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Microviridins D-F,[1] Serine Protease Inhibitors from the Cyanobacterium *Oscillatoria agardhii* (NIES-204)

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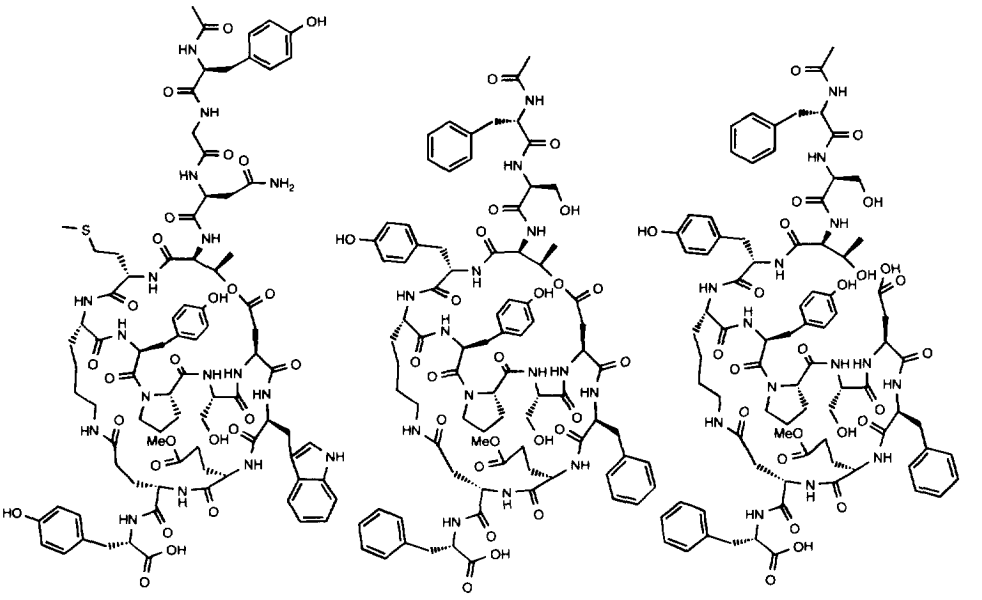
Abstract : New serine protease inhibitors, microviridins D, E and F, were isolated from the cyanobacterium *Oscillatoria agardhii* (NIES-204). Their structures were elucidated to be **1-3** by extensive 2D NMR data and chemical degradation. These dicyclic or cyclic peptides inhibited serine protease potently.

Cyanobacteria commonly found throughout the world under widely varied conditions have proven to be a rich source of biologically active metabolites. These metabolites have been important biomedically as leads to new pharmaceutical compounds, herbicides and pesticides. From the genus *Microcystis*, in particular, potent cyclic heptapeptide hepatotoxins termed microcystins have been isolated. More than forty microcystins produced by some genera of freshwater cyanobacteria such as *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* have been reported thus far.[2] Nodularins, cyclic pentapeptide hepatotoxins related to microcystins, have been isolated from the brackish water cyanobacterium, *Nodularia spumigena* and reported to be tumor promoters.[3] Recent reports suggest that the potent toxicity of the microcystins is attributable to marked inhibition of protein phosphatases type 1 and type 2A, making them important biochemical probes.[4]

A number of novel peptides have been isolated from cyanobacteria and their biological activities have been reported. Hapalosin, a cyclic depsipeptide having multidrug-resistance reversing activity, has been isolated from *Hapalosiphon welwitschii* W. & G. S. West.[5] A fungicidal decapeptide named calophycin has been isolated from *Calothrix fusca*,[6] and puwainaphycins A-E, one of which (C) is a potent cardioactive agent, are cyclic decapeptides that have been isolated from a terrestrial cyanobacterium *Anabaena* sp. BQ-16-1.[7] Microcystilide A, cell-differentiation-promoting depsipeptide has been isolated from *Microcystis aeruginosa*,[8] and microviridin, a tyrosinase inhibitory tricyclic peptide isolated from *M. viridis*.[9]

We have also reported novel serine protease inhibitory peptides such as micropeptins A and B,[10] aeruginosin 298-A,[11] aeruginosins 98-A and B,[12] micropeptin 90,[13] oscillapeptin,[14] and microviridins B and C[15] from freshwater cyanobacteria.

In our continuous screening program for protease inhibitors, *Oscillatoria agardhi* (NIES-204) showed strong activity against elastase and chymotrypsin, and we isolated microviridins D-F (**1-3**). We report here the isolation and their structure elucidation.



1 **2** **3**

The harvested cyanobacterial cells were extracted three times with 80 % MeOH and one time with MeOH. The combined extracts were subjected to solvent partition. The *n*-BuOH layer was fractionated by ODS flash chromatography with aqueous MeOH. Assay-guided fractionation resulted in the isolation of three elastase inhibitory cyclic peptides, microviridins D (**1**, 0.006 % yield, dry weight), E (**2**, 0.009 %), and F (**3**, 0.0055 %).

