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NOTE

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Clonostachysins A and B, New Anti-dinoflagellate Cyclic Peptides from a Marine-derived Fungus

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Abstract The two new anti-dinoflagellates, clonostachysins A and B, were obtained from a marine sponge derived fungus *Clonostachys rogersoniana* strain HJK9. Their chemical structures were determined by spectroscopic studies as highly N-methylated cyclic peptides of the nine amino acids. The absolute stereochemistry was elucidated by the advanced Marfey's method. Both clonostachysins A and B exhibited a selectively inhibitory effect on a dinoflagellate *Prorocentrum micans* at 30 μM, but had no effect on other microalgae and bacteria even at 100 μM.

Keywords clonostachysin, anti-dinoflagellate, cyclic peptides, marine, fungus

Marine microorganisms are recognized as a promising source of novel natural products [1]. We have been screening marine-derived bacteria and fungi for antimicroalgal, antibacterial and anticancer activities to find novel bioactive substances. In the course of this screening, we found that an extract of the marine-derived fungus named HJK9, which had been isolated from a sponge, *Halicondria japonica*, collected in Numazu, Japan, had an inhibitory effect on the dinoflagellate, *Prorocentrum micans*. We have previously reported β-cyanoalanine as a specific inhibitor of cyanobacteria [2] and *N*-(3-hydroxy-1-oxotetradecyl)-leucine as an anti-dinoflagellate substance [3], but as far as we know, only a few substances have been reported outside our group as being anti-microalgal [4, 5]. We describe in this note the isolation and structural determination of two new anti-dinoflagellates, clonostachysins A and B, from a marine-derived fungus.

The clonostachysin-producing fungus (HJK9) was identified as *Clonostachys rogersoniana* from morphological studies and its 18S rDNA sequence. This fungus was cultured in potato dextrose broth, which had been prepared with artificial seawater (Tropic Marine®), for 7 days at 30°C under rotation at 100 rpm. The use of artificial seawater increased the productivity of the active substance by nearly a factor of two. The cultured broth was centrifuged, and the resulting supernatant was extracted with ethyl acetate. The ethyl acetate extract which exhibited anti-dinoflagellate activity was fractionated in a Sep Pak Florisil® column with step-wise elution using chloroform/methanol mixtures (100% chloroform, 100 : 1, 10 : 1, and 100% methanol). The activity was detected in the first fraction (100% chloroform), and the active fraction was further purified by HPLC equipped with an ODS column (TSKgel ODS-80Ts, i.d. 4.6×250 mm, Tosoh Corporation) with 50% aqueous methanol as the solvent. Clonostachysins A (4.0 mg) and B (2.5 mg) were obtained from 8 liter of the culture broth by purifying twice with HPLC. The physico-chemical properties of clonostachysins A and B are summarized in Table 1.

The molecular formula of clonostachysin A was

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Table 1 Physico-chemical properties of clonostachysins A and B

	Clonostachysin A	Clonostachysin B
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{53}H_{87}N_9O_{10}$	$C_{54}H_{89}N_9O_{10}$
ESI-MS (*m/z*)	1010.7 $[M+H]^+$	1024.7 $[M+H]^+$
HR FAB-MS (*m/z*)		
Found	1010.6664 $[M+H]^+$	1024.6831 $[M+H]^+$
Calcd.	1010.6654	1024.6811
UV λ_{max} nm (ε) in MeOH	282 (820)	282 (1000)
IR ν_{max} (KBr) cm^{-1}	3842, 2926, 1655, 1543, 1459, 1244, 1091	3713, 1637, 1543, 1459, 1032
$[\alpha]_D^{25}$	−97° (*c* 0.065, MeOH)	−87° (*c* 0.030, MeOH)

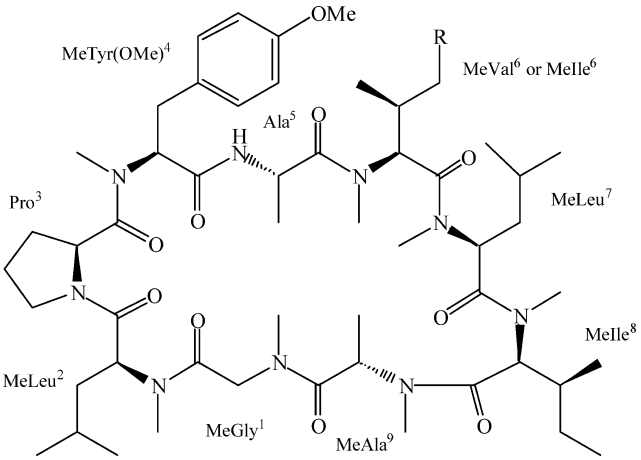

Fig. 1 Structure of clonostachysin A (R=H) and B (R=CH_3).

