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Micropeptin T-20, A Novel Phosphate-containing Cyclic Depsipeptide from the Cyanobacterium *Microcystis aeruginosa*

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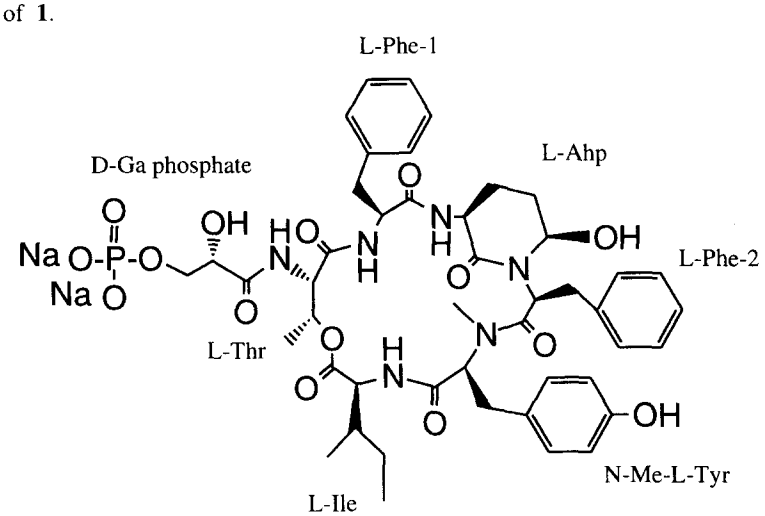
Abstract : Micropeptin T-20, a novel glyceric acid 3-*O*-phosphate and 3-amino-6-hydrooxy-2-piperidone-containing cyclic depsipeptide, was isolated from a cyanobacterium *Microcystis aeruginosa* . The structure was identified as **1** by 2D NMR and chemical degradation analyses. Micropeptin T-20 inhibited chymotrypsin.

Recently, cyanobactera have been reported to produce 3-amino-6-hydroxy-2-piperidone (Ahp)-containing cyclic depsipeptides[1-16]. In many cases, the N-terminals of these cyclic depsipeptides are combined with glyceric acid. Additionally, a sulfate group is attached to the terminus of the glyceric acid (glyceric acid 3'-*O*-sulfate). During investigations into toxins from newly-isolated *Microcystis aeruginosa* strains, we have found a novel glyceric acid 3'-*O*-phosphate-containing cyclic depsipeptide and here present its isolation and structure elucidation of **1**.



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Microcystis aeruginosa was isolated from a bloom of the cyanobacterium *Microcystis aeruginosa*, collected from freshwater Kang Krachan Dam in Thailand. The strain of *Microcystis aeruginosa* was grown axenically in

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batch culture in 10 L volumes of MA medium[17] at 20-25 °C. Cultures were sparged with air at about 1.5 L·min^{-1} and light supplied by white fluorescent tubes giving an irradiance incident on the surface of the vessels of about 250 μmol photon·m^{-2}·s^{-1}. Cells were harvested from stationary phase by centrifugation at 10,000 g for 20 min to give a pellet, which was freeze-dried. Methanol extract from 10 g of freeze-dried cells was evaporated under reduced pressure. The remaining residue was suspended in 5% (v/v) aqueous acetic acid solution. The suspension was centrifuged at 2,000 rpm for 20 min and the supernatant retained. The inhibitor **1** was fractionated by solid-phase extraction using ODS cartridges (Sep-pak ODS). The fractionated inhibitor was purified by reverse-phase HPLC (Mightysil RP-18, 20 mm I.D. x 25 cm) with methanol (60%) containing 0.05 M phosphate buffer (pH 3.0) at 10 ml·min^{-1}. The purified inhibitor was further separated by HPTLC (Merck, Silica gel 60 containing fluorescent indicator) using chloroform / methanol / water (60/40/10, v/v) as the solvent. The yield of the inhibitor **1**, micropeptin T-20, was 17 mg. **1** was assayed for inhibition of chymotrypsin using α-chymotrypsin type II (Sigma C-4129) and *N*-benzoyl-L-tyrosine ethyl ester as a substrate.

