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Non-Toxic Peptides from Toxic Cyanobacteria, *Oscillatoria agardhii*

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Abstract—For the elucidation of the biosynthetic relationship between non-toxic and hepatotoxic peptides produced by cyanobacteria, we compared the secondary metabolites from the toxic and non-toxic cyanobacteria, *Oscillatoria* (*Planktothrix*) *agardhii*. Three groups of peptides, cyclic depsipeptides having a 3-amino-6-hydroxy-2-piperidone moiety, a cyclic nonapeptide and cyclic peptides possessing an ureido linkage were isolated together with microcystins from the toxic *O. agardhii* strains 97 and CYA128. A tricyclic peptide composed of 14 amino acid residues was isolated from the non-toxic *O. agardhii* strains 2 and 18.

Some cyanobacteria produce toxins such as the hepatotoxic cyclic peptides, microcystins and nodularin, and neurotoxic alkaloids, anatoxin-a and -a(s).[1] Microcystins are known to be produced by the strains of four cyanobacterial genera, *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*) and *Nostoc*, and nodularin is produced by *Nodularia spumigena*.[1] They have caused the deaths of wild and domestic animals all over the world, and have led to the deaths of 50 patients from haemodialysis in Brazil in 1996.[2,3] Microcystins are cyclic heptapeptides and are presumed to be non-ribosomally synthesized by peptide synthetases.[4,5] Recently, Börner et al. partially identified microcystin synthetase genes in the toxic cyanobacterial strain and confirmed that microcystins are non-ribosomally synthesized.[5] Furthermore, the synthetic genes of a non-toxic peptide other than microcystins were also isolated from the same strain.[5] Actually, a large number of non-toxic peptides have been isolated from various cyanobacteria.[6]

In our studies, we have focused on the biosynthetic relationship between these peptides and hepatotoxic peptides, and carried out the detection, isolation and structural determination of peptides produced together with hepatotoxic peptides by the toxic cyanobacterial genera, *Microcystis*, *Anabaena*, *Nostoc* and *Nodularia* (Table 1). Aeruginopeptins, cyclic depsipeptides possessing a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety, were isolated from not only the cultured cells of toxic *M. aeruginosa* but also bloom samples.[7,8] Anabaenopeptins, cyclic peptides possessing an ureido linkage, were isolated from *A. flos-aquae* NRC 525-17 that simultaneously produced anatoxin-a(s) and microcystins.[9] Anabaenopeptilides, whose structures are similar to those of aeruginopeptins, were also isolated along with anabaenopeptins from the toxic *Anabaena* sp. strains which co-produced microcystins.[10] On the other hand, no peptide has been detected from the neurotoxic *Anabaena* sp. strains which produced anatoxin-a.[11] Furthermore, we compared the products from the toxic and non-toxic *N. spumigena*. While we isolated two groups of peptides, cyclic peptides such as anabaenopeptins and linear peptides composed of three amino acids and a fatty acid, together with nodularin from toxic *N. spumigena*, two glycosidic compounds were isolated instead of these peptides from non-toxic *N. spumigena*.[11] These results strongly suggested that the toxic strains producing hepatotoxic peptides have the synthetase genes for other groups of peptides and that the production of these

Table 1. Peptides isolated from toxic and non-toxic cyanobacteria (*M*: *Microcystis aeruginosa*, *A*: *Anabaena* sp., *O*: *Oscillatroia* (*Planktothrix*) *agardhii*, *N*: *Nodularia spumigena*)

	Group	Peptide (cyanobacteria)
Toxic strain	Cyclic depsipeptide possessing Ahp moiety	Aeruginopeptins[7,8](*M*), anabaenopeptilides[10] (*A*) oscillapeptilides (*O*), oscillapeptin G[20] (*O*)
	Cyclic peptide possessing ureido linkage	Anabaenopeptins[9,25] (*A*, *O*), nodulapeptins[11] (*N*) oscillamide Y[26] (*O*)
	Cyclic peptide possessing β-amino acid	Nostophycin[12] (*Nostoc sp.*)
	Cyclic peptide	Oscillacyclin (*O*)
	Linear peptide	Spumigins[11] (*N*)
Non-toxic strain	Tricyclic peptide	Microviridin I (*O*)
	Glycosidic compound	Suomilide[11] (*N*)

Keywords: biologically active compound; biosynthesis; cyanobacteria; peptides and polypeptides.
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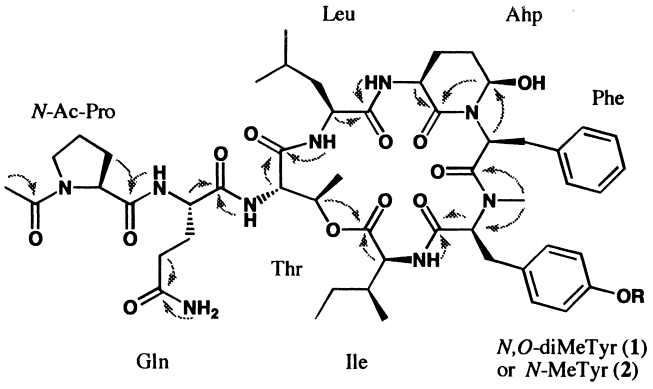
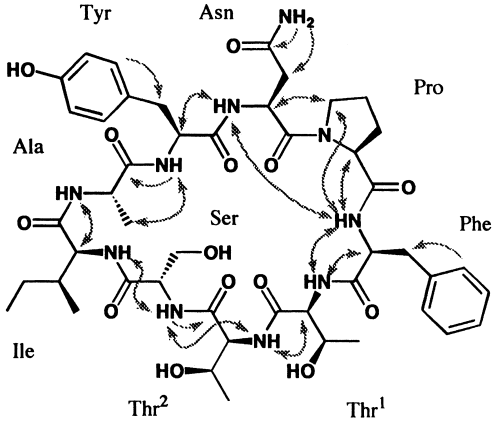
peptides is closely related to that of the hepatotoxic peptides. In addition, it is suggested that nostophycin isolated together with microcystin from the toxic *Nostoc* sp. 152 is biosynthetically related to the microcystins, because both have a β-amino acid and two D-amino acids in common.[12]

