Pergamon

Tetrahedron Vol. 51, No. 39, pp. 10679-10686, 1995
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0040-4020/95 $9.50+0.00

0040-4020(95)00645-1

NEW MICROVIRIDINS, ELASTASE INHIBITORS FROM THE BLUE-GREEN ALGA *MICROCYSTIS AERUGINOSA*

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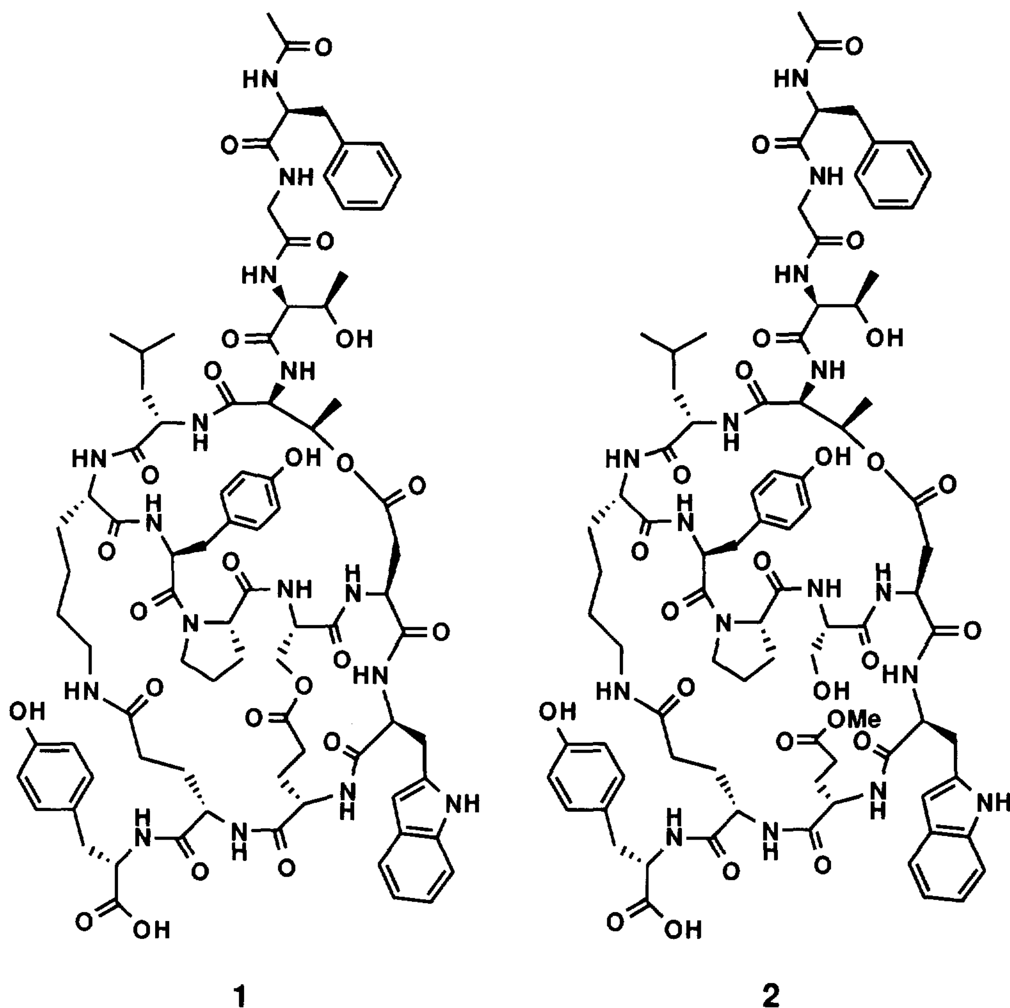
Abstract: Microviridins B and C were isolated from the freshwater blue-green alga *Microcystis aeruginosa* (NIES-298). Their structures were elucidated to be **1** and **2** on the basis of 2D NMR data and chemical degradation. These peptides inhibited elastase potently.

