Kawaguchipeptin B, an Antibacterial Cyclic Undecapeptide from the Cyanobacterium *Microcystis aeruginosa*

Keishi Ishida, Hisashi Matsuda, Masahiro Murakami,* and Katsumi Yamaguchi

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

Received February 26, 1997[⊗]

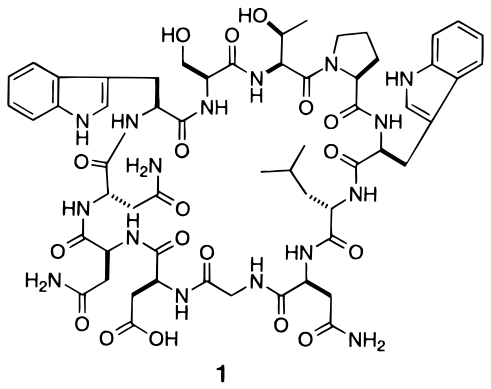
Kawaguchipeptin B, an antibacterial cyclic undecapeptide, was isolated from the cultured cyanobacterium *Microcystis aeruginosa* (NIES-88). Its structure was elucidated as **1** on the basis of 2D NMR data and chemical degradation. Kawaguchipeptin B (**1**) inhibited the growth of the Gram-positive bacterium *Staphylococcus aureus* at a concentration of 1 μg/mL (MIC).

Microcystis aeruginosa has been shown to be a rich source of unique peptides such as microcystins,[1] aeruginopeptins,[2] microcystilide A,[3] cyanopeptolins,[4] micropeptins,[5] microginin,[6] aeruginosins,[7] and microviridins.[8] In the course of our screening of protease inhibitory peptides from cyanobacteria, we recently reported the isolation and structure elucidation of kawaguchipeptin A (**2**),[9] a cyclic undecapeptide containing two prenyl Trp units, from the toxic *M. aeruginosa* (NIES-88). This unique peptide did not, however, show any protease inhibitory activity. In the process of the isolation of protease inhibitory peptides from other fractions, we found a related peptide, kawaguchipeptin B (**1**). Kawaguchipeptin B also had no any protease inhibitory activity, but showed antibacterial activity. Here we describe the isolation and structure elucidation of **1**.

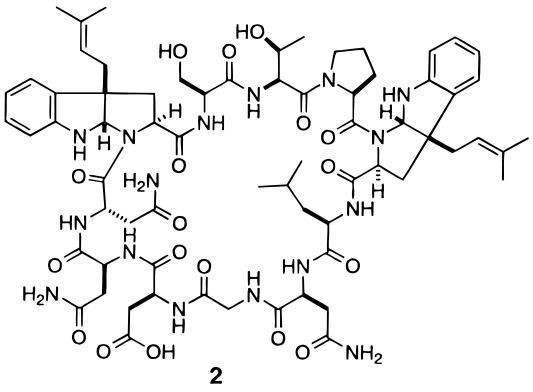
M. aeruginosa (NIES-88)[10] was isolated from a bloom in Lake Kawaguchi and mass-cultured in our laboratory as previously described.[11] The 80% MeOH extract of freeze-dried alga was partitioned between H_2O and Et_2O. The aqueous layer was further extracted with *n*-BuOH and subjected to ODS flash column chromatography followed by reversed-phase HPLC with aqueous MeCN containing 0.05% TFA to yield **1**.

Kawaguchipeptin B (**1**) was isolated as a colorless amorphous powder. The molecular formula of **1** was established as $C_{58}H_{76}N_{16}O_{18}$ by HRFABMS and NMR spectral data (Table 1). Its peptidic nature was suggested by its ^{1}H- and ^{13}C-NMR spectra, and amino acid analysis indicated the presence of Asp, Thr, Ser, Pro, Gly, Leu, and Trp.