Microviridin D (**1**) was isolated as colorless amorphous powder. The molecular formula of **1** was deduced for $C_{84}H_{107}N_{17}O_{26}S$ by HRFAB-MS and detailed analyses of the ^{13}C and ^{1}H NMR spectra (Table 1). Standard amino acid analysis of the acid hydrolyzate of **1** revealed the presence of each one residue of Thr, Ser, Gly, Met, Lys and Pro, each two residues of Asp and Glu, and three residues of Tyr. The extensive 2D NMR analyses of **1** including ^{1}H-^{1}H COSY, HMBC, HMQC, HOHAHA and NOESY spectra indicated the presence of another amino acid, Trp.

Amino acid sequence of **1** was mostly deduced by the interpretation of inter-residual HMBC correlations [Tyr (1) CO/Gly NH, Gly CO/Asn NH, Asn α-CO/Thr NH, Thr CO/Met NH, Met CO/Lys α-NH, Lys CO/Tyr (2) NH, Ser CO/Asp NH, Asp CO/Trp NH, Trp CO/Glu (1) NH, Glu (1) CO/Glu (2) NH, Glu (2) δ-CO/Lys ε-NH, Glu (2) CO/Tyr (3) NH] and NOESY correlations [Tyr (1) α-H/Gly NH, Gly α-H/Asn NH, Asn α-H/Thr NH, Thr α-H/Met NH, Met α-H/Lys α-NH, Lys α-NH /Tyr (2) NH, Tyr (2) α-H/ Pro α-H , Pro α-H/ Ser NH, Ser α-H/Asp NH, Asp α-H/Trp NH, Trp α-H/Glu (1) NH, Glu (1) α-H/Glu (2) NH, Glu (2) γ-H/Lys ε-NH, Glu (2) α-H/Tyr (3) NH].

Table 1. ^{1}H and ^{13}C NMR data of Microviridin D (**1**) in DMSO-d_6

Units	^{1}H (*J* Hz)	^{13}C	HMBC correlations	Units	^{1}H (*J* Hz)	^{13}C	HMBC correlations
Ac				Pro			
1		169.4	Ac 2, Tyr(1) NH	1		172.8	Pro 2, Ser 2
2	1.74 (s)	22.4		2	3.57 (m)	60.4	
Tyr(1)				3	1.44 (m)	31.1	
1		171.9	Tyr(1) 2, Gly NH		1.75 (m)		
2	4.38 (m)	54.5	Tyr(1) 3, NH	4	1.61 (2H, m)	21.6	
3	2.62 (m)	36.6	Tyr(1) 2, 5, 9, NH	5	3.26 (m)	46.5	
	2.89 (dd, 8.8, 4.4)				3.55 (m)		
4		128.1	Tyr(1) 3, 6, 8	Ser			
5,9	7.02 (d, 8.4)	130.0	Tyr(1) 3	1		169.5	Ser 2, Asp NH
6,8	6.62 (d, 8.4)	114.8	Tyr(1) OH	2	4.32 (m)	54.3	
7		155.7	Tyr(1) 5, 9	3	3.54 (m)	61.7	
OH	9.12 (s)				3.58 (m)		
NH	8.04 (d, 8.0)			OH	5.18 (br)		
Gly				NH	7.32 (d, 7.0)		
1		168.7	Gly 2, Asn 2, NH	Asp			