For the elucidation of this biosynthetic relationship, we further investigated the secondary metabolites from the remaining toxic cyanobacterial genus, *Oscillatoria*, which is now also called *Planktothrix*, and additionally compared the secondary metabolites from the toxic and non-toxic strains. The toxic cyanobacteria, *Oscillatoria* (*Planktothrix*) *agardhii* strain 97 isolated from Lake Maarianallas and strain CYA128 isolated from Lake Vesijärvi in Finland, produce [D-aspartic acid[3]]microcystin-RR and [dehydroalanine[7]]microcystin-RR as the main toxin, respectively.[13] The *O. agardhii* strain 2 isolated from Lake Markusbölefjärden and strain 18 (green pigment) isolated from Lake Långsjön in Finland were the non-toxic strains.[14,15] In the present study, novel cyclic depsipeptides, oscillapeptilide 97-A (**1**) and -B (**2**), a new cyclic nonapeptide, oscillacyclin (**4**), and known cyclic peptides possessing an ureido linkage, anabaenopeptins B (**5**) and F (**6**), were isolated from the toxic *O. agardhii* strain 97 (Fig. 1). Likewise, a cyclic depsipeptide, oscillapeptin G (**3**), and cyclic peptides having an ureido linkage, anabaenopeptins F (**6**) and oscillamide Y (**7**), were isolated as known peptides with microcystins from the toxic *O. agardhii* strain CYA128. No such peptides were detected in the extract of the non-toxic *O. agardhii* strains 2 and 18, whereas a tricyclic peptide, microviridin I (**8**) was isolated as a new peptide from these strains. In this paper, we report the isolation and structural determination of these peptides from the toxic and non-toxic *Oscillatoria* (*Planktothrix*) *agardhii* strains (Table 1).

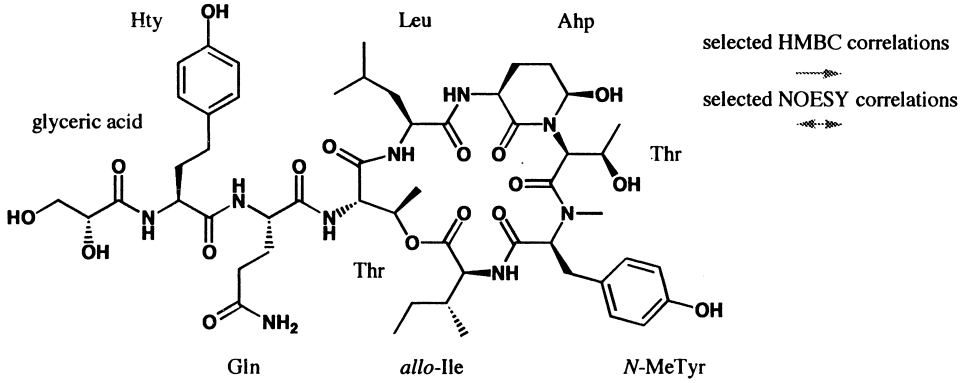
First of all, a method for the differentiation of microcystins from non-toxic peptides other than the microcystins was required in this study. We have already established the analytical methods for microcystins using HPLC with photodiode array detection and Frit-FAB LC/MS.[16,17] These methods have the advantage that peptides other than microcystins can also be detected and characterized at a screening stage, and we have successfully applied these techniques to previous studies.[7–12] In the present study, we also applied these techniques to detect peptides and confirmed the presence of several peptides together with microcystins in the 5% AcOH aqueous extracts of these toxic strains. Additionally, an unknown peptide was detected in both the 5% AcOH aqueous extracts of the non-toxic *O. agardhii* strains 2 and 18. Subsequently, the peptides detected by these techniques were purified from each extract of the cultured cells of the *O. agardhii* strains



oscillapeptilide 97-A (**1**) and 97-B (**2**)



oscillacyclin (**4**)



oscillapeptin G (**3**)

Figure 1.

Table 2. ^{13}C and ^{1}H NMR spectral data for Oscillapeptilide 97-A (**1**) in DMSO-d_6 (The chemical shifts of ^{1}H resonances, which overlapped with other ^{1}H resonances in 1D spectrum, were determined using ^{1}H–^{1}H COSY and HSQC experiments)

Position		Major[a]		Minor[a]		Position		^{13}C	^{1}H (mult; *J*, Hz)
		^{13}C	^{1}H (mult; *J*, Hz)	^{13}C	^{1}H (mult; *J*, Hz)				
Ac						Ahp			
	1	168.5		168.6			2	168.8	
	2	22.2	1.98 (s)	22.0	1.83 (s)		3	48.4	3.63 (m)
Pro							3-NH		7.06 (–[b])
	1	172.0					4	21.6	1.58 (m)
	2	58.9	4.32 (dd; 8.1, 2.9)	60.3	4.36 (dd; 8.6, 2.8)				2.37 (m)
	3	29.4	1.87 (m)	31.7	1.92 (m)		5	29.3	1.52 (m)
			2.20 (m)		2.18 (m)				1.67 (m)
	4	24.2	1.87 (m)	22.5	1.75 (m)		6	73.7	5.99 (t; 3.7)
	5	47.5	3.46 (m)	46.2	3.32 (m)		6-OH		5.05 (br s)
			3.54 (m)		3.42 (m)	Phe			
Gln							1	170.3	
	1	171.8		171.9			2	50.1	4.68 (dd; 11.4, 4.2)
	2	52.4	4.38 (m)	52.2	4.41 (m)		3	35.3	1.78 (–[b])
	2-NH		8.12 (br d; 7.7)		8.27 (br d; 8.1)				2.85 (dd; 13.9, 11.7)
	3	27.3	1.76 (m)	27.4	1.78 (m)		4	136.6	
			1.88 (m)		1.90 (m)		5,9	129.2	6.77 (d; 7.0)
	4	31.3	2.11 (m)	31.4	2.12 (m)		6,8	127.7	7.16 (t; 7.0)
	5	173.9		173.7			7	126.2	7.12 (d; 8.8)
	5-NH_2		7.18 (br s)		6.70 (br s)	*N*,*O*-diMeTyr			
			7.24 (br s)		6.73 (br s)		1	168.9	
Thr							2	60.7	4.92 (dd; 11.4, 1.8)
	1	169.0					2-NCH_3	30.2	2.76 (s)
	2	54.5	4.55 (d; 10.3)	54.6	4.58 (d; 10.3)		3	32.7	2.76 (–[b])
	2-NH		7.69 (br d; 9.2)		7.91 (br d; 9.2)				3.15 (dd; 13.9, 1.8)
	3	71.9	5.37 (–[b])	71.9	5.38 (–[a])		4	129.4	
	4	17.5	1.16 (d; 6.6)	17.6	1.16 (d; 6.6)		5,9	114.0	6.95 (d; 8.8)
Leu							6,8	130.5	7.12 (d; 8.8)
	1	170.0					7	158.1	
	2	50.1	4.12 (m)				7-OCH_3	55.0	3.70 (s)
	2-NH		8.35 (br d; 8.8)			Ile			
	3	39.0	1.70 (m)				1	171.2	
			1.79 (m)				2	55.0	4.73 (d; 4.4)
	4	24.1	1.43 (m)				2-NH		7.41 (–[b])
	5	20.9	0.72 (d; 6.2)				3	37.6	1.79 (m)
	6	23.2	0.83 (d; 7.0)				4	24.2	0.99 (m)
									1.20 (m)
							5	11.4	0.82 (t; 7.5)
							6	16.0	0.85 (d; 7.3)

