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Occurrence of Four Depsipeptides, Aeruginopeptins, Together with Microcystins from Toxic Cyanobacteria

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Abstract: Four novel depsipeptides, aeruginopeptins (1-4) were isolated from toxic cyanobacteria, *Microcystis aeruginosa* TAC 95 and M228 which also produce microcystins LR and YR, respectively. Their structures were mainly determined by 2D-NMR techniques and MS/MS method.

Acute hepatotoxicosis involving the hepatotoxins (liver toxins) is the most commonly encountered toxicosis by cyanobacteria. These toxins are produced by strains of species within the genera *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria* and *Nostoc*. The toxins from these genera include hepta- or pentapeptides. The cyclic heptapeptides are named microcystins and show severe hepatotoxicity.[1] It is also demonstrated that microcystins inhibit protein phosphatases 1 and 2A as strongly as "okadaic acid class" compounds such as okadaic acid, calyculin A and tautomycin, and are a tumor promoter of liver.[2] However, recent advance of researches on biological activity by microcystins suggests that other types of compounds than microcystins, which are simultaneously produced by cyanobacteria, are deeply concerned with the characteristic biological activities caused by toxic cyanobacteria.[3]

Originally, *Microcystis aeruginosa* TAC 95[4] and M228[5] produce microcystins LR and YR, respectively. In order to detect other compounds than microcystins, Frit-FAB LC/MS was applied to the toxic fractions from these strains. Each toxic fraction contained two compounds, which were separated by repeated silica gel and Toyopearl HW-40 chromatographies to give totally four compounds as white amorphous powder. They were designated as aeruginopeptins 95-A (**1**) and -B (**2**), and aeruginopeptins 228-A (**3**) and -B (**4**), whose physicochemical properties are shown in Table 1.

The compound **1** is a peptide with the molecular weight of 1145 and Thr, Glu, Ile and Tyr were identified to be constituent amino acids with L-configuration by Marfey method. A combination of 2D NMR techniques such as ^{1}H-^{1}H COSY, HOHAHA, HMQC and HMBC indicated the presence of other structural units, 4-hydroxyphenyllactic acid (Hpla), *N*-methylphenylalnine (MePhe) and 3-amino-6-hydroxy-2-piperidone (Ahp)[6] composed of Glu-γ-carbonyl-γ-aldehyde and Thr in addition to the protein amino acids mentioned above in **1**. Additionally, there are three Thr in the molecule, one of which is acylated, because H-3 of the Thr is shifted to

Table 1. Physicochemical properties and constituent amino acids of four aeruginopeptins

Peptide	Molecular formula Molecular weight	$[\alpha]_D$	UV (MeOH) λmax nm (ε)	Constituent amino acid***
A (1)	C56H75N9O17*	-35.1°	277 (2672)	L-Thr, L-Glu, L-Ile, L-Tyr
95	1145	(c 0.191, MeOH)	225 (18129)**	
B (2)	C56H79N9O17*	-39.5°	280 (2681)	L-Thr, L-Glu, L-Ile
	1149	(c 0.091, MeOH)	225 (12448)**	
A (3)	C52H68N8O15*	-34.0°	277 (3828)	L-Thr, L-Glu, L-Ile, L-Tyr
228	1044	(c 0.118, MeOH)	225 (22620)**	
B (4)	C52H72N8O15*	-33.0	280 (2096)	L-Thr, L-Glu, L-Ile
	1048	(c 0.091, MeOH)	225 (11528)**	

* These compositions were established by high resolution FABMS.
** Shoulder.
*** These were confirmed by Marfey method.

downfield at 5.57ppm. Glu should be replaced by Gln, because the difference of the molecular weight of **1** from that of the remaining structure units were 128. The absolute configuration of MePhe was also determined to be L by Marfey method.[7]

