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Structural elucidation of cyanobacterial peptides encoded by peptide synthetase gene in *Anabaena* species

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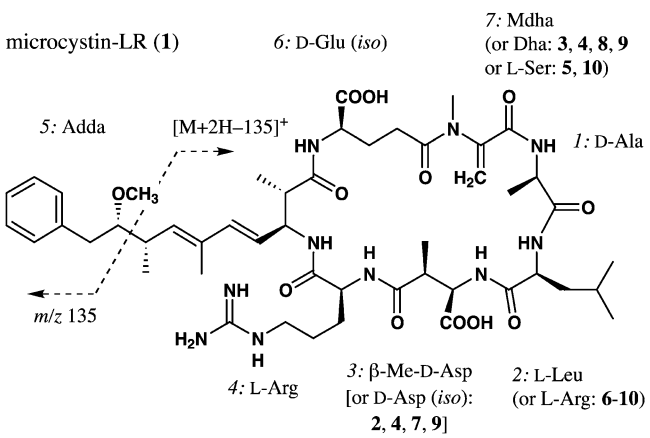
Abstract—During our biosynthesis study of cyanobacterial peptides including microcystins, we investigated the metabolic peptides in the hepatotoxic cyanobacteria, *Anabaena* sp. strains 90 and 202A2, in which the genetic analysis of the peptide synthetase had been carried out. For the exhaustive screening of cyanobacterial peptides, an analytical method using ESI-LC/MS on-line photodiode array detection was successfully developed and applied. Based on the analytical results, two groups of peptides, the cyclic depsipeptides having a 3-amino-6-hydroxy-2-piperidone moiety, anabaenopeptilides, and the cyclic peptides possessing an ureido linkage, anabaenopeptins, were isolated together with microcystins from both strains. Consequently, we confirmed the structures including the stereochemistry of the anabaenopeptilides encoded by the sequencing peptide synthetase genes in *Anabaena* strain 90.

1. Introduction

Cyanobacteria produce a wide variety of bioactive secondary metabolites.[1] The most well-known are the hepatotoxic cyclic peptides, microcystins and nodularins, which have caused the deaths of wild and domestic animals all over the world, and have also led to the deaths of 50 patients due to haemodialysis in Brazil in 1996.[2,3] Microcystins such as microcystin-LR (**1**) are known to be produced by the strains of four cyanobacterial genera, *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc*, and nodularin is produced by *Nodularia spumigena*.[2] More than 60 structural variants of the microcystins, cyclic heptapeptides, have been isolated.[4] In addition, a large number of peptides other than the hepatotoxic peptides have also been isolated from various cyanobacteria.[1]



In our studies, we have focused on the biosynthetic relationship between these peptides with hepatotoxic peptides by the above-mentioned toxic cyanobacterial genera.[5–10] As a result, it was suggested that the hepatotoxic cyanobacteria produce a few peptides other than microcystins. Thus, the isolated peptides were mainly classified into the following five structural groups: cyclic depsipeptides possessing a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety, depsipeptides having a tricyclic ring system, linear peptides composed of three amino acids and a fatty acid, and two groups of cyclic peptides possessing an ureido linkage or a β-amino acid.[1,5–10] Almost all these cyanobacterial peptides including microcystins form a cyclic structure and contain non-protein amino acids such as D-amino acid and *N*-methyl amino acid, suggesting that they are non-ribosomally formed by multifunctional peptide synthetase using the thiotemplate mechanism.[11,12] Recently, microcystin synthetase genes were cloned from the hepatotoxic cyanobacterium, *Microcystis aeruginosa* strain K-139, which produces [Dha[7]]microcystin-LR (**3**) and [D-Asp,[3] Dha[7]]microctsin-LR (**4**) as shown in Table 1.[13,14] The production of the microcystins was knocked out by insertional inactivation of the peptide synthetase gene, demonstrating that microcystins are formed by the peptide synthetase system.[13]

Furthermore, the synthetic genes of a peptide other than microcystins were also found in the same strain.[15] These

Keywords: cyanobacterial peptides; peptide synthetase gene; microcystins; advanced Marfey's method.

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Table 1. Structures and molecular-related ions (*m/z*) of microcystins detected from *Anabaena* sp. 90 and 202 strains

	2	*3*	*7*	$[M+H]^+$	$[M+2H]^{2+}$	$[M+2H-135]^+$
Microcystin-LR (**1**)	L-Leu	D-β-MeAsp	Mdha	995.5	498.3	861.5
[D-Asp^3] microcystin-LR (**2**)	L-Leu	D-Asp	Mdha	981.5	491.3	847.5
[Dha^7] microcystin-LR (**3**)	L-Leu	D-β-MeAsp	Dha	981.5	491.3	847.5
[D-Asp^3, Dha^7] microcystin-LR (**4**)	L-Leu	D-Asp	Dha	967.5	484.3	833.5
[L-Ser^7] microcystin-LR (**5**)	L-Leu	D-β-MeAsp	L-Ser	999.5	500.3	865.5
Microcystin-RR (**6**)	L-Arg	D-β-MeAsp	Mdha	1038.5	519.8	904.5
[D-Asp^3] microcystin-RR (**7**)	L-Arg	D-Asp	Mdha	1024.5	512.8	890.5
[Dha^7] microcystin-RR (**8**)	L-Arg	D-β-MeAsp	Dha	1024.5	512.8	890.5
[D-Asp^3, Dha^7] microcystin-RR (**9**)	L-Arg	D-Asp	Dha	1010.5	505.8	876.5
[L-Ser^7] microcystin-RR (**10**)	L-Arg	D-β-MeAsp	L-Ser	1042.5	521.8	908.5

β-MeAsp: β-methylaspartic acid; Mdha: *N*-methyldehydroalanine; Dha: dehydroalanine.

results strongly suggested that the toxic strains producing hepatotoxic peptides contain the synthetase genes for other groups of peptides and that the production of these peptides is related to that of the hepatotoxic peptides. Particularly, nostophycin isolated together with microcystin from the toxic *Nostoc* sp. strain 152 seems biosynthetically and genetically to be related to the microcystins, because both have a β-amino acid and two D-amino acids in common.[9] At present, the genetic research of multifunctional peptide synthetase using the thiotemplate mechanism is actively being carried out in gramicidin and other antibiotics.[11,12] In these investigations, it is essential to elucidate the structure including the stereochemistry of the corresponding metabolites and to determine the correlation of these structures to the peptide synthetase genes for the functional analysis of these genes.