Blue-green algae are well known to produce unique biologically active peptides. Especially the genus *Microcystis* is a rich source for peptide metabolites and has been paid much attention to as the cause of serious problems to human and livestock health. The hepatotoxic cyclic peptides, microcystins, are responsible for worldwide poisoning caused by blue-green algae[2] and over 40 microcystin-type peptides have been reported to date. Recently other types of peptides have been identified from *Microcystis* spp. The second group is Ahp (3-amino-6-hydroxypiperidone)-containing depsipeptides. Micropeptins were isolated from a nontoxic strain of *M. aeruginosa* (NIES-100) and showed trypsin and plasmin inhibition.[3] Microcystilide A,[4] aeruginopeptins[5] and cyanopeptolins[6] were isolated from toxic strains of *Microcystis* spp. Microcystilide A showed cell-defferentiation-promoting activity, but no biological activities have been reported for aeruginopeptins and cyanopeptolins. In addition, further different types of peptides are produced by *Microcystis* spp. A linear pentapeptide, microginin, was isolated from a nontoxic strain of *M. aeruginosa* (NIES-100) as an angiotensin-converting enzyme inhibitor.[7] A tricyclic peptide, microviridin, was isolated from a toxic strain of *M. viridis*.[8] It consisted of 14 amino acids and inhibited tyrosinase. Thrombin and trypsin inhibitors, aeruginosin 298-A[9] and aeruginosins 98-A and B[10] were isolated from *M. aeruginosa* (NIES-298, NIES-98). Cytotoxic aeruginoguanidine was isolated from *M. aeruginosa* (NIES-98).[11] In the course of our search for protease inhibitors from microalgae, we have also reported a trypsin inhibitor, radiosumin, and an elastase and chymotrypsin inhibitor, nostopeptin A,[12] from cultured freshwater blue-green algae along with *Microcystis* peptides.

We also found potently active elastase inhibitors from *M. aeruginosa* (NIES-298) which produced aeruginosin 298-A and identified them to be microviridin-type peptides. Elastase inhibitors have potential for therapeutic agents of pulmonary emphysema. In this paper the isolation and structure elucidation of the new elastase inhibitors will be described.



M. aeruginosa (NIES-298) was obtained from the NIES-collection and cultured in our laboratory. The 80 % methanol extract of freeze-dried algal cells was subjected to solvent partitioning. Both hydrophilic and lipophilic layers showed elastase inhibitory activities. Each layer was separated by ODS flash chromatography and the active fractions were joined together to be resubjected to ODS flash chromatography. Many active fractions were obtained and each of them was subjected to ODS HPLC. A single peak obtained from the most potent fraction showed a strong inhibition against elastase. But FAB mass spectrum of this peak revealed the presence of two components whose molecular weights were 1754 and 1722. To separate the two compounds, the single peak was subjected to CN HPLC. The yields of two active components, **1** and **2**, were 40 mg and 18 mg, respectively. We designated them to be microviridins B and C. Molecular weights of components in other moderately active fractions were 1700-1800 by FABMS. These results implied the presence of a lot of closely related compounds. Their contents, however, were too low to purify for structure elucidation.