[a] The isomerization is attributable to restricted rotation of the amide *C*–*N* bond of *N*-acetyl Pro.
[b] The multiplicity of ^{1}H resonances were not determined due to the overlapping with other ^{1}H resonances.

by repeated silica gel and TOYOPEARL HW-40F chromatographies on the basis of the results of TLC analysis using iodine vapor as the detection.

Oscillapeptilide 97-A (**1**) is a colorless amorphous powder, which is negative to ninhydrin. The positive ion and negative ion FABMS spectra showed $[M+H]^+$, $[M+H-H_2O]^+$ and $[M-H]^-$ peaks at *m*/*z* 1046, 1028 and 1044, respectively, indicating a molecular weight of 1045 for **1**. The molecular formula of **1** was established to be $C_{53}H_{75}N_9O_{13}$ based on the HRFABMS and NMR spectral data (Table 2). It was suggested that **1** is a peptide compound based on the ^{1}H and ^{13}C NMR spectra. The amino acid analysis of the 6 M HCl hydrolysates of **1** using the advanced Marfey's method[18] revealed the presence of L-isoleucine (Ile), *N*-methyl-L-tyrosine (*N*-MeTyr, from *N*,*O*-dimethyltyrosine), L-phenylalanine (Phe), L-leucine (Leu), L-threonine (Thr), L-glutamic acid (Glu, from glutamine) and L-proline (Pro). Furthermore, the 2D NMR analysis of **1** confirmed the presence of an acetyl group, these amino acids including glutamine (Gln) and *N*,*O*-dimethyltyrosine (*N*,*O*-diMeTyr), and an additional amino acid moiety. However, Phe was suggested to be an *N*,*N*-disubstituted derivative, because its amide proton was not observed. The structure of one more amino acid moiety, 3-amino-6-hydroxy-2-piperidone (Ahp), in **1** was deduced as follows. In the COSY spectra, the connectivity from 3-*N*H (7.06 ppm) to 6-*O*H (5.05 ppm) was easily determined. The carbonyl carbon of C-2 at 168.8 ppm of Ahp was correlated with the protons of H-3 (3.63 ppm) and H-6 (5.99 ppm) of Ahp, and the carbon of C-6 (73.7 ppm) of Ahp was correlated with the proton of H-2 (4.68 ppm) of the Phe derivative in the HMBC spectrum. Consequently, Ahp was deduced to constitute a part of the hemiaminol structure formed from glutamic-γ-carbonyl-γ-aldehyde and Phe. The sequence of the constituent amino acids of **1** has been established with the help of the HMBC spectra as shown in **1**. These obtained results indicated that oscillapeptilide 97-A (**1**) has the following sequence: *N*-acetyl Pro-Gln-Thr-Leu-Ahp-Phe-*N*,*O*-diMeTyr-Ile, and the hydroxy group in Thr is combined with the carboxy group in Ile at the *C*-terminus via an ester bond.

Table 3. ^{13}C and ^{1}H NMR spectral data for Oscillacyclin (**4**) in DMSO-d_6 (The chemical shifts of ^{1}H resonances, which overlapped with other ^{1}H resonances in 1D spectrum, were determined using ^{1}H–^{1}H COSY and HSQC experiments)

Position		^{13}C	^{1}H (mult; *J*, Hz)
Thr[1]			
	1	168.9	
	2	55.8	4.78 (dd; 9.0, 4.6)
	2-NH		7.46 (br d; 8.8)
	3	67.1	4.18 (–[a])
	3-OH		5.10 (br d; 7.3)
	4	17.6	0.98 (d; 6.2)
Thr[2]			
	1	171.5	
	2	59.5	4.13 (–[a])
	2-NH		8.10 (br d; 8.5)
	3	65.5	4.17 (–[a])
	3-OH		4.94 (–[a])
	4	20.5	1.11 (d; 6.6)
Ser			
	1	169.0	
	2	56.3	4.19 (–[a])
	2-NH		7.59 (br d; 7.7)
	3	61.8	3.59 (m)
			3.70 (m)
	3-OH		4.72 (br s)
Ile			
	1	171.3	
	2	56.2	4.21 (–[a])
	2-NH		7.10 (br d; 9.5)
	3	36.1	1.68 (m)
	4	24.1	0.98 (m)
			1.42 (m)
	5	10.5	0.77 (t; 7.3)
	6	14.7	0.91 (d; 7.0)
Ala			
	1	172.5	
	2	49.9	3.73 (m)
	2-NH		8.47 (br d; 2.5)
	3	16.1	1.01 (d; 7.0)
Tyr			
	1	169.5	
	2	56.7	3.67 (m)
	2-NH		8.25 (br d; 7.3)
	3	32.7	3.10 (–[a])
	4	129.0	
	5,9	129.9	6.87 (d; 8.4)
	6,8	114.8	6.62 (d; 8.4)
	7	155.6	
	7-OH		9.11 (br s)
Asp			
	1	170.7	
	2	47.0	4.95 (–[a])
	2-NH		7.62 (br d; 8.4)
	3	37.6	2.20 (dd; 11.0, 3.6)
			3.13 (–[a])
	4	173.6	
	4-NH_2		7.41 (br s)
			7.77 (br s)
Pro			
	1	170.1	
	2	60.4	4.15 (–[a])
	3	28.8	1.37 (m)
			1.85 (m)
	4	23.2	1.12 (m)
			1.65 (m)
	5	47.1	3.50 (m)
			3.75 (m)
Phe			
	1	170.4	
	2	54.6	4.43 (ddd; 13.2, 9.2, 3.6)
	2-NH		8.16 (br d; 9.2)
	3	36.5	2.94 (t; 13.0)
			3.25 (dd; 13.9, 4.0)
	4	138.4	
	5,9	128.0	7.29 (m)
	6,8	129.0	7.27 (m)
	7	126.2	7.19 (t; 7.2)