During the course of our biosynthetic study of cyanobacterial peptides including microcystins, we tried to carry out two approaches from the metabolic and genetic sides using the two hepatotoxic *Anabaena* sp. strains 90 and 202A2.[16] In the genetic approach, an investigation of the peptide synthetase genes was made using *Anabaena* strain 90, and one of peptide synthetase genes other than that for microcystins was recently cloned and sequenced.[17] Furthermore, it was confirmed that the production of anabaenopeptilides was knocked out by insertional inactivation of the peptide synthetase gene in *Anabaena* strain 90, demonstrating that the cloned gene encodes the peptide synthetase for anabaenopeptilide. This result also suggested that cyanobacterial peptides other than microcystins are also formed by the peptide synthease system.[17] In order to identify the resulting metabolic peptides corresponding to the genes, we made an exhaustive inquiry into cyanobacterial peptides with an analytical system using the ESI-LC/MS technique and carried out the isolation and structural elucidation of these peptides as an approach from the metabolic side. As a result, two groups of peptides other than the microcystins were detected by the ESI-LC/MS analysis of *Anabaena* strains 90 and 202A2, in which two cyclic depsipeptides having the Ahp moiety named anabaenopeptilides (**11**–**14**) and a few types of cyclic peptides possessing an ureido linkage, the anabaenopeptins (**15**–**18**) were produced. In this paper, we describe the results of the exhaustive screening for cyanobacterial peptides using ESI-LC/MS, and the isolation and structural determination of the detected peptides from the toxic *Anabaena* strains 90 and 202A2 as the metabolic approaches.

2. Results

2.1. Screening of peptides in the toxic *Anabaena* strains

We have established the extraction and clean-up methods for microcystins using 5% AcOH aq. extraction and ODS cartridge treatment.[18] Furthermore, this method also has the advantage of an efficient extraction for almost all the peptides produced by cyanobacteria without extraction of pigment, which is a serious obstacle to the chromatographic analyses.[18] Therefore, we applied this extraction method to lyophilized cell samples of the toxic cyanobacteria, *Anabaena* sp. strains 90 and 202A2. These axenic strains were isolated from Lake Vesijärvi in Finland and then purified, and were shown to produce several kinds of microcystins as shown in Table 1.[16] Although we required a method for the differentiation of the microcystins from the group of peptides, the analytical methods for microcystins using HPLC with photodiode array detection and Frit-FAB LC/MS have been also established.[19] The HPLC analysis using photodiode array detection and mass chromatography monitored at *m/z* 135 using Frit-FAB LC/MS allow the rapid identification of the microcystins.[19] Under ESI conditions, the characteristic cleavage ion, $[M+2H-135]^+$ in the side-chain of the Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(*E*),6(*E*)-decadienoic acid] together with the fragment ion at *m/z* 135 is also observed as shown in **1**.[20] In this study, we applied the analytical method using ESI-LC/MS on-lined photodiode array detection to the peptide fractions extracted from the two *Anabaena* strains.

Fig. 1 shows the HPLC chromatograms monitored at UV 238 nm for the microcystins and at 280 nm for the other peptides and the reconstructed ion chromatograms (*m/z* 400–2000) of the extract from the *Anabaena* strains 90 (a) and 202A2 (b). The microcystins detected at UV 238 nm could be identified from the ESI-LC/MS spectra. Although the *Anabaena* strains 90 and 202A2 are known for the production of three microcystins (**1**, **2** and **6**) and four microcystins (**3**–**5** and **8**), respectively, other microcystins were also detected on the HPLC and the reconstructed ion chromatograms.[16] On the basis of the *m/z* values of the

