $^1H-^{13}C$, and $^1H-^{15}N$ NMR correlation experiments and the HRFABMS measurement. The absolute stereochemistry of the asymmetric centers was determined by the Marfey's method. This peptide showed a moderate cytotoxicity against P388 murine leukemia cells.

Microcystis aeruginosa has been shown to be a rich source of unique and bioactive secondary metabolites, especially peptides.[1] In the course of our screening program of protease inhibitors from microalgae, we found that *M. aeruginosa* produces protease inhibitors such as microginins,[2−4] micropeptins,[5−9] microviridins,[10] and aeruginosins[11−13] and antibacterial cyclic peptides such as kawaguchipeptins[14,15] and microcystins,[16] which are well known to be protein phosphatase 1 and 2A inhibitors.[17] Recently, Lawton *et al.* isolated the first peptide containing the thiazole unit from the cyanobacterium *M. aeruginosa*.[18] In the process of the isolation of aeruginosin 298-B[13] from the cyanobacterium *M. aeruginosa* (NIES-298), we found a major component that did not have protease inhibitory activity and that differed from aeruginosin,[11,13] microcystin,[19] and microviridin type peptides.[10] Here we describe the isolation, structure, and stereochemistry of microcyclamide (**1**), which is the first cyclic hexapeptide consisting of five-membered heterocycles (two thiazole and one methyloxazole) from the cyanobacterium *M. aeruginosa* (Chroococcales, Chroococcaceae).

M. aeruginosa (NIES-298)[20] was obtained from the NIES collection and cultured in our laboratory to yield 65 g (dry weight) from 300 L of culture. The 80% methanol extract of freeze-dried alga was partitioned between water and diethyl ether. The aqueous layer was further extracted with *n*-butanol and fractionated by ODS flash column chromatography (20−100% MeOH elution) followed by reversed-phase HPLC, using 0.05% TFA in aqueous MeCN to yield microcyclamide (**1**; 52.7 mg).

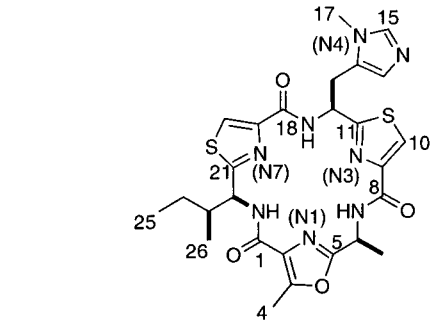
The molecular formula of **1** was established to be $C_{26}H_{30}N_8O_4S_2$ by the HRFABMS and NMR spectral data (Table 1). The peptidic nature of **1** was suggested from three doublet amide protons (δ 8.42 (N8), 8.58 (N2), and 8.94 (N6)), which were not correlated with a carbon atom in the HMQC and six sp^2 carbons in the amide region (δ 159.3−169.0) of the ^{13}C NMR spectrum. Two usual amino acids (isoleucinyl and alanyl) and *N*-methyl histidinyl residues were established by analyses of $^1H-^1H$ COSY, HMQC, and HMBC (Figure 1). The thiazole−methyl histidinyl and thiazole−isoleucinyl units were determined by the HMBC correlations between H-10 and H-12/C-11, and H-20 and H-22/C-21, respectively (Figure 1). The methyloxazole−alanyl unit was suggested on the basis of the HMBC correlations from the aromatic methyl protons to C-2 and C-3 and of the ^1H and ^{13}C chemical shifts that are in good agreement with the values reported for the same units in tenuecyclamides A−D.[22] Although the sequence of three units was mostly deduced by the HMBC correlations, the correlation from NH (N8) to C-2 could not be observed. The $^1H-^{15}N$ HMBC correlation from NH (N8) to N (N1) connected the thiazole−isoleucinyl unit to the methyloxazole−alanyl unit (Figure 1). Moreover, the presence of eight nitrogen atoms and the cyclic hexapeptidic nature of **1** were also supported by the $^1H-^{15}N$ HMBC spectrum (Figure 1 and Table 1).