The sequencing of these structural units was carried out by HMBC technique. First, the carbonyl carbons of the constituent amino acids were assigned by correlation of the carbonyl carbon and its own H-2, assigned *via* the $^2J_{CH}$ coupling (Fig. 1). Next, the relationship between the carbonyl carbon and H-2 of the neighboring amino acid was confirmed by $^3J_{CH}$ coupling (Fig. 1). These experiments indicated that **1** has the following sequence: Thr (II)-Gln-Thr (III)-Tyr-Ahp-Thr (I)-MePhe-Ile and the hydroxy group in Thr (III) is combined with the carboxyl group *via* an ester bond. Although the connectivity of Hpla could not be clarified by this technique, it was shown that it is at *N*-terminus by MS/MS method as described later. Thus, **1** proves to be a 19-membered depsipeptide composed of 8 amino acid residues combined with Hpla as the *N*-terminus, although the stereochemistry at C-3 and -6 of Ahp and at C-2 of Hpla remains unsolved.

The difference of the molecular weights of **1** and **2** is 4 and Tyr was not detected by amino acid analysis of **2**. Although the ^1H and ^{13}C NMR spectra of **2** were quite similar to those of **1**, the difference was only observed in the lower field region, in which aromatic protons due to a *para*-substituted benzene in **1** disappeared and instead two olefinic protons (5.70 and 5.57 ppm, J = 10.1 Hz) appeared newly. These results suggested that **2** possesses a tetrahydrotyrosine (ThTyr) shown in Fig. 3 instead of Tyr in **1**. The presence of this abnormal amino acid was confirmed by DQF-COSY experiment. Actually, the same procedure using 2D-NMR techniques showed that **2** have the same 8 amino acid residues as those of **1** and ThTyr. However, the sequence of these amino acid residues was not able to be determined by HMBC.

In the FAB mass spectra of **1** and **2** several informative fragment ions were observed together with the molecular related ions, which can be classified into two groups, common and different ions to both components. So MS/MS method was applied to sequence determination of the constituent amino acid residues of aeruginopeptins under FABMS conditions. In the product ion spectrum of m/z 1128 $(M+H-H_2O)^+$ of **1**,[8] several ions that appeared in the FAB mass spectrum were prominently observed, which were characterized as the following two series (Fig. 2): the first pathway; the successive release of amino acid residues from the *N*-terminus of the precursor ion produces ***a*** and ***b*** and finally gives the most abundant ion ***c***, and another pathway;