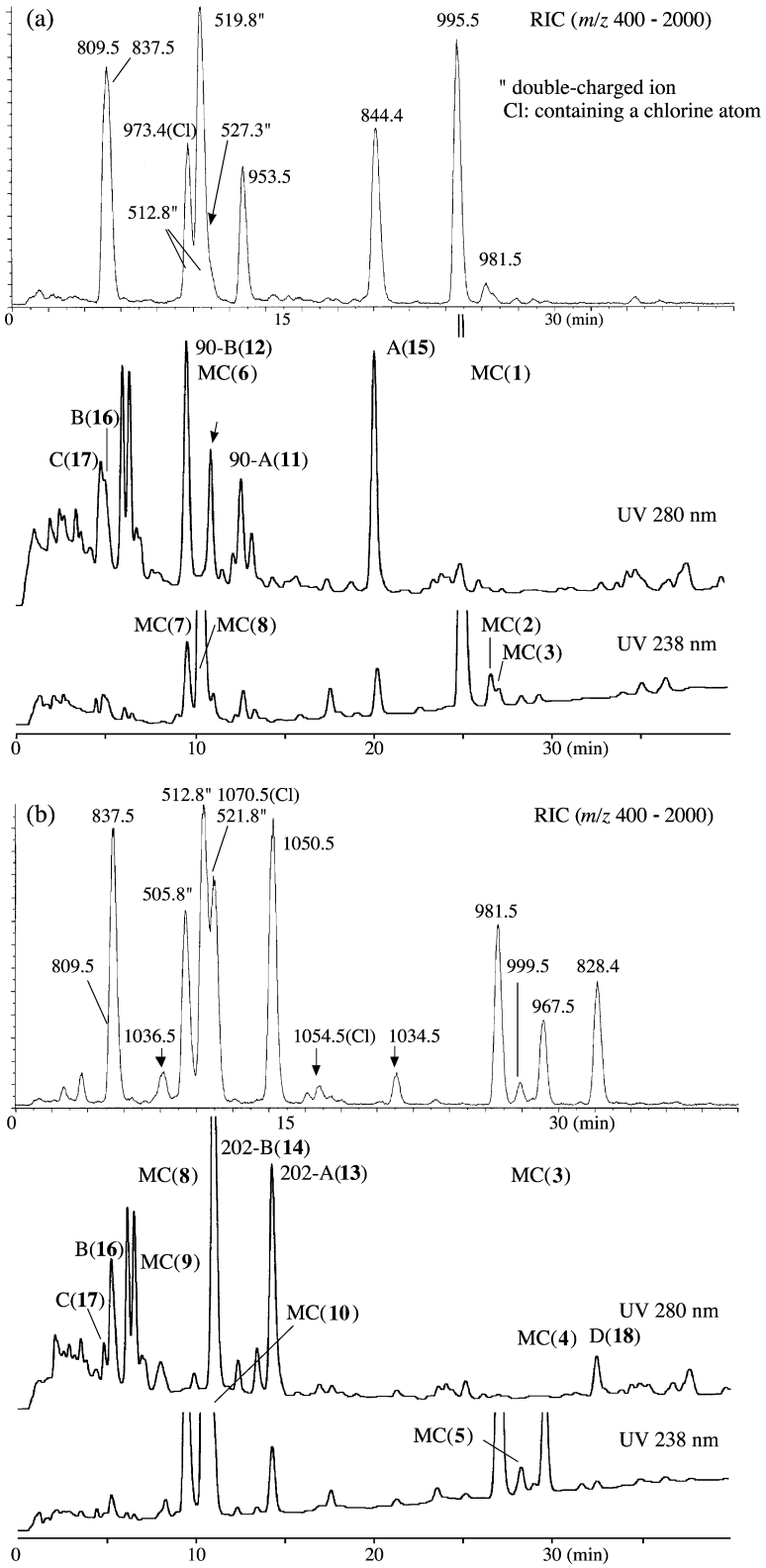
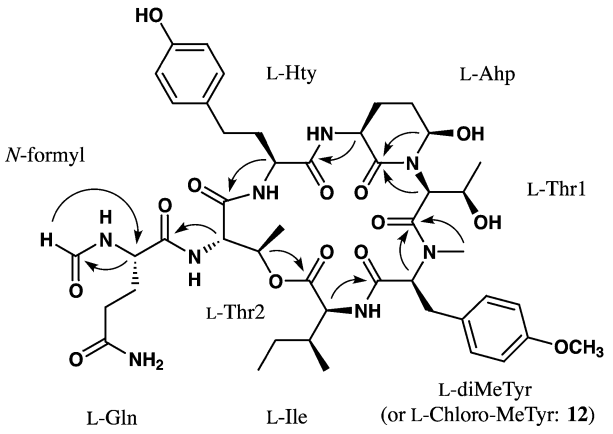


Figure 1. HPLC Chromatograms monitored at UV 238 and 280 nm, and the reconstructed ion chromatograms (*m*/*z* 400–2000) by ESI-LC/MS analysis of the extract from the *Anabaena* strains (a) 90 and (b) 202A 2. MC: microcystins, 90-A, 90-B and 202-A, -B: anabenopeptilides, A–D: anabaenopeptins.

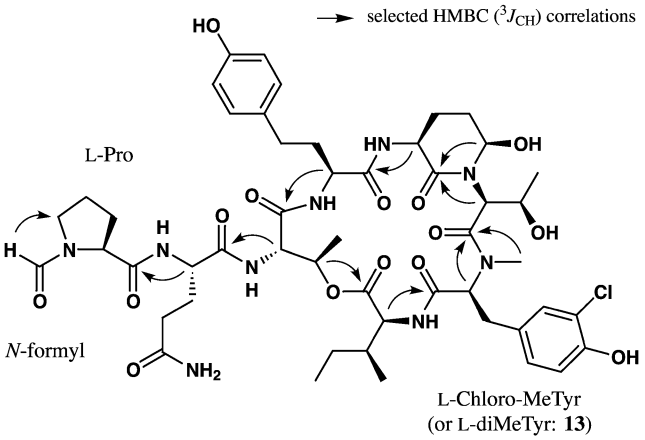
molecular ion species and the retention times of the detected microcystins, it was obvious that the *Anabaena* strain 90 additionally produced **3**, **7** and **8** and the *Anabaena* strain 202A2 additionally produced **9** and **10** (Table 1).

On the other hand, several peptides other than the microcystins could also be clearly detected by the HPLC chromatograms monitored at UV 280 nm and the reconstructed ion chromatograms. In the *Anabaena* strain 90, two

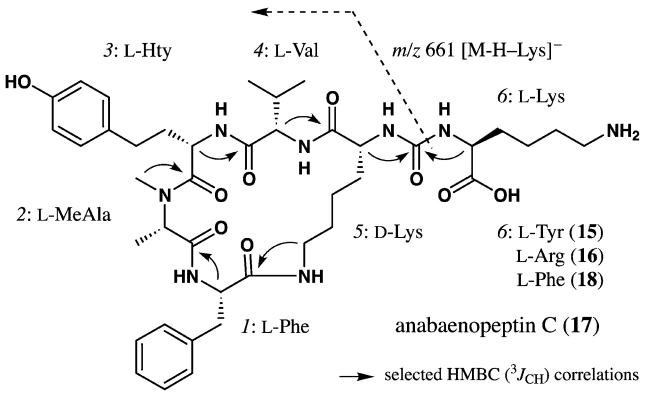
peptides designated as anabaenopeptilides 90-A (**11**) and -B (**12**) were observed at *m/z* 953.5 and 973.4, respectively, for the protonated molecules, $[M+H]^+$ as shown in Fig. 1 (a). Likewise, the two peptides designated as anabaenopeptilides 202-A (**13**) and -B (**14**) were mainly detected at *m/z* 1050.5 and 1070.5, respectively, for the protonated molecules in the *Anabaena* strain 202A2 as shown in Fig. 1 (b). These peptides also showed the characteristic molecular-related ions, $[M+H-H_2O]^+$ and $[M+18]^+$, together with the protonated molecules. In addition, the characteristic isotope patterns of the protonated molecules were observed in **12** and **14**, indicating the presence of a chlorine atom. Furthermore, the peptides detected at *m/z* 844.4 in the *Anabaena* strain 90 and at *m/z* 837.5 in both strains were identified as anabaenopeptins A (**15**) and B (**16**) by their *m/z* values and retention times, respectively, which were isolated from the hepatotoxic and neurotoxic *Anabaena flos-aquae* strain NRC525-17.[7] Additionally, anabaenopeptin C (**17**) was newly detected at *m/z* 809.5 in both strains and anabaenopeptin D (**18**) was detected at *m/z* 828.4 in the *Anabaena* strain 202A2. Some of unknown peptides were purified by repeated silica gel and TOYOPEARL HW-40F chromatographies and their structures were definitely determined as will be shown later.