Table 1. ^{1}H and ^{13}C data of microviridin B (1) in DMSO-d_6

Position	^{1}H	J (Hz)	^{13}C	HMBC (^{1}H)
Ac				
1			169.5	Ac 2, Phe 2, NH
2	1.75	(s)	22.4	
Phe				
1			171.8	Phe 2, 3, Gly 2, NH
2	4.49	(m)	54.2	Phe 3, NH, Gly NH
3	2.76	(d, 13.9)	37.4	Phe 2, 4, 5, 9, NH
	3.02	(dd, 13.9,4.0)		
4			138.1	Phe 2, 3, 6, 8
5,9	7.26	(m)	129.1	Phe 3, 7
6,8	7.25	(m)	128.0	Phe 7
7	7.17	(m)	126.2	Phe 6, 8
NH	8.17	(d, 8.0)		
Gly				
1			168.8	Gly 2, Thr(I) 2, NH
2	3.80	(br)	42.0	Gly NH
NH	8.34	(t, 5.4)		
Thr(I)				
1			170.14	Thr(I) 2, 3, Thr(II) 2, NH
2	4.45	(m)	57.7	Thr(I) 4, OH, NH
3	3.97	(m)	66.7	Thr(I) 2, 4, OH, NH
4	1.02	(d, 6.2)	18.5	Thr(I) 2, OH
OH	5.16	(d, 4.4)		
NH	7.84	(d, 7.9)		
Thr(II)				
1			168.8	Thr(II) 2, Leu 2, NH
2	4.63	(d, 9.0)	54.6	Thr(II) 4
3	5.36	(d, 6.3)	71.2	Thr(II) 2, 4
4	1.14	(d, 6.2)	17.1	Thr(II) 3
NH	7.69	(d, 8.5)		
Leu				
1			170.08	Leu 2, 3, Lys NH
2	4.25	(m)	50.8	Leu 3, 4
3	1.34	(m)	40.5	Leu 2, 4, 5, 5'
	1.56	(m)		
4	1.44	(m)	24.0	Leu 3, 5, 5'
5	0.76	(d, 6.3)	21.6	Leu 3, 4, 5'
5'	0.82	(d, 6.4)	23.1	Leu 3, 4, 5
NH	8.43	(d, 8.3)		
Lys				
1			170.0	Lys 2, Tyr(I) NH
2	4.10	(m)	52.5	Lys 4, NH
3	1.54	(m)	31.9	Lys 2, 4, 5
4	1.20	(m)	21.2	Lys 2, 3
5	1.28	(m)	28.6	Lys 4
	1.40	(m)		
6	2.94	(m)	37.4	Lys 4, 5
	3.19	(m)		
αNH	6.72	(d, 6.6)		
εNH	6.90	(m)		
Tyr(I)				
1			171.0	Tyr(I) 2, 3
2	4.34	(m)	51.7	
3	2.66	(m)	37.4	Tyr(I) 2, 5, 9
	2.73	(m, 10.6)		
4			126.4	Tyr(I) 3, 6, 8
5,9	6.95	(d, 8.2)	130.0	Tyr(I) 3, 5, 9
6,8	6.66	(d, 8.2)	115.1	Tyr(I) OH
7			156.2	Tyr(I) 5, 6, 8, 9, OH
OH	9.28	(s)		
NH	8.38	(d, 7.4)		
Pro				
1				
2	3.50	(m)	60.5	
3	1.60	(m)	30.6	Pro 2
4	1.48	(m)	21.7	Pro 5
	1.64	(m)		
5	3.21	(m)	46.1	
	3.38	(m)		
Ser				
1			170.3	Asp NH
2	4.49	(m)	52.3	
3	4.14	(d, 10.6)	61.7	
	4.75	(d, 8.9)		
NH	6.66	(d, 8.2)		
Asp				
1			172.4	Trp 2, NH
2	4.39	(br)	52.0	Asp 3
3	2.64	(m)	34.4	Asp 2
	2.87	(m)		
4			169.3	Asp 3, Thr(II) 3
NH	9.03	(br)		
Trp				
1			171.5	Trp 2, 3, Glu(I) NH
2	4.47	(m)	54.0	Trp 3
3	3.22	(m)	25.7	Trp 2
1'	10.9	(s)		
2'	7.28	(s)	123.7	Trp 3, 1'
3'			109.1	Trp 2, 3, 1', 4'
4'	7.42	(d, 7.6)	118.1	Trp 5', 6'
5'	6.98	(m)	118.5	Trp 6', 7'
6'	7.05	(dd, 7.5, 7.5)	121.1	Trp 4'
7'	7.27	(m)	111.6	Trp 4', 5', 6'
8'			127.2	Trp 3, 1', 2', 4'
9'			136.2	Trp 1', 2', 4', 6', 7'
NH	7.42	(d, 7.6)		
Glu(I)				
1			169.7	Glu(II) NH
2	3.93	(m)	53.3	
3	1.50	(m)	24.2	
	1.81	(m)		
4	1.05	(m)	29.5	
	2.06	(m)		
5				
NH	6.51	(d, 6.4)		
Glu(II)				
1			170.9	Tyr(II) 2, NH
2	4.09	(m)	51.9	
3	1.89	(m)	31.9	Glu(II) 2
	2.11	(m)		
4	1.53	(m)	28.2	Glu(II) 2, 3
	2.03	(m)		
5			171.1	Lys 6, εNH
NH	7.17	(m)		
Tyr(II)				
1			172.7	Tyr(II) 2, 3
2	4.26	(m)	53.9	Tyr(II) 3, NH
3	2.79	(m)	35.9	Tyr(II) 2, 5, 9, NH
	2.90	(dd, 13.9, 4.7)		
4			127.4	Tyr(II) 2, 3, 6, 8
5,9	7.01	(d, 8.2)	130.0	Tyr(II) 3, 5, 9
6,8	6.63	(d, 8.2)	115.0	Tyr(II) OH
7			155.9	Tyr(II) 5, 6, 8, 9, OH
OH	9.18	(s)		
NH	7.79	(d, 7.4)		