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

The molecular weight and formula of oscillapeptilide 97-B (**2**) was established to be 1031 and $C_{52}H_{73}N_9O_{13}$ based on the FABMS and HRFABMS, respectively, suggesting that **2** is a desmethyl derivative of **1**. The amino acid analysis for **2** revealed the presence of the same amino acids with the absolute configurations as **1**. The 1D NMR spectra of **1** and **2** completely resembled each other except for the lack of the resonances for a methoxy group in that of **1** (^{1}H: 3.70 ppm, ^{13}C: 55.0 ppm). Therefore, oscillapeptilide 97-B (**2**) was suggested to be a desmethyl derivative of **1**. Namely, *N*,*O*-diMeTyr in **1** is replaced by *N*-methyl-L-tyrosine (*N*-MeTyr) in **2**. Throughout the experiments, it was observed that the ^{1}H and ^{13}C resonances for *N*-acetyl Pro in **1** and **2** appeared as the doublet and the difference of ^{1}H and ^{13}C chemical shifts in the doublet resonances was proportional to the distance from the *N*-acetyl group (Table 2), suggesting that this phenomenon is attributable to the restricted rotation of the amide *C–N* bond.[19]

Oscillapeptin G (**3**) was first isolated as a tyrosinase inhibitor from the *O. agardhii* strain NIES-610 (CYA18).[20] In this study, **3** was also isolated from the *O. agardhii* strain CYA128 and was identified using FABMS, HRFABMS, ^{1}H NMR, COSY spectra and amino acid analysis. The absolute configurations of homotyrosine (Hty) and *N*-MeTyr, which were not determined in the first report,[20] were elucidated to be the L-configuration based on the amino acid analysis for **3** using the advanced Marfey's method.[18] Additionally, the amino acid analysis indicated that **3** has L-*allo*-Ile instead of L-Ile.[12]

The cyclic depsipeptides containing the Ahp moiety such as oscillapeptilides 97-A (**1**), -B (**2**) and oscillapeptin G (**3**) have been frequently found as secondary metabolites of the terrestrial cyanobacteria.[6] The absolute configuration at C-3 of Ahp in **1**–**3** 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] Furthermore, the stereochemistry of Ahp in **1**–**3** was determined to be (3*S*,6*R*)-3-amino-6-hydroxy-2-piperidone on the basis of the following NMR spectral experiments. Due to the small coupling of less than 4.0 Hz that was observed between H-6 and each H-5, and the large coupling of more than 11.0 Hz that was observed between H-3 and one of H-4, the hydroxy group at C-6 and H-3 are axially oriented.[22] The proposed absolute stereochemistry of Ahp was coincident with that of micropeptin 90,[22] cyanopeptolins[23] and A90720A.[24]

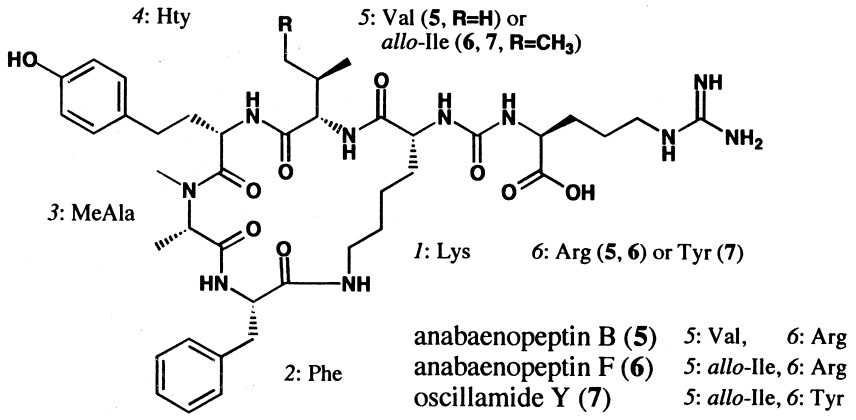
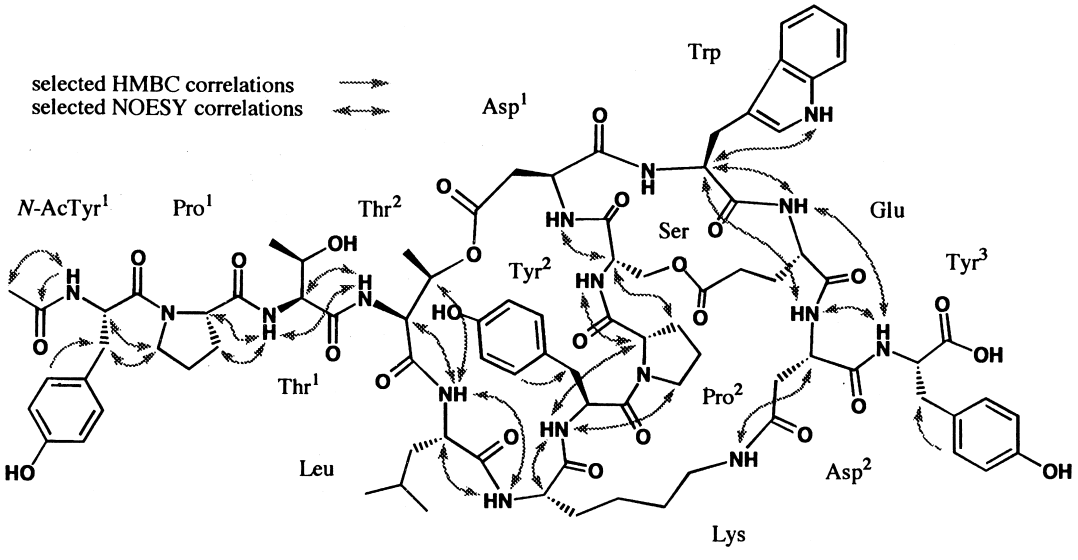


Figure 2.