Table 1. ^1H, ^{13}C, and ^{15}N NMR Data of Microcyclamide (**1**) in DMSO-d_6

	^1H	(mult, *J* in Hz)	^{13}C	mult	^{15}N
1			159.6	(s)	
2			153.5	(s)	
3			127.8	(s)	
4	2.60	(s)	11.2	(q)	
(N1)					242.3
5			161.2	(s)	
6	5.19	(qd, 6.8, 6.0)	44.1	(d)	
7	1.53	(d, 6.8)	19.1	(q)	
(N2)	8.58	(d, 6.0)			116.5
8			159.3	(s)	
9			147.7	(s)	
10	8.48	(s)	125.9	(d)	
(N3)					305.0
11			169.0	(s)	
12	6.01	(ddd, 9.4, 9.0, 5.1)	49.6	(d)	
13	3.36	(dd, 15.4, 9.0)	30.8	(t)	
	3.50	(dd, 15.4, 5.1)			
14			130.2	(s)	
(N4)					174.0
15	8.98	(s)	135.7	(d)	
(N5)					238.1
16	7.40	(s)	119.0	(d)	
17	3.84	(s)	33.2	(q)	
(N6)	8.94	(d, 9.4)			115.0
18			159.3	(s)	
19			147.7	(s)	
20	8.28	(s)	125.2	(d)	
(N7)					305.4
21			167.8	(s)	
22	5.31	(dd, 7.7, 6.0)	54.4	(d)	
23	2.01	(m)	40.2	(d)	
24	1.18	(m)	25.3	(t)	
	1.59	(m)			
25	0.94	(t, 7.3)	11.5	(q)	
26	0.81	(d, 6.4)	14.5	(q)	
(N8)	8.42	(d, 7.7)			117.4

* To whom correspondence should be addressed. Tel.: +81-3-5841-5298. Fax: +81-3-5841−8166. E-mail: amura@mail.ecc.u-tokyo.ac.jp.

10.1021/np000159p CCC: $19.00
Published on Web 07/26/2000



Microcyclamide (**1**)

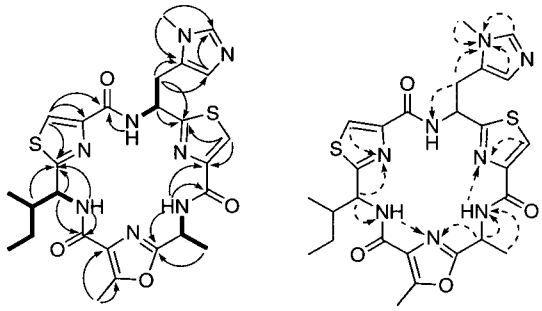


Figure 1. COSY (bold lines), HMBC (arrows), and $^{1}H-^{15}N$ HMBC (dashed arrows) correlations of **1**.

To determine the absolute configuration of microcyclamide, **1** was ozonized followed by acid hydrolysis with 6 N HCl. The amino acid analysis of the acid hydrolysate gave Ala, Asp (oxyproduct of MeHis by ozonolysis), and Ile, and the HPLC analysis of the Marfey[21] derivatives of the acid hydrolysate indicated all to be of L-configuration.

Microcyclamide (**1**) showed a moderate cytotoxicity against P388 murine leukemia cells at an IC_{50} value of 1.2 μg/mL. Several cyclic hexapeptides consisting of five-membered heterocycles, such as nostocyclamide,[23] dendroamides A–C,[24] raocyclamides A and B,[25] and tenuecyclamides A–D,[22] have recently been isolated from orders Nostocales and Stigonematales. Microcyclamide is the first example of a cyclic hexapeptide produced by *M. aeruginosa* (order Chroococcales). These peptides are known to especially show cytotoxicity, and antimicrobial and algicidal activity. Nostocyclamide was reported to increase the photosynthetic evolution of oxygen and respirarotry consumption of oxygen of the cyanobacteria *Anabaena* and *Synechococcus* and of spinach chloroplasts at low concentration.[26] These peptides may play allelopathic roles in the ecosystem.