anabaenopeptilide 90-A (**11**)



anabaenopeptilide 202-B (**14**)



2.2. Structure determination of unknown peptides from the toxic *Anabaena* strains

2.2.1. Anabaenopeptilides. Anabaenopeptilide 90-A (**11**) is a colorless amorphous powder, which was negative to ninhydrin. The positive and negative ion FABMS spectra showed $[M+H-H_2O]^+$, $[M+H]^+$ and $[M-H]^-$ peaks at *m/z* 935, 953 and 951, respectively, indicating a molecular weight of 952 for **11**. The molecular formula of **11** was established to be $C_{46}H_{64}N_8O_{14}$ based on the HRFABMS and NMR spectral data (Tables 2 and 3). It was suggested that **11** was a peptide compound based on the ^1H and ^{13}C NMR spectra in CD_3OD. The amino acid analysis of the 6 M HCl hydrolysates of **11** using the advanced Marfey's method[21] revealed the presence of a mole of L-isoleucine (Ile), *N*-methyl-L-tyrosine (*N*-MeTyr, from *N*,*O*-dimethyltyrosine), L-homotyrosine (Hty) and L-glutamic acid [Glu, from glutamine (Gln)] and two moles of L-threonine (Thr). Furthermore, the 2D NMR analysis of **11** confirmed the presence of a formyl group, these amino acids including *N*,*O*-dimethyltyrosine (diMeTyr) and an additional amino acid moiety. The structure of one more amino acid moiety, Ahp, was deduced as follows. In the COSY spectra, the connectivity from H-3 (4.59 ppm) to H-6 (5.25 ppm) was easily determined. The carbonyl carbon of C-2 at 171.6 ppm of Ahp was correlated with the protons of H-3 (4.59 ppm) and H-6 (5.25 ppm) of Ahp, and the proton of H-2 (4.51 ppm) of one of the Thr1 in the HMBC spectrum. Consequently, Ahp was deduced to constitute a part of the hemiaminol structure formed from glutamic-γ-carbonyl-γ-aldehyde and Thr1. The sequence of the constituent amino acids of **11** has been established with the help of the HMBC spectra. First, the carbonyl carbons of the constituent amino acids were assigned by correlation of the carbonyl carbons and its own α-proton assigned via the $^2J_{CH}$ coupling. Next, the connectivity between neighboring amino acids was confirmed by $^3J_{CH}$ coupling between the α-proton and carbonyl carbon in neighboring amino acid as shown in **11**. These obtained results indicated that anabaenopeptilide 90-A (**11**) had the following sequence: *N*-formyl Gln-Thr2-Hty-Ahp-Thr1-*N*,*O*-diMeTyr-Ile, and the hydroxy group in Thr2 was combined with the carboxy group in Ile at the C-terminus via an ester bond.

The molecular weight and formula of anabaenopeptilide 202-A (**13**) were established to be 1049 and $C_{51}H_{71}N_9O_{15}$ based on the FABMS and HRFABMS, respectively (Table 2). The amino acid analysis for **13** revealed the presence of

Table 2. Physicochemical properties and constituent amino acids of anabaenopeptilides and anabaenopeptins

Peptides	$[a]_D$[a]	Molecular formula (HRFAB MS data)[b]	Constituent amino acids[c]
Anabaenopeptilide 90-A (**11**)	−86.1	$C_{46}H_{64}N_8O_{14}$ (*m/z* 935.4504 Δ −1.1 mmu)	L-(Glu, Thr, Hty, MeTyr, Ile)
Anabaenopeptilide 90-B (**12**)	−99.5	$C_{45}H_{61}ClN_8O_{14}$ (*m/z* 955.3962 Δ −0.7 mmu)	L-(Glu, Thr, Hty, Cl-MeTyr, Ile)
Anabaenopeptilide 202-A (**13**)	−107.3	$C_{51}H_{71}N_9O_{15}$ (*m/z* 1032.5050 Δ +0.8 mmu)	L-(Pro, Glu, Thr, Hty, MeTyr, Ile)
Anabaenopeptilide 202-B (**14**)	−82.6	$C_{50}H_{68}ClN_9O_{15}$ (*m/z* 1052.4520 Δ +2.0 mmu)	L-(Pro, Glu, Thr, Hty, Cl-MeTyr, Ile)
Anabaenopeptin A (**15**)	−64.1	$C_{44}H_{57}N_7O_{10}$ (*m/z* 844.4263 Δ +1.8 mmu)	L-(Phe, MeAla, Hty, Val, Tyr), D-Lys
Anabaenopeptin B (**16**)	−71.4	$C_{41}H_{60}N_{10}O_9$ (*m/z* 837.4629 Δ +0.6 mmu)	L-(Phe, MeAla, Hty, Val, Arg), D-Lys
Anabaenopeptin C (**17**)	−65.2	$C_{41}H_{60}N_8O_9$ (*m/z* 809.4563 Δ +0.1 mmu)	L-(Phe, MeAla, Hty, Val, Lys), D-Lys
Anabaenopeptin D (**18**)	−50.0	$C_{44}H_{57}N_7O_9$ (*m/z* 828.4296 Δ 0.0 mmu)	L-(Phe, MeAla, Hty, Val, Phe), D-Lys