While almost all cyclic depsipeptides containing the Ahp moiety inhibit serine proteases such as trypsin, chymotrypsin, elastase, plasmin and thrombin,[6] oscillapeptilides 97-A (**1**), -B (**2**) and oscillapeptin G (**3**) were also recognized as the elastase (IC_{50} 0.73, 0.41 and 1.12 μg/mL) and chymotrypsin (IC_{50} 12.9, 10.7 and 11.4 μg/mL) inhibitors.

Oscillacyclin (**4**) is a colorless amorphous powder and is negative to ninhydrin. The molecular weight and formula of **4** was established to be 994 and $C_{47}H_{66}N_{10}O_{14}$ based on the FABMS and HRFABMS, respectively. It was suggested that **4** is a peptide compound based on the ^{1}H and ^{13}C NMR spectra (Table 3). The amino acid analysis of the hydrolysate of **4** using the advanced Marfey's method[18] indicated the presence of 1 mol each of serine (Ser), alanine (Ala), aspartic acid (Asp, from asparagine), tyrosine (Tyr), Ile, Pro, Phe and 2 mol of Thr and that these nine constituent amino acids all have the L-configuration. Furthermore, the 2D NMR analysis of **4** confirmed the presence of these amino acids including asparagine (Asn). The molecular formula of **4** required 20 unsaturations. Because 19 unsaturations could be accounted for by the functionalities present in the nine individual amino acids, it was apparent that **4** was cyclic. The sequence of the constituent amino acids of **4** has been established with the help of the HMBC and NOESY spectra as shown in **4**. These obtained results indicated that oscillacyclin (**4**) has the following sequence: *cyclo*(-Ala-Tyr-Asn-Pro-Phe-Thr[1]-Thr[2]-Ser-Ile-).

Cyclic peptides possessing the ureido linkage were isolated as the last group of non-toxic peptides from both toxic strains. Anabaenopeptins B (**5**) and F (**6**) were isolated from the toxic *O. agardhii* strain 97, and oscillamide Y (**7**) and **6** were isolated from the toxic *O. agardhii* strain CYA128 (Fig. 2). The isolation of **5** from *O. agardhii* has been reported and its analogues, anabaenopeptin E, **6** and **7** have also been isolated from *O. agardhii* (NIES-204, 610).[25,26] Although the absolute configurations of Hty and *N*-methylalanine (*N*-MeAla) were not determined and L-Ile was identified as one of the constituent amino acids in **7** in the first report,[26] they were elucidated to be the L-configuration for **7** and L-*allo*-Ile was found to be included in **6** and **7** instead of L-Ile by using the advanced Marfey's method in this study.[12,18]



microviridin I (**8**)

Figure 3.

Table 4. ^{13}C and ^{1}H NMR spectral data for Microviridin I (**8**) in DMSO-d_6 (The chemical shifts of ^{1}H resonances, which overlapped with other ^{1}H resonances in 1D spectrum, were determined using ^{1}H–^{1}H COSY and HSQC experiments. The multiplicity of ^{1}H resonances were not determined due to the broadening and overlapping with other ^{1}H resonances)

Position		^{13}C	^{1}H	Position		^{13}C	^{1}H	Position		^{13}C	^{1}H
Ac				Lys				Trp			
	2	22.2	1.73		2	52.8	4.04		2	53.7	4.53
Tyr[1]					2-NH		6.90		2-NH		7.42
	2	52.5	4.56		3	32.6	1.37,1.50		3	25.7	3.21
	2-NH		8.18		4	22.6	1.05,1.13		1′		10.90
	3	36.1	2.60,2.82		5	28.3	1.26,1.41		2′	124.0	7.24
	4	127.8			6	38.6	2.91		3′	109.2	
	5,9	130.1	7.06		6-NH		7.35		4′	118.2	7.45
	6,8	114.9	6.63	Tyr[2]					5′	118.5	6.94
	7	155.8			2	51.3	4.40		6′	121.0	7.02
	7-OH		9.24[a]		2-NH		8.41		7′	111.5	7.23
Pro[1]					3	37.4	2.66,2.77		8′	127.3	
	2	59.2	4.52		4	126.2			9′	136.2	
	3	28.9	1.92,2.04		5,9	130.0	6.92	Glu			
	4	24.6	1.86,1.90		6,8	115.2	6.66		2	54.6	3.94
	5	46.8	3.46,3.63		7	156.3			2-NH		6.48
Thr[1]					7-OH		9.39[a]		3	24.3	1.38,1.99
	2	58.0	4.34	Pro[2]					4	30.3	1.05,2.02
	2-NH		8.01		2	60.4	3.37	Asp[2]			
	3	66.5	4.03		3	30.6	1.36,1.60		2	50.8	4.42
	3-OH		5.07		4	21.3	1.64		2-NH		7.28
	4	19.0	1.08		5	46.0	3.15,3.37		3	38.1	2.30,2.42
Thr[2]				Ser				Tyr[3]			
	2	54.6	4.60		2	52.2	4.38		2	55.8	4.05
	2-NH		7.60		2-NH		6.46		2-NH		7.32
	3	71.3	5.33		3	61.7	4.52		3	36.6	2.78,2.98
	4	16.7	1.16	Asp[1]					4	129.2	
Leu					2	51.3	4.46		5,9	131.5	6.97
	2	50.8	4.26		2-NH		9.24		6,8	114.7	6.58
	2-NH		8.39		3	34.9	2.67		7	155.3	
	3	40.7	1.34,1.58						7-OH		9.08[a]
	4	24.1	1.40								
	5	23.1	0.82								
	6	21.3	0.76								

[a] ^{1}H resonances may be interchanged.