Micropeptin T-20 (**1**) is a colorless amorphous solid and contained phosphate and sodium[18] : λmax (MeOH) 278 nm(ε: 2800). In the positive HRFABMS using glycerol as the matrix, the $[M + H]^+$ ion was observed at *m/z* 1011.3474, and a dephosphate ion at *m/z* 909.3998 $[M - Na_2PO_3 + H]^+$ was also observed. From the results, the molecular formula of **1** was established to be $C_{46}H_{57}N_6O_{15}PNa_2$ (calcd for [M+H]: 1011.3493, Δ 1.9 mmu). The spectral data (Table 1) of ^{1}H- and ^{13}C-NMR of **1** suggested that micropeptin T-20 is a peptide. Amino acid analysis of the hydrolysate (6 N HCl, 110 °C, 20 h) indicated the presence of 1 mol each of threonine (Thr), isoleucine (ILe) and *N*-methyltyrosine (N-MeTyr)[19] and 2 mol of phenylalanine (Phe).

Table 1. ^{1}H* and ^{13}C ** NMR data for micropeptin T-20 in methanol-d_4.

position		^{1}H	*J* (Hz)	^{13}C	position		^{1}H	*J* (Hz)	^{13}C
Ga-Phos	1			173.6	Phe-2	1			171.8
	2	4.40	(dd 7.0, 4.0)	71.9		2	5.06	(m)	63.2
	3	4.24	(m)	70.8		3	3.40	(m)	34.2
		3.98	(m)				2.76	(m)	
Thr	1			170.0		4			137.6
	2	4.53	(d 9.2)	56.1		5,9	7.21	(d 7.0)	129.8
	3	5.48	(q 6.4)	73.7		6,8	7.16	(t 7.0)	128.4
	4	1.26	(d 6.4)	18.1		7	7.10	(t 7.0)	127.6
Phe-1	1			172.9	N-Me-Tyr	1			173.3
	2	4.70	(dd 4.0, 10.7)	55.9		2	4.94	(m)	52.6
	3	3.40	(m)	37.3		3	2.95	(m)	36.3
		2.75	(m)				2.05	(m)	
	4			138.8		4			128.7
	5,9	7.22	(d 7.0)	131.7		5,9	7.10	(d 8.5)	130.3
	6,8	7.18	(t 7.0)	128.7		6,8	6.82	(d 8.5)	116.8
	7	7.14	(t 7.0)	127.7		7			157.8
Ahp	2			171.2		Me	2.85	(s)	31.7
	3	3.85	(ddd 12.5, 9.6, 6.4)	50.7	Ile	1			174.7
	4	2.59	(m)	22.3		2	4.62	(d 10.8)	57.4
		1.82	(m)			3	1.82	(m)	38.9
	5	1.80	(m)	30.6		4	1.38	(m)	26.3
		1.62	(m)				1.17	(m)	
	6	5.16	(br.s)	75.8		5	0.88	(t 7.8)	11.4
						6	0.91	(d 7.7)	16.5

*Recorded at 500 MHz (δ values). **Recorded at 125 MHz (δ values).

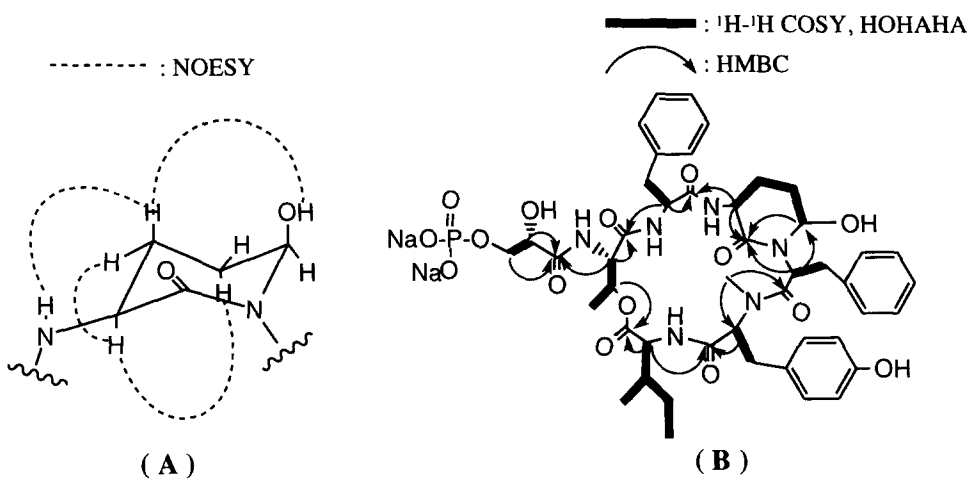

Fig.1. Relative stereochemistry of Ahp in the cyclic peptide in dimethylsulfoxide (DMSO)-d_6 (A) and ^{1}H-^{1}H COSY, HOHAHA, and HMBC correlations of micropeptin T-20 observed in methanol-d_4 (B).