Table 2. ^{1}H and ^{13}C NMR data of microviridin C (2) in DMSO-d_6

Position	^{1}H	J (Hz)	^{13}C	HMBC (^{1}H)
Ac				
1			169.5	Ac 2, Phe 2, NH
2	1.74	(s)	22.4	
Phe				
1			171.8	Phe 2, 3, Gly 2, NH
2	4.48	(m)	54.2	Phe 3, NH
3	2.74	(m)	37.4	Phe 2, 5
	3.00	(m)		
4			138.1	he 2, 3, 6, 7, 8
5,9	7.24	(s)	129.1	Phe 3
6,8	7.25	(s)	128.0	Phe 5, 7, 9
7	7.18	(dd, 8.5,4.3)	126.2	Phe 5, 9
NH	8.17	(d, 8.0)		
Gly				
1			168.8	Gly 2, Thr(I) 2, NH
2	3.77	(br)	42.0	
NH	8.35	(t, 5.5)		
Thr(I)				
1			170.1	Thr(I) 2, 3, Thr(II) NH
2	4.43	(m)	57.6	Thr(I) 4
3	3.97	(m)	66.8	Thr(I) 2, 4
4	1.01	(d, 6.3)	18.9	Thr(I) 2, OH
OH	5.02	(d, 4.9)		
NH	7.78	(d, 7.4)		
Thr(II)				
1			169.0	Thr(II) 2
2	4.59	(d, 9.0)	54.8	Thr(II) 4
3	5.36	(d, 6.0)	70.4	Thr(II) 4
4	1.10	(d, 6.0)	16.5	
NH	7.85	(d, 8.4)		
Leu				
1			170.9	Leu 2, Lys NH
2	4.16	(m)	51.4	
3	1.41	(m)	40.0	Leu 5, 5'
	1.56	(m)		
4	1.50	(m)	24.1	Leu 5, 5'
5	0.75	(d, 6.2)	21.3	Leu 5'
5'	0.84	(d, 6.2)	23.0	Leu 5
NH	8.38	(d, 6.0)		
Lys				
1				
2	4.22	(m)	52.8	
3	1.55	(m)	32.2	
4	1.23	(m)	22.7	
5	1.39	(m)	29.2	
6	3.02	(m)	38.3	
αNH	6.58	(d, 7.1)		
εNH	7.38	(br)		
Tyr(I)				
1				
2	4.45	(m)	51.6	
3	2.69	(m)	38.5	Tyr(I) 5
4			126.2	Tyr(I) 3, 6, 9
5,9	6.92	(d, 8.1)	130.2	Tyr(I) 3
6,8	6.66	(d, 8.1)	115.1	Tyr(I) OH
7			156.2	Tyr(I) 6, 8, OH
OH	9.28	(s)		
NH	8.25	(d, 6.4)		