Experimental Section

General Experimental Procedures. The UV spectrum was recorded on a Hitachi 330 spectrophotometer. Optical rotation was measured on a JASCO DIP-1000 polarimeter. ^{1}H, ^{13}C, and ^{15}N NMR spectra were obtained with either a JEOL JNM-A500 or 600 in DMSO-d_6 at 27.0 °C. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. ^{15}N chemical shifts were referenced to 112 ppm for NH_2CHO. FAB mass spectra, containing high-resolution FABMS, were recorded by employing a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500 A amino acid analyzer. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Wako Pure Chemical Industries (Osaka, Japan). P388 mouse leukemia cells were obtained from Dainippon Pharmaceuticals (Osaka, Japan).

Culture Conditions. *Microcystis aeruginosa* (NIES-298) was obtained from the NIES collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10 L glass bottles containing MA medium [$Ca(NO_3)_2 \cdot 4H_2O$ (5 mg), KNO_3 (10 mg), $NaNO_3$ (5 mg), Na_2SO_4 (4 mg), $MgCl_2 \cdot 6H_2O$ (5 mg), β-Na_2glycerophosphate (10 mg), $Na_2EDTA \cdot 2H_2O$ (0.5 mg), $FeCl_3 \cdot 6H_2O$ (0.05 mg), $MnCl_2 \cdot 4H_2O$ (0.5 mg), $ZnCl_2$ (0.05 mg), $CoCl_2 \cdot 6H_2O$ (0.5 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.08 mg), H_3BO_3 (2 mg), BICINE (50 mg), distilled water (100 mL), pH 8.6] with aeration (filtered air, 0.3 L/min) at 25 °C under illumination of 250 $\mu E/m^2 \cdot s$ on a 12L:12D cycle. Cells were harvested by continuous centrifugation at 10 000 rpm after incubation for 10–14 days. Harvested alga was lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Freeze-dried alga (65 g from 300 L of culture) was extracted with 80% MeOH (1.5 L × 2) and MeOH (1.5 L × 1). The combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension, and this was extracted with ether. The aqueous layer was then extracted with *n*-BuOH. The *n*-BuOH layer (2.1 g) was subjected to ODS flash chromatography (Cosmosil 140C18-PREP, 10 × 10 cm) with aqueous MeOH followed by CH_2Cl_2. The 100% MeOH fraction was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 20–70% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) and reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 25–50% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield microcyclamide (**1**, 52.7 mg).

Microcyclamide (1): colorless amorphous solid; UV (MeOH) λ_{max} 231 (ϵ 26500) nm; $[\alpha]^{23}_D$ −46.3° (*c* 0.1, MeOH); ^{1}H, ^{13}C, and ^{15}N NMR see Table 1; HRFABMS (positive) *m/z* 583.1931 $[M + H]^+$ (calcd for $C_{26}H_{31}N_8O_4S_2$, 583.1910).

Ozonolysis and Acid Hydrolysis of 1. Compound **1** (1.0 mg) in CH_2Cl_2 (1 mL) was ozonized at −10 °C for 20 min. After the removal of solvent by a nitrogen stream down to 100 μL, the reaction mixture was dissolved in 6 N HCl (0.4 mL) and heated at 105 °C for 16 h. The reaction mixture was dried, dissolved in 1.0 mL of 0.02% N HCl, and subjected to amino acid analysis. Retention times (min) of the standard amino acids were Asp (10.4), Ala (34.8), allo Ile (43.2), and Ile (45.0). Retention times of the amino acid analysis of **1** (min) were Asp (10.6), Ala (34.8), and Ile (45.0).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of ozonized **1**, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (L-FDAA) (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added, and the mixture was kept at 80 °C. After 3 min, 50 μL of 2 N HCl and 200 μL of 50% MeCN were added to the reaction mixture, which was then analyzed by reversed-phase ODS-HPLC: column, Cosmosil 5C18-MS (4.6 × 250 mm); gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min; flow rate 1.0 mL/min; UV-detection 340 nm. Retention times of standard amino acids (min): L-Asp (39.6), D-Asp (40.4), l-Ala (43.5), D-Ala (46.1), L-Ile (52.7), and D-Ile (56.8). Retention times of the amino acids of **1** (min): L-Asp (39.6), L-Ala (43.5), and L-Ile (52.7).