[a] $[\alpha]_D^{30}$ (*c* 0.050, MeOH).
[b] Anabaenopeptilides (**11**–**14**): HRFAB data for $[M+H-H_2O]^+$, anabaenopeptins (**15**–**18**): HRFAB data for $[M+H]^+$.
[c] Amino acid analysis using the advanced Marfey's method. Hty: homotyrosine, MeTyr: *N*-methyltyrosine Cl-MeTyr: 3-(3-chloro-4-hydroxyphenyl)-*N*-methylalanine, MeAla: *N*-methylalanine.

the same amino acids with the absolute configurations as **11** in addition to that of L-proline (Pro). The 1D NMR spectra of **11** and **13** completely resembled each other except for the additional resonances for Pro (Table 3). Therefore, anabaenopeptilide 202-A (**13**) was suggested to be a derivative with Pro inserted into **11**. The sequencing of the constituent amino acids in **13** was carried out using HMBC and ROESY techniques. As a result, it was confirmed that **13** had a structure common to **11** except that L-Pro was inserted between the *N*-formyl group and Gln of **11**.

The FABMS, HRFABMS and NMR spectral data established that anabaenopeptilides 90-B (**12**) and 202-B (**14**) had molecular weights of 972 and 1069 and molecular formula of $C_{45}H_{61}ClN_8O_{14}$ and $C_{50}H_{68}ClN_9O_{15}$, respectively, and both isotope patterns of the protonated molecules revealed the presence of a chlorine atom (Tables 2 and 3). The 1D NMR spectra of **12** and **14** closely resembled those of **11** and **13**, respectively except for the lack of resonances for a methoxy group in those of **11** and **13** (^{1}H: 3.72 ppm, ^{13}C: 55.7 ppm). In addition, the resonance for the *p*-substituted benzene ring of diMeTyr in 1D NMR spectra of **11** and **13** were replaced by those for the 1,3,4-trisubstituted benzene ring in those of **12** and **14**, suggesting that diMeTyr in both **11** and **13** was replaced by chlorinated *N*-methyltyrosine (Cl-MeTyr) in **12** and **14**, respectively. The amino acid analysis of **12** and **14** using the advanced Marfey's method revealed the presence of Cl-MeTyr in addition to the same other constituent amino acids as **11** and **13**, and their absolute configurations were determined as all L-configurations. The sequencing of their constituent amino acids was carried out using HMBC and ROESY techniques as shown in **14**. These results indicated that the structural difference in **12** and **14** from **11** and **13** was only one of the constituent amino acids, Cl-MeTyr, instead of diMeTyr in **11** and **13**, respectively. Throughout the experiments, it was observed that the ^{1}H and ^{13}C resonances for *N*-formyl Pro in **13** and **14** appeared as the doublet and the difference of the ^{1}H and ^{13}C chemical shifts in the doublet resonances was proportional to the distance from the *N*-formyl group (Table 3), suggesting that this phenomenon was attributable to the restricted rotation of the amide C–N bond.[22]

The cyclic depsipeptides containing the Ahp moiety such as the anabaenopeptilides have been frequently found as secondary metabolites of cyanobacteria.[1,5,6,10] The absolute configuration at C-3 of Ahp in **11**–**14** was determined on the basis of the amino acid analysis of Pro and pentahomoserine, which were derived from the hydrolysate of their reduced products.[21,23] Furthermore, the stereochemistry of Ahp in **11**–**14** was determined to be (3*S*,6*R*)-3-amino-6-hydroxy-2-piperidone on the basis of the following NMR spectral experiments: the small coupling constant of less than 2.0 Hz was observed between H-6 and each H-5, and the large coupling of constant more than 12.0 Hz that was observed between H-3 and one of H-4, indicating that the hydroxy groups at C-6 and H-3 are axially oriented.[24] The proposed absolute stereochemistry of Ahp was coincident with that of micropeptin 90,[24] cyanopeptolins[25] and A90720A.[26]

2.2.2. Anabaenopeptins. Cyclic peptides possessing an ureido linkage were isolated as another group of cyanobacterial peptides from both *Anabaena* strains. Anabaenopeptins A (**15**), B (**16**) and C (**17**) were isolated from the strain 90, and anabaenopeptins B (**16**) and D (**18**) were isolated from the 202A2 strain. We have isolated **15** and **16** from the hepatotoxic and neurotoxic *Anabaena flos-aquae* strain NRC525-17, which produces microcystins and anatoxin-a(s).[7] The structural determination of the unknown anabaenopeptins C (**17**) and D (**18**) were carried out on the basis of the comparison of the spectral data with those for **15** and **16**. The molecular weights and formula of **17** and **18** were established to be 808, $C_{41}H_{60}N_8O_9$ and 827, $C_{44}H_{57}N_7O_9$ based on the FABMS, HRFABMS and NMR spectral data, respectively (Table 4). The ^{1}D NMR spectra of the isolated anabaenopeptins (**15**–**18**) resembled each other, suggesting that they have the common cyclic pentapeptide moiety, *cyclo*-(ε-Lys-Phe-MeAla-Hty-Val), as shown in Table 4. Additionally, they gave the common characteristic fragment ion at *m/z* 661 under their negative FABMS conditions, and **15**, **16** and **18** were negative to the ninhydrin test, but **17** was positive. These data indicated that **17** and **18** have the same structure as **15** and **16** except that Tyr in **15** and and in Arg **16** were replaced by Lys and Phe in **17** and **18**, respectively. This conclusion was supported by 2D NMR spectral data including HMBC spectra as shown in **17** and Table 4. From these experimental results, it was shown that the anabaenopeptins were unique cyclic peptides that have the common cyclic pentapeptide moiety linked with Tyr, Arg, Lys and Phe, *via* an ureido bond. The elucidation of the stereochemistry of **17** and **18** was carried out using the advanced Marfey's method. As a result, four

