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Micropeptin 103, a Chymotrypsin Inhibitor from the Cyanobacterium *Microcystis viridis* (NIES-103)

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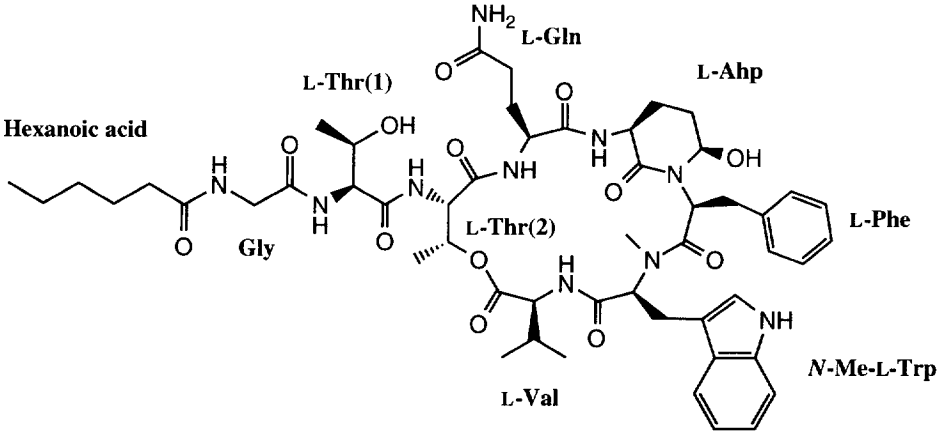
Abstract: Micropeptin 103, a chymotrypsin inhibitor, was isolated from the cultured freshwater cyanobacterium *Microcystis viridis* (NIES-103). So far, this type of peptide has not been reported from *Microcystis viridis*. Its structure was elucidated to be **1** on the basis of 2D NMR data and chemical degradation. Micropeptin 103 inhibited chymotrypsin with IC_{50} of 1.0 μg/mL.

The cyanobacteria *Microcystis* spp. are well known as producers of microcystins,[1] posing serious worldwide problems to human and livestock health. Recently, cyclic depsipeptides containing 3-amino-6-hydroxy-2-piperidone (Ahp) were isolated from varied cyanobacteria; aeruginopeptins,[2] microcystilide A,[3] and micropeptins[4] from *M. aeruginosa,* cyanopeptolins[5] from *Microcystis* sp., A90720A[6] from *Microchaete loktakensis*, and oscillapeptin[7] from *Oscillatoria agardhii.* In the course of our screening program of protease inhibitors from microalgae, we found that *Microcystis viridis* (NIES-103)[8,9] had potent inhibitory activity on chymotrypsin. Here we first describe the isolation and structure elucidation of a cyclic depsipeptide containing Ahp, micropeptin 103, from *M. viridis*.

The 80% methanol extract of freeze-dried alga (119 g) was partitioned between water and diethyl ether. The aqueous layer, which inhibited chymotrypsin potently, was further extracted with *n*-butyl alcohol and subjected to ODS flash column chromatography followed by reversed-phase HPLC (Cosmosil 5C18-MS) with aqueous MeCN (20-60%) containing 0.05% TFA to yield **1** (22.0 mg).[10]



1

Table 1. ^{1}H and ^{13}C NMR Data for Micropeptin 103 in DMSO-d_6

Position		^{1}H	J (Hz)	^{13}C	Position		^{1}H	J (Hz)	^{13}C
Hexanoic acid	1			172.6 (s)	Phe	1			170.5 (s)
	2	2.12	(t,7.5)	35.1 (t)		2	4.72	(dd,9.5,4.4)	50.0 (d)
	3	1.49	(m)	24.9 (t)		3	1.24	(m)	35.1 (t)
	4	1.21	(m)	30.8 (t)			2.62	(m)	
	5	1.25	(m)	21.9 (t)		4			136.4 (s)
	6	0.85	(t,6.0)	13.8 (q)		5,9	6.20	(d,8.1)	129.2 (d)
Gly	1			168.2 (s)		6,8	6.99	(dd,8.1,7.2)	127.5 (d)
	2	3.37	(m)	42.0 (t)		7	7.00	(t,7.2)	125.9 (d)
	NH	8.09	(t,5.9)		*N*-Me-Trp	1			169.6 (s)
Thr(1)	1			170.7 (s)		2	5.10	(dd,11.0,3.7)	60.2 (d)
	2	4.43	(dd,8.4,4.0)	57.6 (d)		3	2.98	(dd,15.0,11.0)	23.3 (t)
	3	4.00	(m)	66.8 (d)			3.37	(m)	
	4	1.05	(d,6.2)	19.5 (q)		1'	11.01	(d,2.6)	
	NH	7.68	(d,8.4)			2'	7.12	(d,2.6)	124.6 (d)
Thr(2)	1			169.2 (s)		3'			109.3 (s)
	2	4.60	(dd,9.2,1.1)	54.8 (d)		4'	7.59	(d,7.7)	118.4 (d)
	3	5.38	(m)	71.9 (d)		5'	7.06	(t,7.7)	118.8 (d)
	4	1.19	(d,6.6)	17.9 (q)		6'	7.12	(m)	121.0 (d)
	NH	7.99	(d,9.2)			7'	7.38	(d,8.1)	111.4 (d)
Gln	1			169.5 (s)		8'			136.2 (s)
	2	4.19	(br)	51.6 (d)		9'			127.0 (s)
	3	1.55	(m)	26.3 (t)		*N*-Me	2.80	(s)	30.1 (q)
		2.32	(m)		Val	1			171.8 (s)
	4	1.98	(m)	31.5 (t)		2	4.72	(dd,8.8,4.4)	55.8 (d)
		2.01	(m)			3	2.08	(m)	30.8 (d)
	5			173.7 (s)		4	0.74	(d,7.0)	17.1 (q)
	NH_2	6.65	(br)			4'	0.88	(d,7.0)	19.2 (q)
		7.12	(br)			NH	7.38	(d,8.8)	
	NH	8.42	(d,8.1)						
Ahp	2			168.7 (s)					
	3	3.50	(ddd,12.3,8.8,6.8)	48.8 (d)					
	4	1.55	(m)	21.9 (t)					
		2.32	(m)						
	5	1.41	(m)	29.2 (t)					
		1.62	(m)						
	6	4.98	(m)	73.7 (d)					
	NH	7.02	(m)						
	OH	5.85	(d,2.9)						