2	3.72 (d, 5.9)	41.8		1		170.1	Asp 2, Trp NH
NH	8.24 (t, 5.5)			2	4.61 (m)	49.1	
Asn				3	2.58 (m)	35.0	
1		171.5	Asn 2, 3, Thr NH		2.79 (dd, 8.5, 3.8)		
2	4.71 (m)	49.4	Asn 3	4		171.5	Asp 3
3	2.41 (m)	37.3	Asn 2	NH	8.61 (d, 8.1)		
	2.57 (m)			Trp			
4		171.6	Asn 3	1		171.2	Trp 2, Glu (1) NH
NH	8.10 (d, 7.7)			2	4.38 (m)	54.5	
NH_2	6.93 (br)			3	2.90 (dd, 8.8, 5.5)	27.6	Trp 2
	7.37 (br)				3.10 (m)		
Thr				1'	10.8 (s)		
1		169.0	Thr 2, Met NH	2'	7.15 (s)	123.7	Trp 3, 1'
2	4.49 (m)	55.1	Thr 4	3'		109.5	Trp 3, 1', 2'
3	5.33 (m)	70.1	Thr 4	4'	7.52 (d, 7.7)	118.2	Trp 6'
4	1.10 (d, 6.2)	16.5		5'	6.95 (m)	118.3	Trp 7'
NH	7.91 (d, 8.4)			6'	7.04 (m)	120.8	Trp 4'
Met				7'	7.30 (d, 8.1)	111.3	Trp 5'
1		170.0	Met 2, Lys NH	8'		127.1	Trp 1', 2', 5', 7'
2	4.17 (m)	52.3		9'		136.1	Trp 2', 4', 6'
3	1.75 (m)	30.6	Met 4	NH	7.62 (d, 7.7)		
	2.01 (m)			Glu(1)			
4	2.35 (m)	29.6	Met 3	1		170.4	Glu(1) 2, 3, Glu(2) NH
	2.40 (m)			2	4.07 (q, 6.6)	52.5	Glu(1) 3
S-Me	2.0 (s)	14.6	Met 4	3	1.78 (m)	26.3	Glu(1) 4
NH	8.47 (d, 7.7)				1.90 (m)		
Lys				4	2.19 (2H, t, 8.5)	29.5	Glu(1) 3
1		170.3	Lys 2, Tyr(2) NH	5		172.8	Glu(1) 3, 4, *O*-Me
2	4.28 (m)	52.6		*O*-Me	3.55 (s)	51.3	
3	1.48 (m)	32.4	Lys 2	NH	8.03 (d, 8.0)		
	1.56 (m)			Glu(2)			
4	1.17 (m)	22.7		1		171.0	Glu(2) 2, Tyr(3) NH
	1.25 (m)			2	4.23 (m)	51.7	Glu(2) 3
5	1.35 (m)	29.2		3	1.59 (m)	28.3	
	1.42 (m)				1.92 (m)		
6	3.02 (2H, m)	38.3		4	2.06 (2H, m)	31.5	
α-NH	6.58 (d, 7.8)			5		171.5	Glu(2) 4, Lys ε-NH
ε-NH	7.35 (m)			NH	7.90 (d, 6.2)		
Tyr(2)				Tyr(3)			
1		170.5	Tyr(2) 3	1		172.8	Tyr(3) 2, 3
2	4.46 (m)	51.6	Tyr(2) 3	2	4.31 (m)	53.8	Tyr(3) 3
3	2.67 (m)	38.6	Tyr(2) 2, 5, 9	3	2.78 (m)	35.8	Tyr(3) 2
	2.72 (m)				2.91 (dd, 8.8, 4.4)		
4		126.2	Tyr(2) 3, 6, 8	4		127.3	Tyr(3) 3, 6, 8
5,9	6.92 (d, 8.4)	130.2	Tyr(2) 3	5,9	6.99 (d, 8.4)	130.0	Tyr(3) 3
6,8	6.66 (d, 8.4)	115.2	Tyr(2) OH	6,8	6.63 (d, 8.4)	115.0	Tyr(3) OH
7		156.2	Tyr(2) 5, 9	7		155.9	Tyr(3) 5, 9
OH	9.29 (s)			OH	9.18 (s)		
NH	8.32 (d, 8.1)			NH	8.00 (d, 7.7)		