Position	^{1}H	J (Hz)	^{13}C	HMBC (^{1}H)
Pro				
1			169.6	Pro 2, Ser 2
2	3.57	(m)	60.4	
3	1.44	(m)	31.1	
	1.74	(m)		
4	1.61	(m)	21.6	
5	3.25	(m)	46.5	
	3.52	(m)		
Ser				
1				
2	4.31	(t, 6.5)	54.2	Ser OH
3	3.55	(m)	61.6	
OH	5.20	(br)		
NH	7.38	(br)		
Asp				
1			170.1	Trp NH
2	4.62	(m)	49.1	
3	2.58	(m)	35.1	
	2.75	(m)		
4			170.49	Thr(II) 3
NH	8.59	(d, 7.1)		
Trp				
1			171.2	Trp 2, Glu(I) NH
2	4.38	(d, 6.2)	54.2	
3	2.89	(m)	27.7	
	3.10	(d, 10.1)		
1'	10.77	(s)		
2'	7.15	(s)	123.8	
3'			109.5	Trp 2, 2', 4'
4'	7.52	(d, 7.8)	118.16	Trp 6'
5'	6.95	(dd, 7.8, 8.0)	118.23	
6'	7.03	(dd, 8.0, 8.0)	120.8	
7'	7.30	(d, 8.0)	111.3	
8'			127.1	Trp 7'
9'			131.1	Trp 4'
NH	7.59	(d, 7.0)		
Glu(I)				
1			170.35	Glu(I) 2, 3
2	4.07	(d, 6.2)	52.4	
3	1.78	(m)	26.3	Glu(I) 2
3	1.78	(m)	26.3	Glu(I) 2
4	2.19	(m)	29.6	
5			172.8	Glu(I) 3, 4, *O*-Me
NH	7.78	(d, 7.4)		
O-Me	3.55	(s)	51.3	
Glu(II)				
1				
2	4.21	(m)	51.8	
3	1.60	(m)	28.3	
	1.92	(m)		
4	2.06	(m)	31.5	
5			171.5	Glu(II) 4, Lys εNH
NH	7.90	(d, 7.6)		
Tyr(II)				
1			172.8	Tyr(II) 3
2	4.29	(m)	54.2	
3	2.78	(dd, 3.6, 8.6)	35.9	Tyr(II) 5, 9
	2.92	(m)		
4			127.6	Tyr(II) 3, 6, 8
5,9	6.98	(d, 8.1)	130.0	Tyr(II) 3
6,8	6.62	(d, 8.1)	114.9	Tyr(II) OH
7			155.8	
OH	9.16	(s)		
NH	7.90	(d, 7.6)		

Microviridin B (**1**) is colorless amorphous powder: $[\alpha]_D^{23}$ +153° (*c* 0.14, MeOH); UV (MeOH) λ_{max} 220 nm (ε 38,000), 279 (5,400). The molecular formula of **1** was established to be $C_{84}H_{106}N_{16}O_{24}$ by the HRFABMS (*m/z* 1723.7632 $[M+H]^+$ Δ -1.2 mmu) and NMR spectral data. Its peptidic nature was suggested by the ^1H and ^{13}C NMR spectra of **1** (Table 1), and the amino acid analysis of the hydrolyzate gave Asp, Thr, Ser, Glu, Gly, Leu, Tyr, Phe, Lys, Pro. The extensive NMR analyses including ^1H-^1H COSY, HOHAHA, HMQC and HMBC spectra could assign all the proton signals and the carbon signals except for carbonyl carbons of Pro and Glu(I), indicating the presence of 1 mole each of Asp, Ser, Gly, Leu, Phe, Lys, Pro and Trp, and 2 moles each of Thr, Glu and Tyr in the molecule.