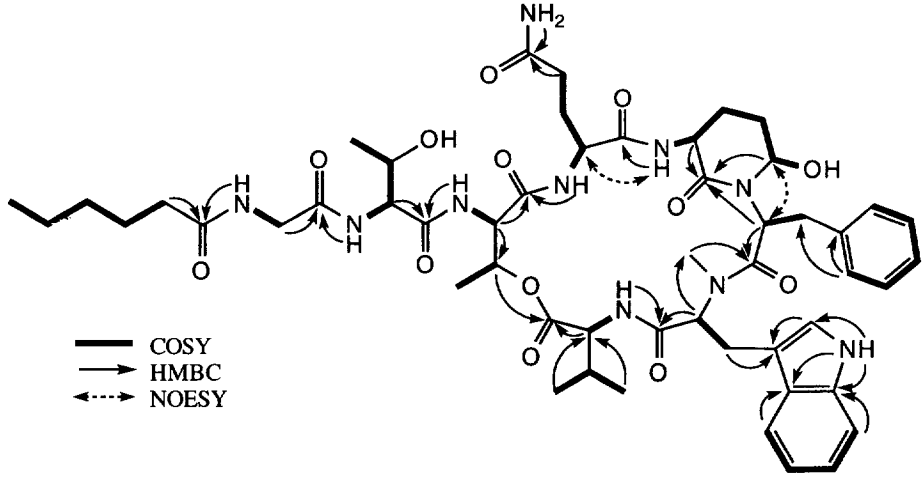


Fig. 1. ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of micropeptin 103

The molecular formula of **1** was established to be $C_{52}H_{72}N_{10}O_{13}$ by HRFABMS and NMR spectral data (Table 1), and amino acid analysis of the hydrolyzate gave Thr, Val, Phe, Glu, and Gly. The extensive NMR analyses of **1** including ^1H-^1H COSY, HMQC,[11] and HMBC[12] spectra indicated the presence of other structural units, *N*-Me-Trp, 3-amino-6-hydroxy-2-piperidone (Ahp), and revealed the spin systems of all units. Similar chemical shifts of protons and carbons also supported the existence of Ahp unit, compared with other related peptides. The HMBC correlation between *N*-methyl proton and C-2 (δ 60.2) clarified the presence of *N*-Me Trp. The sequence of **1** was mostly deduced by HMBC correlations from α-H to C=O (Fig.1), but the correlations from Ahp NH to Gln α-H could not be observed. NOESY correlation (Gln α-H/Ahp NH) connected Gln to Ahp (Fig. 1). HPLC analysis of the acid hydrolyzate (1% phenol in 6 N HCl, 110 °C, 16 h)[13] derivatized with Marfey's reagent[14,15] decided that Gly, Val, Thr(1), Thr(2), Gln, Phe, and *N*-Me-Trp in **1** were all L-form.[16]

NOESY correlations determined the relative stereochemistry of Ahp as in Fig. 2. To decide the absolute stereochemistry of Ahp, first, micropeptin 103 was subjected to the Hoffman rearrengement[17] to change Gln into 2,4-diamino-butyric acid.[18] Then the reaction mixture was oxdized with PCC.[19,20] After filteration, and hydrolysis with 6 N HCl, the reaction mixture afforded Glu which was proved to be L-form from the HPLC analysis by the L-Marfey's method. Therefore, the stereochemistry of Ahp was decided to be (3S,6R)-3-amino-6-hydroxy-2-piperidone (L-Ahp). This absolute stereochemistry was coincident with those of cyanopeptolins,[7] A90720A,[6] and micropeptin 90.[4]

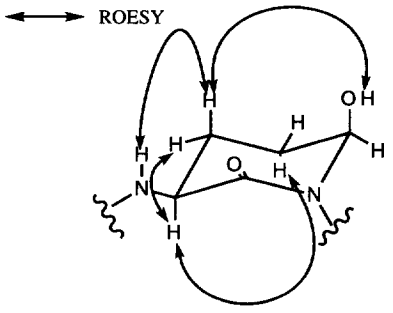


Fig. 2. The relative stereochemistry of micropeptin103

Micropeptin 103 is the first report containing *N*-Me-Trp and inhibited chymotrypsin and thrombin with IC_{50}= 1.0 and 9.0 μg/mL, respectively. This compound did not inhibit trypsin, papain, and elastase at 100 μg/mL.