Interpretation of the ^{1}H−^{1}H COSY and HOHAHA spectra easily led to assignment of Thr, Ser, Pro, Gly, and Leu residues. It was difficult to identify Asp, two Trp, and three Asn residues because of the overlap of NMR signals, but these amino acid residues could be clearly assigned by ^{1}H−^{1}H COSY, HOHAHA, NOESY, HMQC,[12] and HMBC[13] spectra. The presence of three Asn residues was confirmed by HMBC correlations from the primary amide proton and by NOESY cross peaks between the primary amide proton and β-H.[14] The sequence of **1** was deduced by HMBC and NOESY spectra. HMBC correlations were observed for Trp (I) α-H/Trp (I) C═O, Leu α-H/Trp (I) C═O, Leu β-H/Leu C═O, Asn (I) α-H/Asn (I) C═O, Gly α-H/Asn (I) C═O, Gly α-H/Gly C═O, Asp α-H/Gly C═O, Asp NH/Gly C═O, Asp α-H/Asp C═O, Asp β-H/Asp C═O, Asn (II) NH/Asp C═O, Asn (II) α-H/Asn (II) C═O, Asn (II) β-H/Asn (II) C═O, Asn (III) α-H/Asn (II) C═O, Asn (III) α-H/Asn (III) C═O, Asn (III) β-H/Asn (III) C═O, Trp (II) α-H/Trp (II) C═O, Ser NH/Trp (II) C═O, Ser α-H/Ser C═O, Thr α-H/Thr C═O, and Pro β-H/Pro C═O. NOESY cross peaks were observed for Trp (I) α-H/Leu NH, Leu α-H/Asn (I) NH, Asn (I) α-H/Gly NH, Gly α-H/Asp NH, Asp α-H/Asn (II) NH, Asn (II) α-H/Asn (III) NH, Asn (III) α-H/Trp (II) NH, Trp (II) NH/Ser α-H, Ser α-H/Thr NH, Thr α-H/Pro H-5, and Pro α-H/Trp (I) NH.



1



2

Analysis of the hydrolysate as Marfey derivatives[15] and chiral GC analysis of *N*-(trifluoroacetyl isopropyl ester) derivatives of the acid hydrolysate (1% phenol in 6 N HCl, 110 °C, 16 h)[16] of **1** indicated that all the amino acid residues were L.

The different stereochemistry of Leu in **2** (D-form) and that in **1** (L-form) indicated that **1** was not an artifact of **2**. Cyclic undecapeptides have also been isolated from

* To whom correspondence should be addressed. Phone: +81-3-3812-2111 ext. 5298. FAX: +81-3-5684-0622. E-mail: amura@hongo.ecc.u-tokyo.ac.jp.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S0163-3864(97)00146-8 CCC: $14.00

Table 1. ^{1}H- and ^{13}C-NMR Data of Kawaguchipeptin B in DMSO-d_6

		^{1}H J (Hz)	^{13}C			^{1}H J (Hz)	^{13}C
Trp (I)	1		171.25 (s)	Asn (III)	1		171.4 (s)
	2	4.41 (ddd, 10.0, 7.0, 4.0)	53.9 (d)		2	4.49 (ddd, 10.6, 7.3, 5.1)	50.0 (d)
	3	2.92 (dd, 14.6, 10.0)	26.4 (t)		3	2.30 (dd, 15.3, 5.1)	36.3 (t)
		3.17 (dd, 14.6, 4.0)				2.77 (dd, 15.3, 7.3)	
	1′	10.82 (d, 2.2)			4		172.46 (s)
	2′	7.09 (d, 2.2)	123.7 (d)		NH	7.88 (br)	
	3′		109.9 (s)	NH_2	NH_2	7.10 (br)	
	4′	7.56 (d, 8.1)	118.3 (d)			7.57 (br)	
	5′	6.97 (dd, 8.1, 7.0)	118.2 (d)	Trp (II)	1		171.33 (s)
	6′	7.04 (dd, 8.1, 7.0)	120.9 (d)		2	4.33 (m)	54.3 (d)
	7′	7.31 (dd, 8.1)	111.2 (d)		3	2.94 (dd, 14.2, 11.0)	26.1 (t)
	8′		136.1 (s)			3.30 (dd, 14.2, 3.0)	
	9′		127.1 (s)		1′	10.60 (d, 2.4)	
Leu	1		171.8 (s)		2′	7.12 (d, 2.4)	123.6 (d)
	2	4.27 (m)	53.9 (d)		3′		110.3 (s)
	3	1.43 (m)	40.2 (t)		4′	7.48 (d, 8.1)	117.9 (d)
		1.48 (m)			5′	6.96 (dd, 8.1, 7.0)	118.2 (d)
	4	1.55 (m)	24.1 (d)		6′	7.04 (dd, 8.1, 7.0)	120.8 (d)
	5	0.84 (d, 6.6)	21.6 (q)		7′	7.31 (d, 8.1)	111.3 (d)
	5′	0.87 (d, 6.6)	22.9 (q)		8′		135.8 (s)
	NH	7.79 (br)			9′		127.1 (s)
Asn (I)	1		170.8 (s)	Ser	1		169.5 (s)
	2	4.63 (ddd, 8.7, 6.0, 5.8)	50.1 (d)		2	4.34 (m)	56.2 (d)
	3	2.44 (dd, 14.4, 6.0)	39.0 (t)		3	3.70 (m)	61.4 (t)
		2.58 (dd, 14.4, 5.8)			NH	8.07 (d, 8.4)	
	4		170.8 (s)	Thr	1		169.6 (s)
	NH	8.24 (br)			2	4.60 (dd, 8.4, 4.8)	54.3 (d)
	NH_2	7.26 (br)			3	3.94 (m)	66.4 (d)
		7.45 (br)			4	0.98 (d, 6.2)	18.5 (q)
Gly	1		169.0 (s)		NH	7.34 (br)	
	2	3.67 (dd, 16.3, 5.5)	42.8 (t)	Pro	1		171.9 (s)
		3.82 (dd, 16.3, 5.7)			2	4.30 (m)	60.4 (d)
	NH	8.27 (br)			3	1.54 (m)	28.3 (t)
Asp	1		170.4 (s)			1.88 (m)	
	2	4.55 (ddd, 9.8, 5.7, 4.9)	49.5 (d)		4	1.38 (m)	23.9 (t)
	3	2.48 (dd, 16.5, 7.5)	35.9 (t)			1.68 (m)	
		2.78 (dd, 16.5, 4.9)			5	3.52 (m)	47.2 (t)
	4		171.8 (s)				
	NH	8.17 (d, 5.7)					
Asn (II)	1		170.2 (s)				
	2	4.30 (m)	50.2 (d)				
	3	2.42 (dd, 15.8, 8.5)	36.4 (t)				
		2.85 (dd, 15.8, 6.4)					
	4		172.56 (s)				
	NH	7.91 (d, 7.7)					
	NH_2	7.12 (br)					
		7.48 (br)					