HMBC correlations from α-H and NH to C=O (Table 1) determined partial sequences of Ac-Phe-Gly-Thr(I)-Thr(II)-Leu-Lys-Tyr(I)- and -Trp-Glu(I)-. NOESY peaks (Ac H-2/Phe NH; Phe H-2/Gly NH; Gly NH; H-2/Thr(I) NH; Thr(I) NH/Thr(II) NH; Thr(I) H-2/Thr(II) NH; Thr(II) H-2/Leu NH; Thr(II) H-3/Leu NH; Leu NH/Lys NH; Leu H-2/Lys NH; Lys H-2/Tyr(I) NH; Trp NH/Glu(I) NH; Trp H-2/Glu(I) NH) supported these partial sequences. HMBC peak between Thr(II) H-3 (the lowfield proton; δ 5.36) and Asp β-C=O suggested that Thr(II) and Asp were esterified. NOESY correlations (Tyr(I) H-2/Pro H-2; Tyr(I) H-3/Pro H-2; Ser H-2/Asp NH; Ser H-3/Asp NH; Asp NH/Trp NH; Glu(I) NH/Glu(II) NH; Glu(I) H-2/Glu(II) NH; Glu(II) NH/Tyr(II) NH; Glu(II) H-2/Tyr(II) NH) led to partial sequences of -Tyr(I)-Pro- and -Ser-Asp-Trp-Glu(I)-Glu(II)-Tyr(II). Furthermore the linkage between Lys ε-NH and Glu(II) γ-C=O was confirmed by the NOESY peaks (Lys H-6/Glu(II) H-3; Lys H-6/Glu(II) H-4; Lys ε-NH/Glu(II) H-3; Lys ε-NH/Glu(II) H-4). These findings suggested that **1** had a closely related structure to microviridin (microviridin A[13]) isolated from *Microcystis viridis*.[8] Finally a sequence of -Pro-Ser- and esterification between Ser and Glu(I) were suggested by the lowfield chemical shifts of Ser H-3 (δ 4.14, 4.75) and the structure similarity to microviridin. The highfield chemical shift of Glu(I) H-3 (δ 1.05) might be due to anisotropy of an indole ring of Trp. To confirm the stereochemistry of amino acids including Trp, **1** was hydrolyzed by 6 N HCl and 1 % HCO_2H. Chiral GC analysis (Chirasil-Val, Alltech) of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolyzate clarified that all of amino acid residues in **1** were L-form. These results proposed the structure of microviridin B (**1**).

Microviridin C (**2**) is colorless amorphous powder: $[\alpha]_D^{23}$ -2° (*c* 0.06, MeOH); UV (MeOH) λ_{max} 218 nm (ε 30,000), 280 (4,400). The molecular formula of **2** was established to be $C_{85}H_{110}N_{16}O_{25}$ by the HRFABMS (*m/z* 1755.7947 $[M+H]^+$ Δ +4.0 mmu) and NMR spectral data (Table 2), suggesting number of unsaturation of **2** decreased by 1 than that of **1**. ^1H and ^{13}C NMR spectra of **2** suggested that **2** was closely related to **1**. The amino acid analysis of **2** indicated that **2** had the same amino acid composition as **1**. The detailed analyses of ^1H-^1H COSY, HOHAHA, HMQC and HMBC spectra also supported this result. The major difference of **2** from **1** was the presence of a methoxy group (δ_H 3.55, δ_C 51.3) and one more hydroxyl group (δ_H 5.20) correlating with Ser H-2 in COSY spectrum. Therefore Ser and Glu(I) were suggested not to be esterified and γ-carboxylic acid of Glu(I) was expected to exist as a methyl ester. The sequence of **2** was confirmed by analyses of HMBC and NOESY spectrum. HMBC correlations of **2** (Table 2) determined partial sequences of Ac-Phe-Gly-Thr(I)-Thr(II)-, -Leu-Lys-, -Pro-Ser- and -Trp-Glu(I)-. The sequence of Pro-Ser gave information supporting for the ambiguous sequence, Pro-Ser, of **1**. The linkages between Lys ε-NH and Glu(II) γ-C=O and between *O*-Me and Glu(I) were also determined by HMBC spectrum. NOESY correlations