Assays for Cytotoxic Activity. P388 murine leukemia cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in 95% air, with RPMI-1640 media containing 10% fetal bovine serum, 500 units/mL penicillin, and 50 μg/mL streptomycin. The cells (2.5 × 104 cells/mL) were incubated with samples (ranging in concentration from 25 to 0.01 μg/mL) in 0.2 mL of cultured medium at 37 °C for 3 days. After incubation, the cells were treated with tetrazolium salt (MTT) for 4 h, the plate was centrifuged at 3000*g* for 5 min, and the resulting supernatant was removed by suction. The precipitates were dissolved in dimethyl sulfoxide, and absorbance was measured by a microtiter plate reader at a wavelength of 570 nm (reference wavelength at 630 nm). Cytotoxic activity was evaluated on the basis of a comparison of the survival ratio of the cells with the control value.

Acknowledgment. We are indebted to Associate Prof. S. Matsunaga, the University of Tokyo, for valuable advice on structure determination. This work was partly supported by a Grant-in-Aid for "Research for the Future" Program from

the Japan Society for the Promotion of Science. K.I. is financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

References and Notes

(1) Namikoshi, M.; Rinehart, K. L. *J. Indust. Microbiol.* **1996**, *17*, 373−384.
(2) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 501−504.
(3) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1997**, *53*, 10281−10288.
(4) Ishida, K.; Matsuda, H.; Murakami, M. *Tetrahedron* **1997**, *54*, 13475−13484.
(5) Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamagauchi, K. *Tetrahedron Lett.* **1993**, *34*, 8131−8134.
(6) Ishida, K.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 3535−3538.
(7) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1996**, *37*, 9225−9226.
(8) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 184−187.
(9) Ishida, K.; Matsuda, H.; Murakami, M. *Tetrahedron* **1998**, *54*, 5545−5556.
(10) Okino, T.; Matsuda, M.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1995**, *51*, 10679−10686.
(11) Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, *35*, 3129−3132.
(12) Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 2785−2788.
(13) Ishida, K.; Okita, Y.; Matsuda, H.; Okino, T.; Murakami, M. *Tetrahedron* **1999**, *55*, 10971−10988.
(14) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1996**, *52*, 9025−9030.
(15) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 724−726.
(16) Carmichael, W. W. *Sci. Am.* **1994**, *270*, 78−86.
(17) MacKintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P.; Codd, G. A. *FEBS Lett.* **1990**, *264*, 187−192.
(18) Lawton, L. A.; Morris, L. A.; Jaspars, M. *J. Org. Chem.* **1999**, *64*, 5329−5332.
(19) Carmichael, W. W. *J. Appl. Bacteriol.* **1992**, *72*, 445−459.
(20) Watanabe, M. M.; Hiroki, M. In *NIES-Collection List of Strains*, 5th ed.; *Microalgae and Protozoa Microbial Culture Collection;* National Institute of Environmental Studies: Tsukuba, Japan, 1997; pp 79−80.
(21) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(22) Banker, R.; Carmeli, S. *J. Nat. Prod.* **1998**, *61*, 1248−1251.
(23) Todorova, A. K.; Jüttner, F.; Linden, A.; Pluss, T.; von Philipsborn, W. *J. Org. Chem.* **1995**, *60*, 7891−7895.
(24) Ogino, J.; Moore, R. E.; Patterson, G. M. L.; Smith, C. D. *J. Nat. Prod.* **1996**, *59*, 581−586.
(25) Admi, V.; Afek, U.; Carmeli, S. *J. Nat. Prod.* **1996**, *59*, 396−399.
(26) Smith, G. D.; Doan, N. T. *J. Appl. Phycol.* **1999**, *11*, 337−344.

NP000159P