the terrestrial cyanobacterium *Anabaena laxa*[17] and the marine cyanobacterium *Hormothamnion entermorphoides*,[18] but kawaguchipeptins A and B are a new class of cyclic undecapeptides. Kawaguchipeptins A and B inhibited the growth of the Gram-positive bacterium *Staphylococcus aureus* at a concentration of 1 μg/mL (MIC).

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Hitachi 330 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained with 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. FABMS were recorded by a JEOL JMS SX-102 mass spectrometer. Amino acid analyses were carried out with a Hitachi L 8500-A amino acid analyzer.

Culture Conditions. Culture conditions were the same as used for the production of kawaguchipeptin A.[9]

Extraction and Isolation. Freeze-dried alga (122 g from 590 L of culture) was extracted with 80% MeOH (2 L × 2) and MeOH (2 L × 1). The combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension, and this was extracted with Et_2O. The aqueous layer was then extracted with *n*-BuOH. The *n*-BuOH layer (7.0 g) was subjected to ODS flash chromatography (YMC-GEL, YMC, 10 × 12 cm) with aqueous MeOH followed by CH_2Cl_2. The 40% MeOH fraction was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm, Nacarai tesque; 30−70% 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; 30% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield kawaguchipeptin B (**1**, 11.0 mg). Kawaguchipeptin A (**2**) was isolated by MPLC and HPLC on ODS from MeOH fraction.

Kawaguchipeptin B (1): colorless amorphous solid; UV (MeOH) λ_{max} 276 (sh, ϵ 6940), 282 (7390), 290 (6270) nm; $[\alpha]_D$ −42.6° (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS (positive) *m*/*z* 1285.5623 (calcd for $C_{58}H_{77}N_{16}O_{18}$, 1285.5602).

Acid Hydrolysis of Kawaguchipeptin B. For amino acid analysis, 100 μg of **1** was dissolved in 0.5

mL of 6 N HCl containing 1% phenol[16] and heated at 110 °C for 16 h. The reaction mixture was dried, dissolved in 0.6 mL of 0.02 N HCl, and subjected to amino acid analysis. Retention times in the amino acid analysis of **1** (min): Asp (10.53), Thr (15.22), Ser (16.82), Pro (32.08), Gly (33.60), Leu (48.05), and Trp (66.66).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of a 100-μg portion of the peptide, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in Me_2CO (L-FDAA) (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added, and the mixture was kept at 80 °C for 3 min. To the reaction mixture, 50 μL of 2 N HCl and 200 μL of 50% MeCN were added and analyzed by reversed-phase ODS-HPLC: column, Cosmosil 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; UV (340 nm). Retention times of standard amino acids (min): D, L-Ser (35.8), L-Thr (36.5), L-alloThr (36.8), L-Asp (37.2), D-Asp (38.0), D-alloThr (38.2), Gly (39.1), D-Thr (39.8), L-Pro (41.6), D-Pro (42.8), L-Trp (50.4), L-Leu (51.7), D-Trp (53.0), and D-Leu (55.8). Retention times of the amino acids of **1** (min): Ser (35.8), Thr (36.5), Asp (37.2), Gly (39.1), Pro (41.6), Trp (50.4), and Leu (51.7).