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

Units	^{1}H (*J* Hz)	^{13}C	HMBC correlations	Units	^{1}H (*J* Hz)	^{13}C	HMBC correlations
Ac				Pro			
1		169.0	Ac 2, Phe 2, NH	1		171.0	Pro 2
2	1.72 (s)	22.4		2	3.61 (m)	60.4	
Phe(1)				3	1.33 (m)	31.2	Pro 2
1		171.7	Phe 2, 3, Ser(1) 2, NH		1.70 (m)		
2	4.58 (m)	53.7	Phe 3, NH, Ser(1) NH	4	1.62 (2H, m)	21.5	
3	2.71 (m)	37.6	Phe 2, 4, 5, 9, NH	5	3.24 (m)	46.5	
	3.02 (m)				3.59 (m)		
4		138.1	Phe 2, 3, 6, 8	Ser(2)			
5,9	7.21 (m)	129.0	Phe 3, 7	1		169.4	Ser(2) 2, Asp(1) NH
6,8	7.22 (m)	127.9	Phe 7	2	4.22 (m)	54.7	
7	7.18 (m)	126.4	Phe 6, 8	3	3.54 (m)	61.8	
NH	8.07 (d, 8.5)				3.58 (m)		
Ser(1)				OH	5.22 (t, 5.3)		
1		170.0	Ser(1) 2, 3, Thr NH	NH	7.45 (d, 8.2)		
2	4.39 (m)	55.0	Ser(1) 3, OH, NH	Asp(1)			
3	3.54 (m)	61.4	Ser(1) 2, OH	1		170.2	Asp(1) 2, Phe(2) NH
	3.58 (m)			2	4.52 (m)	50.0	Asp(1) 3
OH	4.95 (t, 5.3)			3	2.68 (m)	35.2	Asp(1) 2
NH	8.25 (d, 7.6)				2.91 (m)		
Thr				4		170.4	Asp(1) 3
1		168.6	Thr 2, 3, Tyr(1) NH	NH	8.49 (d, 7.9)		
2	4.55 (m)	54.4	Thr 3, 4, NH	Phe(2)			
3	5.43 (d, 6.5)	69.8	Thr 4	1		170.8	Phe(2) 2, 3, Glu NH
4	1.03 (d, 6.4)	15.7	Thr 3	2	4.33 (m)	54.6	Phe(2) 3
NH	7.72 (d, 8.6)			3	2.79 (m)	37.2	Phe(2) 2, 5, 9
Tyr(1)					2.96 (m)		
1		170.3	Tyr(1) 2, 3, Lys NH	4		137.5	Phe(2) 2, 3, 6, 8
2	4.27 (m)	55.2	Tyr(1) 3,	5,9	7.26 (m)	129.2	Phe(2) 3, 7
3	2.67 (m)	36.3	Tyr(1) 2, 5, 9	6,8	7.23 (m)	128.0	Phe(2) 7
	2.94 (m)			7	7.16 (m)	126.1	Phe(2) 6, 8
4		127.7	Tyr(1) 3, 6, 8	NH	7.41 (d, 7.0)		
5,9	6.95 (d, 8.4)	129.7	Tyr(1) 3, 5, 9	Glu			
6,8	6.61 (d, 8.4)	115.0	Tyr(1) OH	1		170.4	Glu 2, Asp(2) NH
7		155.7	Tyr(1) 5, 6, 8, 9, OH	2	3.89 (m)	52.9	Glu 3
OH	9.14 (s)			3	1.78 (m)	25.8	Glu 2, 4
NH	8.42 (d, 7.0)				1.90 (m)		
Lys				4	2.23 (2H, m)	29.7	Glu 3
1		170.4	Lys 2	5		172.8	Glu 3, 4, *O*-Me
2	4.24 (m)	53.3	Lys NH	*O*-Me	3.58 (s)	51.3	
3	1.52 (m)	32.0	Lys 2, 4, 5	NH	8.03 (d, 5.8)		
	1.58 (m)			Asp(2)			
4	1.19 (m)	23.2	Lys 2, 3	1		171.0	Phe(3) NH
	1.28 (m)			2	4.51 (m)	49.7	Asp(2) 3
5	1.36 (m)	29.0	Lys 4	3	2.30 (m)	38.0	Asp(2) 2
	1.50 (m)				2.41 (m)		
6	2.98 (m)	38.4	Lys 4, 5	4		168.5	Asp(2) 3, Lys ε-NH
	3.11 (m)			NH	7.68 (d, 7.9)		
α-NH	6.58 (d, 7.3)			Phe(3)			
ε-NH	7.42 (m)			1		172.6	Phe(3) 2, 3
Tyr(2)				2	4.35 (m)	53.6	Phe(3) 3
1		170.5	Tyr(2) 2, 3	3	2.89 (m)	36.5	Phe(3) 2, 5, 9
2	4.42 (m)	51.8			3.01 (m)		
3	2.71 (m)	38.2	Tyr(2) 2, 5, 9	4		137.3	Phe(3) 2, 3, 6, 8
	3.02 (m)			5,9	7.25 (m)	129.1	Phe(3) 3, 7
4		126.0	Tyr(2) 3, 6, 8	6,8	7.24 (m)	128.1	Phe(3) 7
5,9	6.93 (d, 8.4)	130.2	Tyr(2) 3, 5, 9	7	7.17 (m)	126.2	Phe(3) 6, 8
6,8	6.67 (d, 8.4)	115.2	Tyr(2) OH	NH	8.11 (d, 7.6)		
7		156.3	Tyr(2) 5, 6, 8, 9, OH				
OH	9.32 (s)						
NH	8.45 (d, 7.9)						

The N-terminus of **1** was acetylated, which was confirmed by the HMBC cross peak from Tyr (1) NH to the carbonyl carbon (δ 169.4) of Ac and NOESY correlation from Tyr (1) NH to methyl proton (δ 1.74) of Ac. The HMBC cross peak between β-H of Thr and the carbonyl carbon of Asp was not observed, but the chemical shift of β-H (δ 5.33) of Thr and structure similarity of **1** to microviridins A-C confirmed ester formation between Thr OH and Asp-γ-COOH. Actually, there were two possibilities in ester formation; one was between Thr OH and Asp-γ-COOH, the other was between Thr OH and Tyr (3)-COOH. Because there were no significant differences in chemical shifts between **1** and microviridins A-C, we confirmed it to be former. Hydroxyl groups of Thr and Asp were not observed in ^{1}H NMR, and this fact also supported the ester formation. All these data led to the structure of microviridin D (**1**). The structure of **1** was closely related to microviridins A-C. Microviridin D (**1**) had Asn and Met residues instead of Gly and Phe of microviridin A, respectively. The γ-carboxylic acid of Glu (1) existed as a methyl ester and no esterification was formed between Glu (1) and Ser. Therefore microviridin D was a dicyclic peptide different from tricyclic peptides microviridins A and B. The absolute stereochemistry of amino acid residues of **1** was determined to be L-form by chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolyzate.