Table 3. ^{13}C and ^{1}H NMR spectral data for anabaenopeptilides 90-A (**11**), -B (**12**) and 202-A (**13**), -B (**14**) in CD_3OD (the chemical shifts of ^{1}H signals, which overlapped with other ^{1}H signals in 1D spectrum, were determined using $^{1}H-^{1}H$ COSY and HSQC experiments)

Anabaenopeptilides 90-A (**11**) and 90-B (**12**)				Anabaenopeptilides 202-A (**13**) and 202-B (**14**)							
				Major[a]				Minor[a]			
Position		^{13}C	^{1}H (mult; *J*, Hz)	Position		^{13}C	^{1}H	Position		^{13}C	^{1}H
Ile	1	175.4		Ile	1	175.3		Ile	1	175.4	
	2	58.0	4.46 (d; 8.0)		2	57.9	4.51		2	58.0	4.47
	3	37.9	1.90 (m)		3	38.0	1.90		3	37.9	1.90
	4	26.5	1.21 (m), 1.45 (m)		4	26.4	1.21, 1.45		4	26.4	1.21, 1.45
	5	10.6	0.88 (t; 7.4)		5	10.7	0.88		5	10.6	0.88
	6	16.3	0.96 (d; 6.8)		6	16.3	0.95		6	16.2	0.95
diMeTyr in **11**	1	172.0		diMeTyr in **13**	1	171.9		diMeTyr in **13**	1	172.0	
	2	63.1	5.01 (dd; 3.0, 11.4)		2	63.1	5.01		2	63.1	5.01
	3	34.4	2.75 (m), 3.36 (dd; 3.2, 14.4)		3	34.4	2.77, 3.36		3	34.4	2.77, 3.36
	4	130.5			4	130.5			4	130.5	
	5, 9	131.8	7.17 (d; 8.8)		5, 9	131.8	7.17		5, 9	131.8	7.17
	6, 8	115.5	6.82 (d; 8.8)		6, 8	115.5	6.82		6, 8	115.5	6.82
	7	160.3			7	160.3			7	160.3	
	NMe	31.2	2.81 (s)		NMe	31.2	2.81		NMe	31.2	2.81
	OMe	55.7	3.72 (s)		OMe	55.7	3.72		OMe	55.7	3.72
Cl-MeTyr in **12**	1	171.8		Cl-MeTyr in **14**	1	171.7		Cl-MeTyr in **14**	1	171.8	
	2	62.9	4.99 (dd; 3.2, 11.6)		2	63.1	5.01		2	63.1	5.01
	3	34.1	2.72 (–), 3.33 (–)[b]		3	34.1	2.75, 3.33		3	34.1	2.75, 3.33
	4	130.3			4	130.5			4	130.5	
	5	131.9	7.13 (d; 2.0)		5	132.0	7.15		5	132.0	7.15
	6	122.0			6	121.9			6	121.9	
	7	154.0			7	153.7			7	153.7	
	8	118.3	6.81 (d; 8.0)		8	118.2	6.83		8	118.2	6.83
	9	130.3	7.05 (dd; 1.8, 8.2)		9	130.3	7.07		9	130.3	7.07
	NMe	31.3	2.80 (s)		NMe	31.2	2.81		NMe	31.2	2.81
Thr1	1	173.2		Thr1	1	173.2		Thr1	1	173.2	
	2	56.5	4.51 (d; 7.6)		2	56.5	4.51		2	56.5	4.51
	3	67.0	3.65 (dq; 6.4, 7.2)		3	67.0	3.65		3	67.0	3.65
	4	19.7	0.42 (d; 6.0)		4	19.7	0.43		4	19.7	0.43
Ahp	2	171.6		Ahp	2	171.6		Ahp	2	171.5	
	3	51.0	4.59 (dd; 6.4, 12.4)		3	51.0	4.60		3	51.0	4.60
	4	22.2	1.85 (m), 2.75 (m)		4	22.2	1.85, 2.77		4	22.2	1.85, 2.77
	5	30.9	1.83 (m), 2.00 (t; 14.0)		5	30.8	1.84, 2.02		5	30.8	1.84, 2.02
	6	77.2	5.25 (s)		6	77.2	5.26		6	77.2	5.26
Hty	1	174.3		Hty	1	174.2		Hty	1	174.2	
	2	54.3	4.28 (dd; 2.8, 11.2)		2	54.3	4.29		2	54.3	4.29
	3	33.5	1.88 (m), 2.42 (m)		3	33.5	1.88, 2.43		3	33.5	1.88, 2.43
	4	31.9	2.54 (m) 2.70 (m)		4	31.9	2.51 2.70		4	31.7	2.51 2.70
	5	132.6			5	132.6			5	132.6	
	6, 10	130.7	7.01 (d; 8.4)		6, 10	130.7	6.99		6, 10	130.7	6.99
	7, 9	116.2	6.66 (d; 8.8)		7, 9	116.3	6.67		7, 9	116.3	6.67
	8	156.7			8	156.7			8	156.7	
Thr2	1	172.0		Thr2	1	171.7		Thr2	1	171.9	
	2	57.6	4.78 (–)[b]		2	57.3	4.78		2	57.4	4.78
	3	73.1	5.62 (dq; 6.4, 6.8)		3	73.2	5.60		3	73.2	5.60
	4	18.7	1.43 (d; 6.4)		4	18.7	1.41		4	18.7	1.41
Gln	1	174.3		Gln	1	174.5		Gln	1	174.5	
	2	52.6	4.64 (t; 7.2)		2	54.3	4.54		2	54.6	4.54
	3	29.4	2.12 (m)		3	29.1	2.15		3	28.8	2.15
	4	32.4	2.38 (t; 7.6)		4	32.5	2.42		4	32.5	2.42
	5	177.8			5	177.8			5	177.8	
				Pro	1	173.9		Pro	1	174.1	
					2	59.1	4.41		2	61.2	4.52
					3	31.0	2.08, 2.3		3	31.0	2.08, 2.30
					4	25.2	1.92		4	24.0	1.87, 2.00
					5	48.2	3.51		5	45.5	3.67
Formyl group		163.5	8.10 (s)	Formyl group		163.5	8.26	Formyl group		164.3	8.17

diMeTyr: *N*,*O*-dimethyltyrosine; Cl-MeTyr: 3-(3-chloro-4-hydroxyphenyl)-*N*-methylalanine; Ahp: 3-amino-6-hydroxy-2-piperidone; Hty: homotyrosine.