(Ac H-2/Phe NH; Phe H-2/Gly NH; Gly NH/Thr(I) NH; Gly H-2/Thr(I) NH; Thr(I) H-2/Thr(II) NH; Thr(I) H-3/Thr(II) NH; Thr(I) H-4/Thr(II) NH; Leu NH/Lys NH; Leu H-2/Lys NH; Pro H-4/Ser NH; Trp NH/Glu(I) NH; Trp H-2/Glu(I) NH; Trp H-3/Glu(I) NH; Lys H-6/Glu(II) H-4; Lys ε-NH/Glu(II) H-4) supported partial sequences obtained from HMBC spectrum. Furthermore partial sequences of -Thr(II)-Leu-Lys-Tyr(I)-Pro-, -Ser-Asp-Trp- and -Glu(I)-Glu(II)- were determined by NOESY peaks (Thr(II) H-2/Leu NH; Thr(II) H-3/Leu NH; Thr(II) H-4/Leu NH; Lys H-2/Tyr(I) NH; Tyr(I) H-2/Pro H-2; Tyr(I) H-3/Pro H-2; Tyr(I) H-5/Pro H-2; Tyr(I) H-9/Pro H-2; Ser H-2/Asp NH; Asp NH/Trp NH; Asp H-2/Trp NH; Glu(I) NH/Glu(II) NH; Glu(I) H-2/Glu(II) NH). The structural information of **1** suggested the linkage of Glu(II)-Tyr and ester bond between Thr(II) and Asp, though no information was given by NMR spectra of **2**. To determine the stereochemistries of **2**, **2** was also hydrolyzed by 6 N HCl and 1 % HCO_2H. Chiral GC analysis (Chirasil-Val, Alltech) of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolyzate clarified that all of amino acid residues in **2** were L-form. The structure of microviridin C was proposed to be **2**.

Microviridins B and C inhibited elastase potently (IC_{50}=0.044 and 0.084 μg/mL, respectively). Chymotrypsin (IC_{50}=2.5, 4.9 μg/mL) and trypsin (IC_{50}=58, 32 μg/mL) were inhibited weakly by microviridins B and C. These compounds did not inhibit thrombin and plasmin at 100 μg/mL. In this connection, microviridin A, which was isolated from *M. aeruginosa* (NIES-89), did not show these serine protease inhibitory activities,[13] although it was reported to inhibit tyrosinase.[8]

Microviridins B (**1**) and C (**2**) were thus proved to be tricyclic and dicyclic peptides, respectively. The structure of **1** only differs from microviridin A in having Phe, Thr and Leu in place of Tyr, Gly and Phe, respectively. Almost the same IC_{50} against elastase of **1** and **2** suggests that the linkage of Ser-O-Glu(I) or the ester bond between Ser and Glu(I) is apart from reactive site or does not influence their conformation.

Experimental

General methods: NMR spectra were recorded on a Bruker AM600 NMR spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C. ^1H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. Optical rotations were determined by a JASCO DIP-371 digital polarimeter. Ultraviolet spectra were measured on a Hitachi 330 spectrophotometer. FAB mass spectra were measured by using glycerol as a matrix on a JEOL SX102 mass spectrometer. Amino acid analyses were carried out with a Hitachi 835 amino acid analyzer.

Culture of *Microcystis aeruginosa*: *Microcystis aeruginosa* (NIES-298) was obtained from the NIES-collection 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 at 25 °C under illumination of 250 $\mu E/m^2 \cdot s$ on a 12L:12D cycle. After 10-14 days, the algal cells were harvested by continuous flow centrifugation at 11,000 rpm. The cells were yielded at 0.3 g/L on an average and kept in a freezer at -20 °C until extracted.

Extraction and isolation: Freeze-dried alga (130 g for 362 L of culture) was extracted with $MeOH/H_2O$ (8:2), concentrated and partitioned between Et_2O and H_2O. The H_2O soluble fraction was partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was subjected to ODS flash chromatography with increasing amounts of MeOH in water. The Et_2O soluble fraction was partitioned between *n*-hexane and $MeOH/H_2O$ (9:1) and subsequently between CCl_4 and $MeOH/H_2O$ (8:2). The 80 % MeOH layer was subjected to ODS flash chromatography with increasing amounts of MeOH in water. The active fractions obtained from both layers by ODS chromatography were joined together and resubjected to ODS flash chromatography. The fraction eluted with 80 % MeOH was separated by HPLC on Capcell Pak C_{18} ($MeCN/H_2O/TFA$ 32:68:0.05) followed by HPLC on Develosil CN-5 ($MeOH/H_2O/TFA$ 55:45:0.05) to yield microviridins B (**1**, 40 mg) and C (**2**, 18 mg).