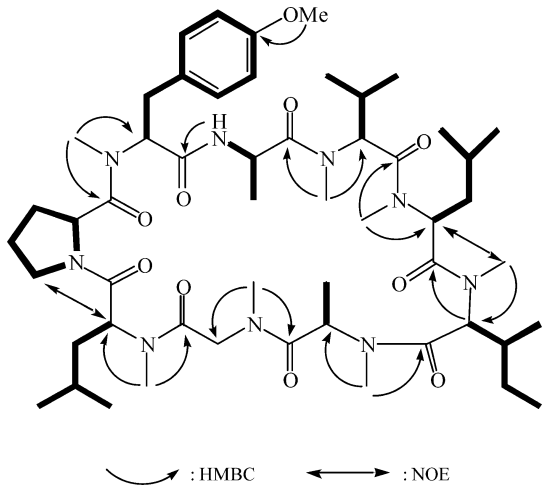

Fig. 2 Partial structures of clonostachysin A and their connection. Bold lines reveal partial structures determined from analyses of ^{1}H NMR, ^{13}C NMR, COSY, HSQC and TOCSY data. The connection of each amino acid was determined from the HMBC and NOE signals.

determined to be $C_{53}H_{87}N_9O_{10}$ from HRFAB-MS data (see in Table 1) for the protonated ion and ^{13}C NMR spectral data. Signals in the ^{1}H NMR spectrum at around 4.5～5.5 ppm were connected to carbons whose chemical shift of around 40～60 ppm in the gHSQC spectrum could have been due to the α-protons of amino acid residues. The seven singlet methyl signals observed at δ_H 2.55～3.00 could have been methyl groups connected to nitrogen atoms because of their carbon chemical shifts of δ_C 28.26～35.29. Nine carbonyl carbon signals at around 167～172 ppm were observed in the ^{13}C NMR spectrum. These results suggested clonostachysin A to be a highly methylated peptide or peptide-like compound. Detailed analyses of the ^{1}H and ^{13}C NMR, and 2D NMR data, including COSY, TOCSY, gHSQC and gHMBC, enabled the structure of clonostachysin A to be determined as a cyclic peptide composed of the nine amino acid residues, MeGly[1], MeLeu[2], Pro[3], MeTyr(OMe)[4], Ala[5], MeVal[6], MeLeu[7], MeIle[8], and MeAla[9] (Figure 2). The ^{1}H and ^{13}C NMR data for clonostachysin A are summarized in Table 2. All of the amino acid residues except Pro and Ala were N-methylated. The connections of nine amino acid residues were mainly determined by the HMBC signals between the methyl protons of the α-amino groups and carbonyl carbons, and between the methyl protons of the α-amino groups and neighboring α-protons of amino acids (Figure 2). The connection of MeLeu[2] and Pro[3] was determined by the NOE signal between the α-proton of MeLeu[2] and the δ-methylene protons of Pro[3], enabling the gross structure of clonostachysin A to be determined. The partial sequence of the peptide residues of clonostachysin A, [-MeLue[2]-MeGly[1]-MeAla[9]-MeIle[8]-MeLeu[7]-MeVal[6]-], was further confirmed by an LC-ESI MS/MS analysis (data not shown).