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REFERENCES AND NOTES

1. Namikoshi, M.; Rinehart, K, L.; Sakai, R.; Stotts, R. R. ; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W. *J. Org. Chem.* **1992**, *57*, 866-872.
2. Harada, K-I.; Mayumi, T.; Shimada, T.; Suzuki, M. *Tetrahedron Lett.* **1993**, *34*, 6091-6094.
3. Tsukamoto, S.; Painuly, P.; Young, K. A.; Yang, X.; Shimizu, Y. *J. Am. Chem. Soc.* **1993**, *115*, 11046-11047.
4. a) Okino, T.; Murakami, M.; Munekata, M.; Haraguchi, 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.
5. Martin, C.; Oberer, L.; Ino, T.; König, A. W.; Busch, M.; Weckesser, J. *J. Antibiot.* **1993**, *46*, 1550-1556.
6. Lee, A. Y.; Smitka, T. A.; Bonjouklian, R.; Clardy, J. *Chemistry & Biology* **1994**, *1*, 113-117.

7. Shin, H. J.; Murakami, M.; Matsuda, H.; Ishida, K.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 5235-5238.
8. *M. viridis* (NIES-103) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan)[9] 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_2·EDTA $2H_2O$ 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] under illumination of 250 μE/m^2s on a 12L:12D cycle.
9. Watanabe, M. M.; Satake, K. N. In *NIES-collection List of strains, Natl. Inst. Envion. Stud.*, Tsukuba, Japan, **1994**, p30-31.
10. Micropeptin 103 is colorless amorphous powder. ; $[\alpha]_D$ -32.8° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 290 (ε 3700); HRFABMS [*m/z* 1045.5416 $(M-H)^-$ Δ +5.5 mmu].
11. Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565-569.
12. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093-2094.
13. Muramoto, K.; Kamiya, H. *Anal. Biochem.* **1990**, *189*, 223-230.
14. Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 1177-1181.
15. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591-596.
16. To the acid hydrolyzate of a 100 μg portion of **1**, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in acetone (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added, and the reaction mixture was kept at 80 °C for 3 min. To the reaction mixture, 50 μL of 2 N HCl and 300 μL of 50% MeCN were added and the reaction mixture was analyzed by reversed-phase ODS-HPLC : column Cosmosil MS (Nacalai Tesque) (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 the standard amino acids (min) : L-Thr (36.2), D-Thr (39.2), L-allo-Thr (36.5), D-allo-Thr (37.5), L-Glu (38.2), D-Glu (39.7), Gly (38.4), L-Val (46.4), D-Val (50.3), L-Phe (50.4), D-Phe (53.8). Retention times of the amino acids of micropeptin 103 (min) : Thr (36.2), Glu (38.2), Gly (38.4), Val (46.4), Phe (50.4). *N*-Me-L-Trp was derivatized with D- and L-FDAA as described above, respectively.[14,15] The derivatives were analyzed by reversed-phase ODS-HPLC: column Cosmosil MS (4.6 × 250 mm) as described above. Retention times of the standards (min); *N*-Me L-Trp-L-FDAA (47.2), *N*-Me L-Trp-D-FDAA (48.4). Retention time of *N*-Me Trp-L-FDAA in the acid hydrolyzate of micropeptin 103 (min): 47.2.
17. Yamamoto, Y.; Yatagai, H.; Maruyama, K. *J. Org. Chem.* **1979**, *44*, 1746-1747.
18. Micropeptin 103 was dissolved in 2 mL of DMF/H_2O (1:1), and then *1,1*-bis(trifluoroacetoxy) iodobenzene was added to the solution with stirring at room temparature for 6 h.
19. Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1996**, *37*, 9225-9226.
20. The reaction mixture which was subjected to the Hoffman rearrengment was dissolved in CH_2Cl_2, and then PCC (pyridinium chlorochromate)/Al_2O_3 in CH_2Cl_2 was added to the solution with stirring at room temperature. After stirring for 8 h, diethyl ether and an excess amount of anhydrous $MgSO_4$ were added, and the reaction mixture was stirred at room temperature for 20 min. After filteration, the solution was evaporated and dissolved in 6 N HCl, and heated at 110 °C for 6 h to yield Glu. The obtained Glu was derivatized with L-FDAA as described above.[15] The derivatives were analyzed by reversed-phase ODS-HPLC as described above.[16] Retention time of Glu-L-FDAA (min): 38.2.

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