Microviridin E (**2**) was also isolated as colorless amorphous powder. The molecular formula of **2** was deduced for $C_{82}H_{100}N_{14}O_{24}$ by HRFAB-MS and detailed analyses of the ^{13}C and ^{1}H NMR spectra (Table 2). Standard amino acid analysis of the acid hydrolyzate of **2** revealed the presence of each one residue of Thr, Lys, Glu and Pro, each two residues of Ser, Asp and Tyr, and three residues of Phe. The NMR signals were all readily assigned by extensive analysis of ^{1}H-^{1}H COSY and HMQC spectra, expect for aromatic region of three phenylalanines, whose signals were overlapped. Amino acid sequence of **2** was also mostly deduced by the interpretation of inter-residual HMBC correlations [Phe (1) CO/Ser (1) NH, Ser (1) CO/Thr NH, Thr CO/Tyr (1) NH, Tyr (1) CO/Lys α-NH, Ser (2) CO/Asp (1) NH, Asp (1) CO/Phe (2) NH, Phe (2) CO/Glu NH, Glu CO/Asp (2) NH, Asp (2) γ-CO/ Lys ε-NH, Asp (2) CO/Phe (3) NH] and NOESY correlations [Phe (1) α-H/Ser (1) NH, Ser (1) α-H/Thr NH, Thr α-H/Tyr (1) NH, Tyr (1) α-H/Lys α-NH, Lys α-H/Tyr (2) NH, Tyr (2) α-H/Pro α-H, Pro α-H/Ser (2) α-H, Ser (2) α-H/Asp (1) NH, Asp (1) α-H/Phe (2) NH, Phe (2) α-H/Glu NH, Glu α-H/Asp (2) NH, Asp (2) β-H/Lys ε-NH, Asp (2) α-H/Phe (3) NH]. The linkage between acetic acid (Ac) and Phe (1) was also confirmed by the HMBC cross peak from Phe (1) NH to the carbonyl carbon (δ 169.0) of Ac and NOESY correlation from Phe (1) NH to methyl proton (δ 1.72) of Ac. The HMBC cross peak between β-H of Thr and the carbonyl carbon of Asp (1) was not observed, but the chemical shift of β-H (δ_H 5.43, δc 69.8) of Thr confirmed ester formation between Thr OH and Asp (1)-γ-COOH. All these data led to the structure of microviridin E (**2**). The structure of **2** was closely related to microviridins A-C. Microviridin E (**2**) had three Phe instead of Tyr (1), Tyr (3), and Trp of **1**. The most noticeable difference of **2** from **1** and microviridins A-C was that **2** was one residue smaller in side chain than other microviridins and had Asp instead of Glu in the second residue from C-terminus. The absolute stereochemistry of amino acid residues of **2** was determined to be L-form by chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolyzate.

Microviridin F (**3**) was also isolated as colorless amorphous powder. The molecular formula of **3** was deduced for $C_{82}H_{102}N_{14}O_{25}$ by HRFAB-MS and detailed analyses of the ^{13}C and ^{1}H NMR spectra. The molecular weight of **3**, 18 larger than that of **2**, suggested that **3** would be a hydrolyzed derivative of **2**. In the COSY spectrum, the correlation between β–methine proton (δ 3.97) and hydroxyl group (δ 4.83) of Thr confirmed no esterification between Thr and Asp (1), and this was the major difference of **3** from **2** and other microviridins. Except for β–methine proton and hydroxyl group of Thr, the signal patterns and chemical shifts of protons and carbons of **3** were very similar to those of **2**. The result of amino acid analysis of **3** was the same as that of **2**. The NMR signals for all these amino acids were also readily assigned by extensive analysis of ^{1}H-^{1}H COSY and HMQC spectra, expect for aromatic region of three phenylalanines because of overlapping. Amino acid sequence of **3** was deduced by the interpretation of inter-residual HMBC correlations and NOESY correlations in a similar manner to **2**. The linkage between acetic acid (Ac) and Phe (1) was also confirmed by the HMBC cross peak from Phe (1) NH to the carbonyl carbon (δ 169.2) of Ac and NOESY correlation from Phe (1) NH to the methyl proton (δ 1.73) of Ac. All these data led to the structure of microviridin F (**3**). The absolute stereochemistry of amino acid residues of **3** was also determined to be L-form.

Microviridins D-F inhibited elastase potently with the IC_{50} of 0.7, 0.6, and 5.8 μg/mL, respectively. Microviridins D and E also inhibited chymotrypsin with the IC_{50} of 1.2 and 1.1 μg/mL, respectively, but microviridin F had no chymotrypsin inhibitory activity at 100 μg/mL. At a concentration of 100 μg/mL, microviridins D-F had no inhibitory activity against thrombin, plasmin, trypsin and papain.

Experimental section

General methods

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. FAB mass spectra were measured by using polyethyleneglycol sulfate or glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analyses were carried out with a Hitachi L-8500A amino acid analyzer. 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). High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6A liquid chromatograph with ODS L-column (10 × 250 mm, Chemicals Inspection and Testing Institute). Ultraviolet spectrum was measured on a Hitachi 330 spectrometer. Optical rotations were determined with a JASCO DIP-140 digital polarimeter.