Kaya et al. reported that oscillamide Y (**7**) is a potent chymotrypsin inhibitor.[26] However, any anabaenopeptins including anaaenapeptin A, which has L-Val in place of L-Ile in **7**, showed no inhibitory activity to chymotrypsin at 100 μg/mL. Very recently, Bradley et al. accomplished the total synthesis of **7** and tested the inhibitory activity, but not only the synthetic **7** but also the authentic **7** were found not to inhibit chymotrypsin.[27] The peptide **7** isolated in the present study also showed no inhibitory activity at 100 μg/mL. These results indicated that **7** has no inhibitory activity to chymotrypsin and that its activity may be derived from the contamination of chymotrypsin inhibitors such as osillapeptin G (**3**).[20]

Microviridin I (**8**) is a colorless amorphous powder which is negative to ninhydrin (Fig. 3). The molecular weight and formula of **8** was established to be 1764 and $C_{86}H_{108}N_{16}O_{25}$ based on the FABMS and HRFABMS, respectively. It was suggested that **8** is a peptide compound based on the ^{1}H and ^{13}C NMR spectra (Table 4). Amino acid analysis of the hydrolysate of **8** using the advanced Marfey's method[18] indicated the presence of 1 mol each of lysine (Lys), tryptophan (Trp), Leu, Ser, Glu, 2 mol of Thr, Pro and Asp, and 3 mol of Tyr and that these 14 constituent amino acids all have the L-configuration. Furthermore, the 2D NMR analysis of **8** confirmed the presence of an acetyl group and all these amino acids. The sequence of the constituent amino acids of **8** has been established mainly with the help of NOESY spectra as shown in **8**, because it was difficult to assign each carbonyl carbon of the constituent amino acids due to complicated overlapping. On the basis of the NOESY correlations, the following two partial sequence were determined: *N*-acetyl-Tyr[1]-Pro[1]-Thr[1]-Thr[2]-Leu-Lys-Tyr[2]-Pro[2]-Ser-Asp[1]- and -Trp-Glu-Asp[2]-Tyr[3]-. Additionally, the linkage between ε-*N*H of Lys and the β-carbonyl carbon of Asp[2] was confirmed by the NOESY correlation between ε-*N*H of Lys and H-2 of Asp[2]. These findings suggested that **8** had a closely related structure to microviridins A∼H isolated as a potent elastase inhibitor except for microviridin A from cyanobacteria, *Microcystis viridis, M. aeruginosa, O. agardhii and Nostoc minutum* (NIES-102, 100, 204 and 26).[28–31] Finally, the presence of ester bonds between Ser and Glu and between Thr[2] and Asp[1] was suggested by the downfield shifts at H-3 (4.52 ppm) of Ser and at H-3 (5.33 ppm) of Thr[2]. The sequence of -Asp[1]-Trp- of **8** was suggested by their similar chemical shifts to that of microviridins A,[28] B[29] and G,[31] which contain a part of the common sequence, -Lys-Tyr[2]-Pro[2]-Ser-Asp[1]-Trp-Glu-, including an ester bond between Ser and Glu. These results proposed the structure of microviridin I (**8**). The peptide **8** showed inhibitory activity to elastase with an IC_{50} of 0.34 μg/mL as well as the other microviridins.[29–31]

In the present study, we investigated the secondary metabolites produced by the toxic and non-toxic cyanobacteria, *Oscillatoria agardhii*, in connection with a study for the elucidation of the biosynthetic relationship between hepatotoxic peptides and other groups of peptides from cyanobacteria. We found that the toxic cyanobacterial genus, *O. agardhii* produces non-toxic peptides together with microcystins. Namely, three groups of peptides, 19-membered depsipeptides containing the Ahp moiety, 19-membered cyclic peptides containing an ureido linkage (anabaenopeptins) and a cyclic nonapeptide, were isolated. On the other hand, although such peptides were not isolated from the non-toxic *O. agardhii*, one of the tricyclic depsipeptides, microviridins, was isolated from the non-toxic strains. Recently, Namikoshi et al. summarized the bioactive compounds produced by cyanobacteria and reported that cyanobacteria produce mainly four groups of non-toxic peptides in addition to the hepatotoxic peptides: depsipeptides containing the Ahp moiety, linear peptides, anabaenopeptins and microviridins.[6] While these peptides are produced by almost all toxic cyanobacteria that co-produce hepatotoxic peptides, no such peptides were detected from the non-toxic and neurotoxic cyanobacteria producing anatoxin-a. These accumulated results suggested that toxic cyanobacteria possess several peptide synthetase genes for non-toxic peptides together with those for hepatotoxic peptides and that the production of such non-toxic peptides is characteristic for the toxic cyanobacteria. On the other hand, while microviridin I was isolated from the non-toxic strain in this study, a few microviridins were isolated together with microcystins from the toxic strains.[28,29] Likewise, the isolation of some non-toxic peptides from both toxic and non-toxic strains has also been reported.[6] The production of non-toxic peptides may have no direct relevance to that of the hepatotoxic peptides, and the non-toxic peptides may be independently produced, because these peptides were produced by the mutant cell's disrupted microcystin synthetase gene.[5] On the basis of these results, we considered the toxic and non-toxic strains as follows: the toxic strains producing hepatotoxic peptides invariably have the synthetase genes for both non-toxic and hepatotoxic peptides and express them, and the non-toxic strains are classified into two types with or without peptide synthetase genes including those for hepatotoxic peptides, but the expression of synthetase genes for hepatotoxic peptides does not occur for some reason or other. At present, the genetic analysis of the microcystin synthetase genes is in the process of being completed,[32] and the investigation of the presence of their genes in various cyanobacteria including the non-toxic strains is being carried out using molecular biological techniques.[14] In order to clearly elucidate the biosynthetic relationship between hepatotoxic peptides and non-toxic peptides, which is our final goal, further study is required particularly, a genetic study.