[a] The isomerization is attributable to restricted rotation of amide C–N bond of *N*-formyl Pro.

[b] The multiplicity of ^{1}H signals were not determined due to the overlapping with other ^{1}H signals.

Table 4. ^{13}C and ^{1}H NMR spectral data for anabaenopeptins C (**17**) and D (**18**) in CD_3OD. (The chemical shifts of ^{1}H signals, which overlapped with other ^{1}H signals in 1D spectrum, were determined using ^{1}H–^{1}H COSY and HSQC experiments)

Anabaenopeptin C (**17**)				Anabaenopeptin D (**18**)			
Position		^{13}C	^{1}H	Position		^{13}C	^{1}H
Phe	1	174.9		Phe	1	174.9	
	2	57.6	4.60		2	57.6	4.58
	3	39.7	2.85, 3.38		3	39.7	2.82, 3.37
	4	139.8			4	139.9	
	5, 9	130.9	7.10		5, 9	130.9	7.11
	6, 8	130.5	7.25		6, 8	130.5	7.21
	7	128.4	7.15		7	128.4	7.16
MeAla	1	173.1		MeAla	1	173.1	
	2	57.2	4.69		2	57.2	4.72
	3	15.1	1.14		3	15.1	1.15
	NMe	29.1	1.85		NMe	29.1	1.85
Hty	1	174.6		Hty	1	174.6	
	2	51.0	4.71		2	51.0	4.69
	3	35.5	1.81, 2.07		3	35.4	1.80, 2.09
	4	32.9	2.58, 2.74		4	32.9	2.58, 2.73
	5	133.6			5	133.7	
	6, 10	131.3	7.05		6, 10	131.3	7.05
	7, 9	117.1	6.70		7, 9	117.1	6.70
	8	160.3			8	160.3	
Val	1	175.7		Val	1	175.6	
	2	62.3	3.87		2	62.1	3.86
	3	32.0	2.13		3	32.0	2.12
	4	21.5	1.02		4	21.4	1.03
	5	20.6	1.16		5	20.6	1.15
Lys	1	177.0		Lys	1	176.8	
	2	57.2	4.11		2	56.9	4.10
	3	33.4	1.73, 1.91		3	33.4	1.69, 1.88
	4	22.6	1.31, 1.48		4	22.5	1.25, 1.42
	5	30.0	1.60		5	30.0	1.58
	6	41.0	2.98, 3.69		6	40.9	2.93, 3.69
ureido C		157.7		ureido C		157.7	
Lys	1	180.6		Phe	1	179.4	
	2	56.7	4.13		2	57.6	4.42
	3	35.1	1.65		3	39.9	2.94, 3.15
	4	24.2	1.44		4	139.9	
	5	29.6	1.65		5, 9	130.9	7.22
	6	41.6	2.85		6, 8	130.5	7.22
					7	128.4	7.21

MeAla: *N*-methylalanine; Hty: homotyrosine.

constituent amino acids in common, Val, Phe, Hty and MeAla, have the L-configurations. Application of this method using LC/MS provided an additional advantage that it can easily detect trace amounts of the remaining constituent amino acids, two moles of Lys for **17** and Lys and Phe for **18**, because the amounts of amino acids forming an ureido bond were limited in the usual acid hydrolysate.[7,21] Furthermore, this technique clearly indicated that **17** possesses each D- and L-Lys and **18** has D-Lys and L-Phe. Finally, the absolute configuration of Lys in the cyclic moiety of **17** was determined as the D-configuration, because the ^{1}H and ^{13}C resonances of the cyclic moiety in **17** showed the same data as that of the other anabaenopeptins, indicating that they have the cyclic moiety with a common stereochemistry.

3. Discussion

In the present study, we tried to correlate the secondary metabolites produced by the hepatotoxic cyanobacteria to the corresponding peptide synthetase genes. First, an analytical method using ESI-LC/MS on-line photodiode array detection was developed for the exhaustive screening of cyanobacterial peptides in two *Anabaena* strains, 90 and 202A2 and it was successfully applied to the peptide fractions extracted from these strains. As a result, eight peptides other than microcystins could be clearly detected in these fractions. The established method was advantageous over conventional ones using MALDI-TOFMS[17] (matrix assisted laser desorption ionization-time of flight mass spectrometry) and usual HPLC,[13] because more structural information could be obtained and it is easier to distinguish microcystins from other peptides using this method. Small amounts of peptides could also be detected by this method. The established method will contribute to the investigation of the relationship between genes encoding synthetases for peptides and secondary metabolic peptides.

Based on analysis results using this screen method, we isolated and characterized four cyclic depsipeptides, anabaenopeptilides 90-A (**11**), -B (**12**), and 202-A (**13**), -B (**14**), and four cyclic peptides, anabaenopeptins A–D (**15**–**18**) together with microcystins from the extracts of two *Anabaena* strains. The anabaenopeptilides and anabaenopeptins have characteristic depsipeptide and cyclic peptide structures composed of an Ahp moiety and ureido linkage, respectively. Particularly, the advanced Marfey's method was quite effective during the structure determination step, because it was possible to determine the absolute configuration of the constituent amino acids without standard samples. For the biosynthetic study, the screening and the structural elucidation including stereochemistry for the cyanobacterial metabolites are extremely important.