Chiral GC Analysis of Kawaguchipeptin B. Compound **1** (100 μg) was dissolved in 0.5 mL of 6 N HCl containing 1% phenol and heated at 110 °C for 16 h. The reaction mixture was evaporated in a stream of nitrogen, dissolved in 10% HCl in *i*PrOH (200 μL), and heated at 100 °C for 30 min. The resulting mixture was evaporated, dissolved in trifluoroacetic anhydride (100 μL) and CH_2Cl_2 (100 μL), reacted at 100 °C for 10 min, and evaporated. The residues was dissolved in CH_2Cl_2 and analyzed by GC with Chirasil-L-Val (Chrompak) column. The oven temperature was maintained for 5 min at 60 °C and raised to 200 °C at 4 °C/min, which was maintained for 10 min. Retention times for standard amino acids (min): D-Ser (15.04) and L-Ser (15.82). Retention time of derivatives of the acid hydrolysate of **1** (min): Ser (15.83).

Antibacterial Assay. Filter paper disks saturated with test solutions were placed on agar plate seeded with *S. aureus*. Assay for *S. aureus* was carried out in the beef extract medium; 1% Bacto-peptone, 1% beef extract, 0.5% NaCl, and 1.2% agar. The plate was incubated overnight at 37 °C. The quantity of antimicrobial activity was evaluated by a minimum inhibitory concentration (MIC) of μg/mL.

Acknowledgments. We are indebted to Y. Nogata, The University of Tokyo, for antimicrobial assay. This work was partly supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan. K. Ishida and H. Matsuda are financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

References and Notes

(1) Carmichael, W. W. *J. Appl. Bacteriol.* **1992**, *72*, 445−459.
(2) Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett.* **1993**, *34*, 6091−6094.
(3) Tsukamoto, S.; Painuly, P.; Young, K. A.; Yang, X.; Shimizu, Y. *J. Am. Chem. Soc.* **1993**, *115*, 11 046−11 047.
(4) Martin, C.; Oberer, L.; Ino, T.; König, W. A.; Busch, M.; Weckesser, J. *J. Antibiot.* **1993**, *46*, 1550−1556.
(5) (a) Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 8131−8134. (b) Ishida, K.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 3535−3538. (c) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1996**, *37*, 9225−9226.
(6) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1992**, *34*, 501−504.
(7) Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 3535−3538.
(8) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1995**, *51*, 10 679−10 686.
(9) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1996**, *52*, 9025−9030.
(10) Watanabe, M. M.; Kasai, F.; Hiwatari, T.; Suda, S.; Nei, T. *Jpn. J. Freez. Dry.* **1984,** *30*, 23−26.
(11) Watanabe, M. M.; Satake, K. N. *In NIES-Collection List of Strains*, 3rd ed., *Microalgae and Protozoa Microbial Culture Collection*; National Institute of Environmental Studies: Tsukuba, Japan, 1991; p 32.
(12) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *647*, 565−569.
(13) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(14) HMBC correlations were observed from Asn (I) NH_2 to 2−C═O and Asn (II) NH_2 to 2−C═O and C-2. NOESY cross peaks were observed for Asn (I) NH_2/H-3 and Asn (III) NH_2/H-3.
(15) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(16) Muramoto, K.; Kamiya, H. *Anal. Biochem.* **1990**, *189*, 223−230.
(17) (a) Frankmölle, W. P.; Larsen, L. K.; Caplan, F. R.; Patterson, G. M. L.; Knübel, G.; Levine, I. A.; Moore, R. E. *J. Antibiot.* **1992**, *45*, 1451−1457. (b) Frankmölle, W. P.; Knübel, G.; Moore, R. E.; Patterson, G. M. L. *J. Antibiot.* **1992**, *45*, 1458−1466.
(18) Gerwick, W. H.; Jiang, Z. D.; Agarwal, S. K.; Farmer, B. T. *Tetrahedron* **1992**, *48*, 2313−2324.

NP970146K