Culture conditions

Oscillatoria agardhii (NIES-204) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10 L glass

bottles containing CB medium[16] with aeration (filtered air, 0.3 L/min) at 25℃ under illumination of 250 $\mu E/m^2 \cdot s$ on a 12L:12D cycle. Cells were harvested after 10-14 days by continuous centrifugation at 10,000 rpm. Harvested cells were lyophilized and kept in a freezer at −20℃ until extraction.

Extraction and isolation

Freeze-dried cells (138 g from 400 L of culture) were extracted three times with 80 % MeOH and concentrated to give a crude extract. This extract was partitioned between ether and water. The water soluble fraction was further partitioned between *n*-BuOH and water. The *n*-BuOH layer was subjected to ODS flash chromatography and eluted sequentially with 20 %, 30 %, 40 %, 50 %, 60 %, 100 % MeOH and CH_2Cl_2. Final purification of active 40 and 50 % MeOH fractions was achieved by reversed-phase HPLC on ODS L-column (linear gradient of CH_3CN in H_2O containing 0.05 % TFA, 20 % to 70 %; flow rate 2.5 mL/min; UV detection at 210 nm) to yield microviridin D (**1**, 8.8 mg), E (**2**, 12.6 mg) and F (**3**, 7.6 mg).

Microviridin D (**1**): $[\alpha]_D^{20}$ +66° (*c* 0.1, MeOH); UV (MeOH) λmax 221 nm (ε 45,300), 279 (7,100); FAB-MS *m/z* 1802 $(M + H)^+$; HRFAB-MS *m/z* 1802.7419 $(M + H)^+$ calcd. for $C_{84}H_{108}N_{17}O_{26}S$ (△ + 4.7 mmu).

Microviridin E (**2**): $[\alpha]_D^{20}$ +12° (*c* 0.1, MeOH); UV (MeOH) λmax 222 nm (ε 17,700), 278 (2,000); FAB-MS *m/z* 1665 $(M + H)^+$, 1558, 1517, 1105; HRFAB-MS *m/z* 1665.7130 $(M + H)^+$ calcd. for $C_{82}H_{101}N_{14}O_{24}$ (△ + 1.7 mmu).

Microviridin F (**3**) : $[\alpha]_D^{20}$ −29° (*c* 0.1, MeOH); UV (MeOH) λmax 222 nm (ε 24,200), 278 (3,000); FAB-MS *m/z* 1683 $(M + H)^+$, 1668, 1535, 1494, 1143, 1128; HRFAB-MS *m/z* 1683.7153 $(M + H)^+$ calcd. for $C_{82}H_{103}N_{14}O_{25}$ (△ −6.5 mmu); ^1H NMR (DMSO-d_6) **Ac**: δ1.73(s, H-2); **Phe**(1): 4.56 (m, H-2), 2.71 (m, H-3a), 3.02 (m, H-3b), 7.20 (m, H-5, 9), 7.24 (m, H-6, 8), 7.16 (m, H-7), 8.07 (d, 8.6, NH); **Ser**(1): 4.35 (m, H-2), 3.56 (m, H-3a), 3.62 (m, H-3b), 5.05 (t, 5.2, OH), 8.18 (d, 7.3, NH); **Thr**: 4.17 (m, H-2), 3.97 (m, H-3), 0.97 (d, 6.1, H-4), 4.83 (d, 5.2, OH); **Tyr**(1): 4.43 (m, H-2), 2.62 (m, H-3a), 2.87 (m, H-3b), 6.97 (d,8.2, H-5, 9), 6.59 (d, 8.2, H-6, 8), 9.11 (s, OH), 7.77 (d, 8.8, NH); **Lys**: 4.27 (m, H-2), 1.42 (m, H-3a), 1.68 (m, H-3b), 1.22 (m, H-4a), 1.26 (m, H-4b), 1.23 (m, 2H, H-5), 2.98 (m, H-6a), 3.03 (m, H-6b), 7.81 (d, 7.6, α-NH), 7.82 (m, ε-NH); **Tyr**(2): 4.46 (m, H-2), 2.69 (m, H-3a), 2.89 (m, H-3b), 7.12 (d, 8.3, H-5, 9), 6.66 (d, 8.3, H-6, 8), 9.18 (s, OH), 8.21 (d, 7.6, NH); **Pro**: 4.40 (m, H-2), 1.47 (m, H-3a), 1.60 (m, H-3b), 1.80 (m, H-4a), 1.90 (m, H-4b), 3.61 (m, 2H, H-5); **Ser**(2): 4.29 (m, H-2), 3.61 (m, H-3a), 3.72 (m, H-3b), 5.45 (t, 4.5, OH), 8.34 (d, 7.0, NH); **Asp**(1): 4.41 (m, H-2), 2.36 (m, H-3a), 2.55 (m, H-3b); **Phe**(2): 4.48 (m, H-2), 2.78 (m, H-3a), 2.96 (m, H-3b), 7.20-4 (m, H-5, 9), 7.24 (m, H-6, 8), 7.16 (m, H-7), 7.87 (d, 7.3, NH); **Glu**: 4.53 (m, H-2), 1.77 (m, H-3a), 1.92 (m, H-3b), 2.29 (m, 2H, H-4), 3.58 (s, *O*-Me), 8.01 (d, 8.2, NH); **Asp**(2): 4.54 (m, H-2), 2.41 (m, H-3a), 2.56 (m, H-3b), 8.33 (d, 7.0, NH); **Phe**(3): 4.37 (m, H-2), 2.91 (m, H-3a), 3.01 (m, H-3b), 7.21 (m, H-5, 9), 7.24 (m, H-6, 8), 7.16 (m, H-7), 8.0 (d, 8.2, NH).