As mentioned earlier, Rouhiainen et al., reported genes encoding the synthetases of the anabaenopeptilides in *Anabaena* strain 90.[17] Four genes encoding putative anabaenopeptilide synthetase domains were characterized, three of which, *apdA*, *apdB* and *apdD*, contained two, four and one modules, respectively. They encoded a total of seven modules for activation and peptide bond formation of seven L-amino amino acids. The remaining gene (*apdC*) was considered for halogenation. Furthermore, the production of two anabaenopeptilides 90-A (**11**) and -B (**12**) was knocked out by insertional inactivation of the corresponding peptide synthetase gene in the *Anabaena* strain 90.[17] The results from the present study structurally supported their conclusions including the stereochemistry. The Ahp moiety is one of the structural characteristic features for anbaenopeptilides, micropeptins and aeruginopeptins and is considered to be composed of Gln and Thr.[17] Although the formation of Ahp needs a reduction step of Gln, no such module was found in the reported organization and modular structure. In many cases, toxic cyanobacteria simultaneously produce several components of peptides such as anabaenopeptilides and anabaenopeptins as shown in this study. For example, *Anabaena* strain 90 simultaneously produced anabaenopeptilides 90-A (**11**) and -B (**12**), which raise the question of how the strain regulates the production of these components. At present, it is impossible to obtain such information from only the results of the gene analysis.

Consequently, the present study showed that these *Anabaena* strains individually had at least three peptide synthetase genes for microcystins, anabaenopeptilides and anabaenopeptins, although the genes encoding the synthetases of the anabaenopeptins have not yet been cloned. The relationship of these genes is still unknown and they seem to be independent of each other. Rouhiainen et al. tried to detect the synthetase (ApdB) corresponding to *apdB* by western blots using antibodies.[17] Probably, some of problems raised above will be solved in a protein level study in the very near future.

4. Experimental

4.1. General procedure

Optical rotations were recorded at 30°C at the sodium D line. FABMS and HRFABMS spectra were obtained using glycerol as the matrix on a JEOL JMS HX-110 mass spectrometer. NMR spectra were measured on a JEOL JNM A400 NMR spectrometer operating at 400 MHz for the ^{1}H and at 100 MHz for the ^{13}C in CD_3OD. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks (^{1}H: 3.30 and ^{13}C: 49.0 ppm in CD_3OD). The amino acid analyses were carried out by the advanced Marfey's method using HPLC and ESI-LC/MS.[21,23]

4.2. Materials

Anabaena sp. strains 90 and 202A2 (isolation and strain history described in Ref. 16 were cultivated in the defined inorganic nutrient culture medium, called Z8, minus its normal concentration of nitrogen ingredients. Cells were harvested after two weeks of cultivation and freeze-dried.

4.3. Extraction of peptide fraction for HPLC and ESI-LC/MS analysis

Each dried cell (50 mg) was extracted four times with 5% AcOH(aq) (10 mL) for 30 min while stirring. The combined extracts were centrifuged at 5000 rpm for 10 min, and the supernatant was applied to a preconditioned ODS silica gel cartridge (0.5 g, Sep-Pak tC18) after filtration through a glass microfiber filter (GF/C). The cartridge was rinsed with water (5 mL) and 20% MeOH aq. (5 mL) and then eluted with 90% MeOH(aq) and MeOH (each 5 mL) to give the desired peptide fraction. After dilution with 400 μL of mobile phase for the ESI-LC/MS analysis, 1 μL of the extracted samples was analyzed by HPLC and ESI-LC/MS analyses.

4.4. HPLC and ESI-LC/MS conditions for the extracted peptide fraction

The separation of the peptides was performed on a TSK gel ODS-80Ts (150×2.0 mm i.d., TOHSO) column maintained at 40°C using an HP1100 HPLC system (Aglent). Methanol–water containing 0.1% formic acid was used as the mobile phase under a linear gradient elution mode (methanol, 45–65%, 40 min) at a flow rate of 0.2 mL/min. The mass spectrometer used was an API Qstar Pulser-*i* (MDS Sciex, Toronto, Canada). All mass spectra were acquired using TOFMS. A mass range of *m/z* 300–2000 was covered with a scan time of 1 s, and data were collected in the positive ion mode. The high performance liquid chromatograph and mass spectrometer were interfaced with a laboratory-made flow splitter and an IonSpray ion source (MDS Sciex). The effluent from the HPLC was split at a ratio of 1:40, and a smaller portion of the effluent was introduced into the ion source at a flow rate of 5 μL/min. The IonSpray voltage was 5 kV with the nebulizer gas air pressure and curtain gas nitrogen pressure set at 20 and 25 psi, respectively.

4.5. Isolation

Each dried cell [strains 90 (4.7 g) and 202A2 (5.0 g)] was extracted four times with 5% AcOH(aq) (200 mL) for 30 min while stirring. The combined extracts were centrifuged at 9000 rpm for 30 min, and the supernatant was applied to a preconditioned ODS silica gel cartridge (20 g, Chromatorex ODS) after the filtration through a glass microfiber filter (GF/A). The cartridge was rinsed with water (200 mL) and 20% MeOH (aq.) (200 mL) and then eluted with MeOH (400 mL) to provide a fraction containing the desired peptides. The fraction (82 mg) from strain 90 was separated to give **11** (3.8 mg), **12** (6.6 mg), **15** (6.5 mg), **16** (4.1 mg) and **17** (2.6 mg) using the following chromatographies: silica gel [Silica gel 60 (230-400 mesh)] using AcOEt/2-PrOH/H_2O (4:3:7, upper layer), $CHCl_3$/MeOH/H_2O (65:35:10 or 65:25:5, lower phase) and TOYOPEARL HW-40F (890×11 mm i.d.; flow rate, 0.3 mL/min; detection, UV230 nm) using MeOH. The fraction (90 mg) from strain 202A2 was separated to give **13** (10.5 mg), **14** (8.5 mg), **16** (6.4 mg) and **18** (1.5 mg) using the following chromatographies: silica gel using AcOEt/2-PrOH/H_2O (4:3:7, upper layer), $CHCl_3$/MeOH/H_2O (65:35:10, lower phase) and TOYOPEARL HW-40F using MeOH.

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