Thr, ILe, Phe, and N-MeTyr were shown to have the L-configuration by HPLC analysis of the derivatives of the acid hydrolysate with L- Marfey's reagent[15].

In the ^{1}H-NMR spectrum, a broad singlet was observed at 5.16 ppm. In the HMQC spectrum of **1**, this broad singlet connected with a carbon at 75.8 ppm. This chemical shift of the carbon suggested that the carbon was substituted with *O* - and *N* -functional groups. After extensive two-dimensional NMR analyses, this broad singlet was elucidated as a signal of H-6 in Ahp unit. The ^{1}H- and ^{13}C-NMR data of Ahp unit in **1** resembled closely those of micropeptins 478-A, -B[15] and A90720A[10]. To confirm the relative stereochemistry of Ahp of **1**, the NOESY correlations were observed in DMSO-d_6 between Ahp NH and H-4, H-4 and OH, H-4 and H-3, and H-3 and H-5 [Fig.1 (A)]. Furthermore, after the PCC oxidation of **1** followed by hydrolysis with HCl[15], L-Glu was identified in the hydrolysate by HPLC analysis of its derivative with L-Marfey's reagent. Therefore, the configuration of Ahp unit in **1** was determined to be [(3*S*, 6*R*)-3-amino-6-hydroxy-2-piperidone (L-Ahp)]. The signal of H-3 in Thr was observed at 5.48 ppm. This chemical shift suggested that the hydroxy group of Thr was acylated. The proton signals around 4.4 ppm to 3.9 ppm were assigned to those of glyceric acid unit by two-dimensional NMR analyses. The doublet of doublet at 4.40 ppm in the ^{1}H-NMR spectrum was correlated with C-2 (71.9 ppm) of glyceric acid by the HMQC spectrum. These chemical shifts suggest that the hydroxy group of C-2 was free. If the hydroxy group of C-2 is esterfied, the signal of H-2 ought to appear around at 4.7 ppm[15]. Moreover, from the FABMS and NMR analyses, 3-hydroxy group of the glyceric acid was linked up with phosphate as ester. The glyceric acid was shown to have the D-configuration by chiral HPLC analysis[20].

The sequence of **1** was mostly deduced by HMBC correlation [Fig.1 (B)] from α-H to carbonyl carbon. The HMBC correlation from H-3 of Thr to C=O of Ile confirmed the ester formation between Thr and Ile. The methyl proton of N-MeTyr correlated with C=O of Phe-2 in the HMBC spectrum. Furthermore, Phe-2 and Ahp was connected as a hemiaminal as mentioned above. From the results, the structure of micropeptin T-20 was established as **1**. Micropeptin T-20 strongly inhibited chymotrypsin[7,8] with IC_{50} of 2.5 x 10^{-9} M, and weakly inhibited tyrosinase with IC_{50} of 5 x 10^{-3} M.

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18. After desalting using a Sep-pak ODS cartridge and purification by HPTLC, micropeptin T-20 (0.96 μmol) was hydrolyzed with 70% $HClO_4$. Phosphate (0.81 μmol) in the hydrolysate was determined by colorimetric method (Harris, W.D. and Popat, P., J. Am. Oil Chem. Soc. **1954**, 31, 124-128.). Sodium was determined by ion chromatography (Kuchida, K., *et al.* J. Environ. Chem. **1993**, 3, 577-579.).
19. D- and L-*N*-methyltyrosine were prepared from D- and L-*N*-methylphenylalanine (Sigma Chemicals), respectively, by the oxidation reaction at room temperature using aluminum chloride and hydrogen peroxide. The yields of D- and L-*N*-methyltyrosine were about 10%.
20. Glyceric acid in the acid hydrolysate of micropeptin T-20, and D- and L-glyceric acid were converted to phenethyl esters using phenethyl alcohol in sulfuric acid-containing dioxane. These esters were analyzed by chiral HPLC [column: CHIRALCEL OD-R, 4.6 x 250 mm; mobile phase: water / acetonitrile / trifluoroacetic acid (86 / 14 / 0.1, v/v); flow rate: 0.5 ml / min.; wave length of UV detector: 210 nm.]. D- and L-Phenethylglycerate were eluted at 33.2 and 32.1 min, respectively.