Amino acid analyses: Compounds **1** and **2** (each 100 μg) were dissolved in 6 N HCl (1 mL) and sealed in test tubes. The test tube was heated at 110 °C for 16 h. The solution was evaporated and redissolved in 0.1 N HCl to subject on a Hitachi 835 amino acid analyzer.

The hydrolyzates by 6 N HCl and 1 % HCO_2H at 110 °C for 16 h of **1** and **2** were heated in 10 % HCl in *i*-PrOH (0.5 mL) at 100 °C for 30 min and then treated with trifluoroacetic anhydride/CH_2Cl_2 (1:1, 0.6 mL) at 100 °C for 5 min. Chiral GC was carried out by using a Chirasil Val III capillary column (0.32 mm x 25 m) with a flame ionization detector (FID). Column temperature was kept at 50 °C for 10 min and increased to 200 °C at a rate of 4 °C/min. Helium was used as carrier gas. Retention times (minutes): D-Thr (19.6), L-Thr (21.1), D-Pro (22.9), L-Pro (23.1), D-Ser (23.8), D-Leu (23.9), D-*a*Thr (24.1), L-Ser (24.8), L-*a*Thr (25.4), L-Leu (25.8), D-Asp (30.0), L-Asp (30.4), D-Phe (34.2), D-Tyr (34.7), L-Phe (35.1), L-Tyr (35.6), D-Glu (39.6), L-Glu (40.4), D-Lys (45.1), L-Lys (45.7), D-Trp (47.9), L-Trp (48.4).

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES and NOTES

1. Present address: Fusetani Biofouling Project, ERATO, Niigata Engineering Co., Ltd. Isogo-ku, Yokohama 235, Japan.
2. Carmichael, W. W. *J. Appl. Bacteriol.* **1992**, 72, 445-459.
3. Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, 34, 8131-8134.
4. Tsukamoto, S.; Painuly, P.; Young, K. A.; Yang, X.; Shimizu, Y.; Cornell, L. *J. Am. Chem. Soc.* **1993**, 115, 11046-11047.
5. Harada, K.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett.* **1993**, 34, 6091-6094.
6. Martin, C.; Oberer, L.; Ino, T.; König, W. A.; Busch, M.; Weckesser, J. *J. Antibiot.* **1993**, 46, 1550-1556.
7. Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, 34, 501-504.

8. Ishitsuka, M. O.; Kusumi, T.; Kakisawa, H.; Kaya, K.; Watanabe, M. M. *J. Am. Chem. Soc.* **1990,** 112, 8180-8182.
9. Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, 35, 3129-3132.
10. Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Letters* **1995**, 36, 2785-2788.
11. Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *36th Symp. Chem. Nat. Prod. (Hiroshima) Symp. Papers* **1994**, 493-500.
12. Matsuda, H.; Okino, T.; Haraguchi, R.; Murakami, M.; Yamaguchi, K. *35th Symp. Chem. Nat. Prod. (Kyoto) Symp. Papers* **1993**, 654-661.
13. The active compounds **1** and **2** were related peptides to microviridin and isolated not from *M. viridis* but from *M. aeruginosa*. The name of microviridin apparently originated from *M. viridis*. If we designate **1** and **2** as other names, it may confuse the recognition of these peptides. We isolated microviridin from the toxic strain of *M. aeruginosa* (NIES-89), so we rename this peptide as microviridin A. Accordingly, the new elastase inhibitors described in this paper are designated to be microviridins B and C.

(*Received in Japan* 21 *June* 1995; *accepted* 2 *August* 1995)