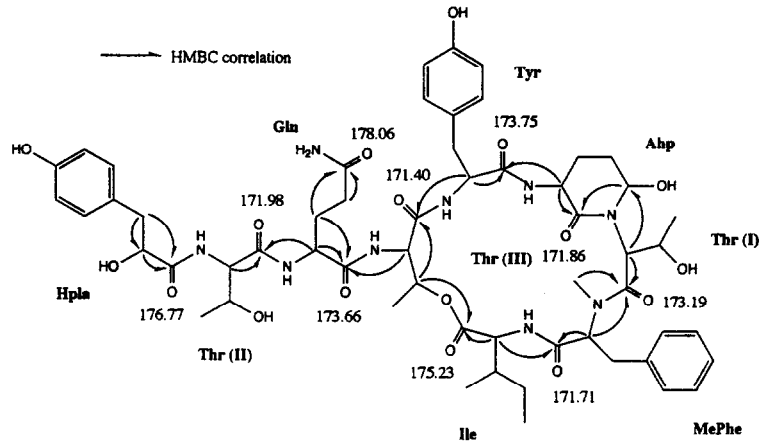


Fig. 1

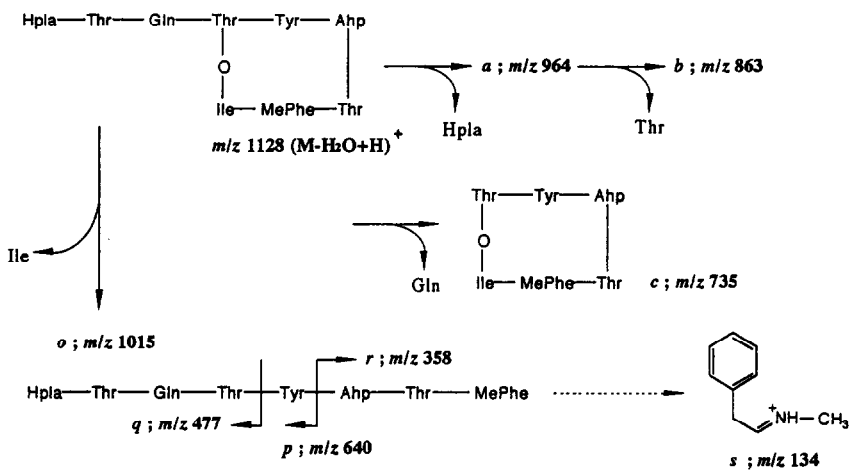
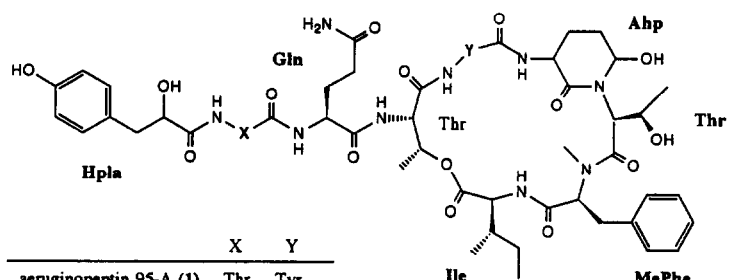
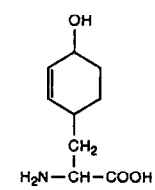


Fig. 2



	X	Y
aeruginopeptin 95-A (1)	Thr	Tyr
aeruginopeptin 95-B (2)	Thr	ThTyr
aeruginopeptin 228-A (3)	—	Tyr
aeruginopeptin 228-B (4)	—	ThTyr



ThTyr : tetrahydrotyrosine

Ahp : 3-amino-6-hydroxy-2-piperidone
Hpla : 4-hydroxyphenyllactic acid
MePhe : *N*-methylphenylalanine

Fig. 3

the loss of Ile from the precursor ion forms a linearlized ion, *o*, from which *C*-terminus ions *r* and *s* , and *N*-terminus ions, *p* and *q* are produced. The appearance of *a*, *p* and *q* confirmed the position of Hpla and the obtained sequence information by MS/MS were completely consistent with that by HMBC method, indicating that MS/MS experiment should be available for amino acid sequencing of aeruginopeptins. The application of this method to **2** concluded that **2** possesses the structure shown in Fig. 3, in which Tyr in **1** is only replaced by ThTyr.

The compounds **3** and **4** are also peptides with the molecular weights of 1044 and 1048, respectively and their amino acid compositions are quite similar to those of **1** and **2**. The respective difference of the molecular weights of **3** and **4** from **1** and **2** corresponds to one molar Thr. In the product ion spectrum of **3** by MS/MS the common ions to **1**, *b*, *c*, *r* and *s* are found along with *o*, *p* and *q* whose *m/z* values have the difference with 101 mass units from those of **1**, indicating that **3** possesses the structure shown in Fig. 3, in which Thr (II) is lost from **1**. Similarly, the structure of **4** was determined as shown in Fig. 3.

The proposed structures for aeruginopeptins are similar to those of dolastatin 13[6] and micropeptins A and B[8], because these compounds have commonly 19-membered depsipeptide moieties. Their biological activities have been reported to be an inhibitor of an enzyme and cytotoxic.[6,9] Although biological activity of the present peptides has not been investigated in detail, a simultaneous administration of **1** with microcystin LR enhances the toxicity compared with an administration of microcystin LR alone. The detailed results will be reported elsewhere.

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