Amino acid analysis

(A) Hydrolysis with 1 % phenol in 6 N HCl. Microviridin D (100 μg) was dissolved in 1 % phenol in 6 N HCl (500 μL) and sealed in a reaction vial. The vial was heated at 100℃ for 10 hours. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl to subject to amino acid analysis.

(B) Hydrolysis with 6 N HCl. Each 100 μg of microviridins E and F was dissolved in 6 N HCl (500 μL) and sealed in reaction vials. The vials were heated at 110℃ for 16 hours. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl to subject to amino acid analysis.

Chiral GC analysis of amino acids

A solution of 10 % HCl in isopropyl alcohol was added to each of the hydrolyzates of **1**-**3** in reaction vials and heated at 100℃ for 30 min. The solvent was removed in a stream of dry nitrogen. Trifluoroacetic anhydride (300 μL) in CH_2Cl_2 (300 μL) was added to the residues, the vials were capped, and the solution was heated at 100℃ for 5 min and evaporated in a stream of dry nitrogen. The residues were dissolved in CH_2Cl_2 (500 μL) and immediately analyzed by chiral GC using Chirasil-Val capillary column with a flame ionization detector (FID). Column temperature was kept at 80℃ for 3 min and then increased at a rate of 4℃/min to 200℃. Retention times (min) of standard amino acids were found as follows: D-Thr (7.4), L-Thr (8.1), D-Pro (9.9), L-Pro (10.1), D-Ser (10.8), L-Ser (11.7), D-Asp (16.4), L-Asp (16.7), D-Met (18.6), L-Met (19.8), D-Phe (20.8), L-Phe (21.7), D-Glu (21.2), L-Glu (22.1), D-Tyr (26.4), L-Tyr (27.1), D-Lys (32.2), L-Lys (32.8), D-Trp (34.2), L-Trp (34.6).

Protease inhibitory activity assay

Serine and cysteine protease inhibitory activities were determined by the modified method of Cannell *et al.*[17] Each assay mixture containing 30 μL (100 μL for plasmin) of Tris-HCl buffer (pH 8.6, 8.3, and 7.6 for elastase, plasmin, and other enzymes, respectively), 50 μL (30 μL for plasmin) of enzyme solution and 20 μL of test solution were added to each microtiter plate well and preincubated at 37℃ for 5 min. Each reaction was started with the addition of 100 μL (50 μL for plasmin) of substrate. The absorbance of the well was immediately read at 405 nm. The developed color was measured after 30 min-incubation (except for plasmin for 10 min) at 37℃. For the thrombin inhibitory activity assay, following stock solutions were prepared for Tris-imidazole buffer; 1. A mixture of equal volumes of 0.1 M imidazole and 0.1 M NaCl. 2. A mixture of equal volumes of 0.1 M imidazole and 0.1 M Tris, both in 0.1 M NaCl. These two stock solutions were then mixed to obtain pH 8.2 and diluted with a 20-fold volume of the Tris-imidazole buffer. Assay mixture containing 90 μL of enzyme solution (1.3 U/mL in the Tris-imidazole buffer) and 20 μL of test solution was added to each microtiter plate well and preincubated at 37℃ for 5 min. Then 90 μL of substrate solution was added to start the reaction. The absorbance of the well was immediately read at 405 nm. The developed color was measured after 30 min-incubation (except for plasmin for 10 min) at 37℃.