Experimental

General aspects

Optical rotations were recorded at 27°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 A600 NMR spectrometer operating at 600 MHz for the ^{1}H and at 150 MHz for the ^{13}C in DMSO-d_6. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks (^{1}H: 2.49 and ^{13}C: 39.5 ppm in DMSO-d_6). Amino acid analyses were carried out by the advanced Marfey's method using HPLC and ESI LC/MS (see Refs. 12,18). Serine protease inhibitory activity assay was carried out according to the method of Ref. 30.

Materials

Oscillatoria (*Planktothrix*) *agardhii* strain 97, CYA128, 2 and 18 (isolation and strain history described in Ref. 15) 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.

Isolation

Each dried cell [strains 97 (5.2 g), CYA128 (4.9 g), 2 (4.2 g) and 18 (4.9 g)] was extracted three times with 5% AcOH(aq) (400 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 filtration through a glass microfiber filter (GF/C). The cartridge was rinsed with water (200 mL) and 20% MeOH aq. (200 mL) and then eluted with MeOH (400 mL) to give a fraction containing the desired peptides. The fraction (138.2 mg) from strain 97 was separated to give **1** (13.5 mg), **2** (3.2 mg), **4** (3.9 mg), **5** (0.3 mg) and **6** (1.3 mg) using the following chromatographies: silica gel [Silica gel 60 (230–400 mesh)] using AcOEt/*i*-PrOH/H_2O (4:3:7, upper layer), $CHCl_3$/MeOH/H_2O (65:35:10 or 65:15:5, lower phase) and TOYOPEARL HW-40F (890×11 mm I.D.; flow rate, 0.3 mL/min; detection, UV230 nm) using MeOH. The fraction (95.5 mg) from strain CYA128 was separated to give **3** (6.6 mg), **6** (0.5 mg) and **7** (2.4 mg) using the following chromatographies: silica gel using AcOEt/*i*-PrOH/H_2O (4:3:7, upper layer), $CHCl_3$/MeOH/H_2O (65:20:5, lower phase) and TOYOPEARL HW-40F using MeOH. The fractions (175.1 and 81.4 mg) from strains 2 and 18 were separated to give **8** (7.2 mg) using the following chromatography: silica gel using $CHCl_3$/MeOH/H_2O (65:35:5, lower phase), AcOEt/*i*-PrOH/H_2O (4:3:7, upper layer) and TOYOPEARL HW-40F using MeOH.

Oscillapeptilide 97-A (1). Amorphous powder; $[\alpha]_D = -49.8°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 1046 $[M+H]^+$, *m/z* 1028 $[M+H-H_2O]^+$, negative FABMS (glycerol) *m/z* 1044 $[M-H]^-$; HRFABMS *m/z* 1028.5460 $[M+H-H_2O]^+$, calcd for $C_{53}H_{73}N_9O_{12}$, Δ +0.3 mmu; ^{1}H and ^{13}C NMR (see Table 2); inhibition activity (IC_{50}) elastase: 0.73 μg/mL, chymotrypsin: 12.9 μg/mL.

Oscillapeptilide 97-B (2). Amorphous powder; $[\alpha]_D = -58.3°$ (*c* 0.100, MeOH); positive FABMS (glycerol)

m/z 1032 $[M+H]^+$, *m/z* 1014 $[M+H-H_2O]^+$, negative FABMS (glycerol) *m/z* 1030 $[M-H]^-$; HRFABMS *m/z* 1014.5314 $[M+H-H_2O]^+$, calcd for $C_{53}H_{73}N_9O_{12}$, Δ +1.3 mmu; ^1H and ^{13}C NMR spectra data were completely consistent with those of **1** except for the loss of the signal for a methoxy group (^{13}C: 55.0, ^1H: 3.70 ppm) in **1** (see Table 2); inhibition activity (IC_{50}) elastase: 0.41 μg/mL, chymotrypsin: 10.7 μg/mL.

Oscillapeptin G (3). Amorphous powder; $[\alpha]_D=-62.3°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 1112 $[M+H]^+$, *m/z* 1094 $[M+H-H_2O]^+$, negative FABMS (glycerol) *m/z* 1110 $[M-H]^-$; HRFABMS *m/z* 1094.5450 $[M+H-H_2O]^+$, calcd for $C_{53}H_{76}N_9O_{16}$, Δ +4.0 mmu; inhibition activity (IC_{50}) elastase: 1.12 μg/mL, chymotrypsin: 11.4 μg/mL.

Oscillacyclin (4). Amorphous powder; $[\alpha]_D=-100.8°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 995 $[M+H]^+$, negative FABMS (glycerol) *m/z* 993 $[M-H]^-$; HRFABMS *m/z* 995.4836 $[M+H]^+$, calcd for $C_{47}H_{67}N_{10}O_{14}$, Δ −0.2 mmu; ^1H and ^{13}C NMR (see Table 3).

Anabaenopeptin B (5). Amorphous powder; positive FABMS (glycerol) *m/z* 837 $[M+H]^+$, negative FABMS (glycerol) *m/z* 835 $[M-H]^-$, *m/z* 661; HRFABMS *m/z* 837.4645 $[M+H]^+$, calcd for $C_{41}H_{61}N_{10}O_9$, Δ +2.2 mmu.

Anabaenopeptin F (6). Amorphous powder, $[\alpha]_D=-63.8°$ (*c* 0.200, MeOH); positive FABMS (glycerol) *m/z* 851 $[M+H]^+$, negative FABMS (glycerol) *m/z* 849 $[M-H]^-$, *m/z* 675; HRFABMS *m/z* 851.4778 $[M+H]^+$, calcd for $C_{42}H_{63}N_{10}O_9$, Δ −0.2 mmu.

Oscillamide Y (7). Amorphous powder, $[\alpha]_D=-58.7°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 858 $[M+H]^+$, negative FABMS (glycerol) *m/z* 856 $[M-H]^-$, *m/z* 675; HRFABMS *m/z* 858.4428 $[M+H]^+$, calcd for $C_{45}H_{60}N_7O_{10}$, Δ +2.6 mmu.