All the enzymes and substrates used in this study were purchased from Sigma Chemical Co. Trypsin and chymotrypsin were dissolved in 50 mM Tris-HCl (pH 7.6) to prepare 150 U/mL and 15 U/mL solutions, respectively. Papain was dissolved in 50 mM Tris-HCl, 1 mM cysteine-HCl, and 2 mM EDTA (pH 7.6) to prepare 10 U/mL enzyme solution. Elastase was dissolved in 50 mM Tris-HCl (pH 8.6) to prepare 2.5 U/mL enzyme solution. Plasmin was dissolved in 0.9 % NaCl/glycerol (1:1) solution to prepare 1.5 mU/mL enzyme solution. Substrates and buffer or solvent conditions were as follows: *N*-benzoyl-D,L-arginine-*p*-nitroanilide (43.3 mg)/1 mL of dimethyl sulfoxide (DMSO)/100 mL of 50 mM Tris-HCl (pH 7.6) for trypsin and papain; *N*-succinyl-phenylalanine-*p*-nitroanilide/50 mM Tris-HCl (pH 7.6, 1 mg/mL) for chymotrypsin; *N*-succinyl-(L-Ala)$_3$-*p*-nitroanilide/50 mM Tris-HCl (pH 8.6, 1 mg/mL) for elastase; D-Val-L-Leu-L-Lys-*p*-nitroanilide/H_2O (1.4 mg/mL) for plasmin; Bz-L-Phe-L-Val-L-Arg-*p*-nitroanilide/DMSO (5 mg/mL)/20-fold volume of Tris-imidazole buffer for thrombin.

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References and notes

1. The name of microviridin (we call it microviridin A in this paper) originated from *Microcystis viridis*, because it was first isolated from *M. viridis*. Although we isolated microviridins D-F from *Oscillatoria agardhii*, we named it microviridins not to confuse its name.
2. (a) Rinehart, K. L.; Harada, K-I.; Namikoshi, M.; Chen, C.; Harvix, C. A.; Munro, M. H. G.; Blunt, J. W.; Mulligan, P. E.; Beasley, V. R.; Dahlem, A. M.; Carmichael, W. W. *J. Am. Chem. Soc.* **1988**, 110, 8557-8558. (b) Kiviranta, J.; Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rinehart, K. L. *Toxicon* **1992**, 30, 1093-1098. (c) Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Sun, F.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. *Toxicon* **1992**, 30, 1457-1464. (d) Sivonen, K.; Skulberg, O. M.; Namikoshi, M.; Evans, W. R.; Carmichael, W. W.; Rinehart, K. L. *Toxicon* **1992**, 30, 1465-1471. (e) Namikoshi, M.; Sivonen, K.; Evans, W. R.; Sun, F.; Carmichael, W. W.; Rinehart, K. L. *Toxicon* **1992**, 30, 1473-1479. (f) Sivonen, K.; Namikoshi, M.; Evans, W. R.; Gromov, B. V.; Carmichael, W. W.; Rinehart, K. L. *Toxicon* **1992**, 30, 1481-1485. (g) Namikoshi, M.; Sun, F.; Choi, B. W.; Rinehart, K. L. *J. Org. Chem.* **1995**, 60, 3671-3679.
3. (a) Falconer, I. R.; Buckley, T. H. *Med. J. Aust.* **1989**, 150, 351. (b) Honkanen, R. E.; Zwiller, J.; Moore, R. E.; Daily, S. L.; Khatra, B. S.; Dukelow, M.; Boynton, A. L. *J. Biol. Chem.* **1990**, 265, 19401-19404. (c) Yoshizawa, S.; Matsushima, R.; Watanabe, M. F.; Harada, K-I.; Ichihara, A.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1990**, 116, 609-614. (d) Nishiwaki-Matsushima, R.; Ohta, T.; Nishiwaki, S.; Suganuma, M.; Kohyama, K.; Ishikawa, T.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1992**, 118, 420-424.
4. Eriksson, J. E.; Toivola, D.; Meriluoto, J. A. O.; Karaki, H.; Han, Y-G.; Hartshorne, D. *Biochim. Biophys. Acta.* **1990**, 173, 1347-1353.

5. Stratmann, K.; Burgoyne, D. L.; Moore, R. E.; Patterson. G. M. L. *J. Org. Chem.* **1994**, 59, 7219-7226.
6. Moon, S-S.; Chen, J-L.; Moore, R. E.; Patterson, G. M. L. *J. Org. Chem.* **1992**, 57, 1097-1103.
7. Gregson, J. M.; Chen, J-L.; Patterson, G. M. L.; Moore, R. E. *Tetrahedron* **1992**, 48, 3727-3734.
8. Tsukamoto, S.; Painuly, P.; Young, K. A.; Yang, X.; Shimizu, Y. *J. Am. Chem. Soc.* **1993**, 115, 11046-11047.
9. Ishitsuka, M. O.; Kusumi, T.; Kakisawa, H.; Kaya, K.; Watanabe, M. M. *J. Am. Chem. Soc.* **1990**, 112, 8180-8182.
10. Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, 34, 8131-8134.
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.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, 36, 3535-3538.
14. Shin, H. J.; Murakami, M.; Matsuda, H.; Ishida, K.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, 36, 5235-5238.
15. Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1995**, 51, 10679-10686.
16. Watanabe, M. M.; Satake, K. N. *NIES-collection List of strains*, Natl. Inst. Environ. Stud., Tsukuba, Japan, **1994**, 30-31.
17. Cannell, R. J. P.; Kellam, S. J.; Owsianka, A. M.; Wallker, J. M. *Planta Medica* **1988**, 54, 10-14.

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