Microviridin I (8). Amorphous powder; $[\alpha]_D=+1.6°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 1765 $[M+H]^+$, negative FABMS (glycerol) *m/z* 1763 $[M-H]^-$; HRFABMS *m/z* 1765.7764 $[M+H]^+$, calcd for $C_{86}H_{109}N_{16}O_{25}$, Δ +1.4 mmu; ^1H and ^{13}C NMR (see Table 4); inhibition activity (IC_{50}) elastase 0.34 μg/mL, chymotrypsin 21.7 μg/mL, trypsin: 26.2 μg/mL.

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References

1. Carmichael, W. W. *Sci. Am.* **1994**, *270*, 64–72.
2. Jochimsen, E. M.; Carmichael, W. W.; An, J. C.; Cardo, D. M.; Cookson, S. T.; Holmes, C. E. M.; Antunes, M. B. C.; Filho, D. A. M.; Lyra, T. M.; Barreto, V. S. T.; Azevedo, S. M. F. O.; Jarvis, W. R. *N. Engl. J. Med.* **1998**, *338*, 873–878.
3. Pouria, S.; Andrade, A.; Barbosa, J.; Cavalcanti, R. L.; Barreto, V. T. S.; Ward, C. J.; Preiser, W.; Poon, G. K.; Neild, G. H.; Codd, G. A. *Lancet* **1998**, *352*, 21–26.
4. Krishnamurthy, T.; Szafraniec, L.; Hunt, D. F.; Shabanowitz, J.; Yates, J. R.; Hauer, C.; Carmichael, W. W.; Skulberg, O.; Codd, G. A.; Missler, S. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 770–774.
5. Dittman, E.; Neilan, B. A.; Erhard, M.; Döhren, H.; Börner, T. *Mol. Microbiol.* **1997**, *26*, 779–787.
6. Namikoshi, M.; Rinehart, K. L. *J. Ind. Microbiol.* **1996**, *17*, 373–384.
7. Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett.* **1993**, *34*, 6091–6094.
8. Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Park, H.; Watanabe, M. F. 35th Symposium on the Chemistry of Natural Products, Symposium Paper, Kyoto, 1993; pp. 377–384.
9. Harada, K.-I.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett.* **1995**, *36*, 1511–1514.
10. Fujii, K.; Harada, K.-I.; Suzuki, M.; Kondo, F.; Ikai, Y.; Oka, H.; Carmichael, W. W.; Sivonen, K. *Harmful and Toxic Algal Blooms*; Yasumoto, T.; Oshima, Y.; Fukuyo, Y.; Eds., Intergovernmental Oceanographic Commission of UNESCO: Sendai, 1996; pp. 559–562.
11. Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, Y.; Shimizu, Y.; Sano, H.; Hirayama, M.; Suzuki, M.; Harada, K.-I. *Tetrahedron Lett.* **1997**, *38*, 5525–5528 see also pp. 5529–5532.
12. Fujii, K.; Sivonen, K.; Kashiwagi, T.; Hirayama, M.; Harada, K.-I. *J. Org. Chem.* **1999**, *64*, 5777–5782.
13. Luukkainen, R.; Sivonen, K.; Namikoshi, M.; Färdig, M.; Rinehart, K. L.; Niemelä, S. I. *Appl. Environ. Microbiol.* **1993**, *59*, 2204–2209.
14. Neilan, B. A.; Dittmann, E.; Rouhiainen, L.; Bass, R. A.; Schaub, V.; Sivonen, K.; Börner, T. *J. Bacteriol.* **1999**, *181*, 4089–4097.
15. Sivonen, K. *Appl. Environ. Microbiol.* **1990**, *56*, 2658–2666.
16. Harada, K.-I.; Suzuki, M.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W.; Rinehart Jr., K. L. *Toxicon* **1988**, *26*, 433–439.
17. Kondo, F.; Ikai, Y.; Oka, H.; Matsumoto, H.; Yamada, S.; Ishikawa, N.; Tsuji, K.; Harada, K.-I.; Shimada, T.; Oshikata, M.; Suzuki, M. *Nat. Toxins* **1995**, *3*, 41–49.
18. Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K.-I. *Anal. Chem.* **1997**, *69*, 3346–3352 see also pp. 5146–5151.
19. Ishibashi, M.; Moore, R. E.; Patterson, G. M. L. *J. Org. Chem.* **1986**, *51*, 5300–5306.
20. Sano, T.; Kaya, K. *J. Nat. Prod.* **1996**, *59*, 90–92.
21. Harada, K.-I.; Fujii, K.; Hayashi, K.; Suzuki, M.; Ikai, Y.; Oka, H. *Tetrahedron Lett.* **1996**, *37*, 3001–3004.
22. Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1996**, *37*, 9225–9226.
23. Martin, C.; Oberer, L.; Ino, T.; König, W. A.; Busch, M.; Weckesser, J. *J. Antibiotics* **1993**, *46*, 1550–1556.
24. Bonjouklian, R.; Smitka, T. A.; Hunt, A. H.; Occolowitz, J. L.;

Perun Jr., T. J.; Doolin, L.; Stevenson, S.; Knauss, L. K.; Szewczyk, S.; Patterson, G. M. L. *Tetrahedron* **1996**, *52*, 395–404.
25. Shin, H. J.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 139–141.
26. Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, *36*, 5933–5936.
27. Marsh, I. R.; Bradly, M.; Teague, S. J. *J. Org. Chem.* **1997**, *62*, 6199–6203.
28. Ishituka, M. O.; Kusumi, T.; Kakisawa, H. *J. Am. Chem. Soc.* **1990**, *112*, 8180–8181.
29. Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1995**, *51*, 10 679–10 686.
30. Shin, H. J.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron* **1996**, *52*, 8159–8168.
31. Murakami, M.; Sun, Q.; Ishida, K.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Phytochemistry* **1997**, *45*, 1197–1202.
32. Nishizawa, T.; Asayama, M.; Fujii, K.; Harada, K.-I.; Shirai, M. *J. Biochem.* **1999**, *126*, 520–529.