The absolute configuration of clonostachysin A was

Table 2 NMR data for clonostachycins A and B in d_6-DMSO

Clonostachycin A			Clonostachycin B		
Position	^{13}C*	^{1}H**	Position	^{13}C*	^{1}H**
MeGly[1]			MeGly[1]		
α	50.84	5.40 d (18.1), 4.04 d (18.1)	α	50.83	5.40 d (18.1), 4.04 d (18.1)
CO	169.44		CO	169.44	
NCH_3	35.29	2.81 s	NCH_3	35.29	2.81 s
MeLeu[2]			$MeLeu_2$		
α	51.91	5.23 dd (12.2, 3.5)	α	51.91	5.23 dd (12.3, 3.6)
β	35.84	1.72 m, 1.35 m	β	35.84	1.72 m, 1.35 m
γ	24.44	1.46 m	γ	24.44	1.45 m
δ	23.01	0.93 d (6.8)	δ	23.01	0.93 d (6.8)
δ	20.78	0.86 d (6.5)	δ	20.78	0.86 d (6.5)
CO	169.97		CO	169.97	
NCH_3	29.98	3.00 s	NCH_3	30.00	3.00 s
Pro[3]			Pro[3]		
α	55.28	4.74 t (7.0)	α	55.28	4.74 t (7.0)
β	28.01	1.35 m, 0.80 m	β	28.01	1.35 m, 0.80 m
γ	25.10	1.89 m, 1.66 m	γ	25.05	1.89 m, 1.66 m
δ	47.24	3.67 m, 3.41 m	δ	47.22	3.67 m, 3.41 m
CO	171.57		CO	171.59	
MeTyr(OMe)[4]			MeTyr(OMe)[4]		
α	60.20	4.93 m	α	60.20	4.92 dd (10.5, 4.8)
β	32.72	2.90 m, 2.85 m	β	32.76	2.90 m, 2.85 m
1′	129.24		1′	129.24	
2′,6′	130.33	7.16 d (8.6)	2′,6′	130.33	7.16 d (8.6)
3′,5′	113.50	6.82 d (8.6)	3′,5′	113.51	6.82 d (8.6)
4′	157.75		4′	157.75	
4′-OCH_3	54.99	3.71 s	4′-OCH_3	54.99	3.71 s
CO	168.73		CO	168.71	
NCH_3	28.26	2.70 s	NCH_3	28.27	2.70 s
Ala[5]			Ala[5]		
α	46.13	4.52 dq (7.2, 5.1)	α	46.14	4.52 dq (7.2, 5.0)
β	16.05	1.16 d (7.2)	β	16.08	1.16 d (7.2)
CO	173.14		CO	173.12	
NH		8.11 d (5.0)	NH		8.11 d (5.0)
MeVal[6]			MeIle[6]		
α	56.18	5.09 d (10.5)	α	55.37	5.12 d (10.5)
β	27.85	2.25 m	β	34.65	2.01 m
γ	20.78	0.82 d (6.6)	γ	26.22	1.22 m, 0.86 m
γ	19.74	0.87 d (6.6)	γ	14.39	0.84 d (6.4)
CO	171.57		δ	12.00	0.90 t (7.2)
NCH_3	29.10	3.08 s	CO	169.33	
			NCH_3	29.23	3.08 s
MeLeu[7]			MeLeu[7]		
α	56.91	4.93 m	α	56.92	4.91 dd (11.2, 2.2)
β	40.09	0.96 m, 2.36 m	β	40.66	0.96 m, 2.35 m
γ	25.05	1.55 m	γ	25.12	1.55 m
δ	23.73	0.90 d (6.6)	δ	23.73	0.89 d (6.8)
δ	21.77	1.01 d (6.6)	δ	21.75	0.99 d (6.8)
CO	167.71		CO	167.72	
NCH_3	29.17	2.62 s	NCH_3	29.23	2.62 s

Table 2 (continued)

Clonostachycin A			Clonostachycin B		
Position	^{13}C*	^{1}H**	Position	^{13}C*	^{1}H**
MeIle[8]			MeIlu[8]		
α	57.22	5.11 d (10.5)	α	57.17	5.10 d (10.5)
β	32.33	2.00 m	β	32.32	2.00 m
γ	24.22	1.25 m, 0.97 m	γ	24.22	1.25 m, 0.97 m
γ	15.20	0.79 d (6.6)	γ	15.29	0.78 d (6.8)
δ	10.29	0.84 t (7.4)	δ	10.26	0.83 t (7.5)
CO	170.11		CO	170.11	
NCH_3	29.24	2.55 s	NCH_3	29.18	2.55 s
MeAla[9]			MeAla[9]		
α	47.24	4.71 q (7.0)	α	47.22	4.71 q (7.0)
β	15.20	1.08 d (7.0)	β	15.20	1.08 d (7.0)
CO	171.67		CO	171.66	
NCH_3	29.98	2.94 s	NCH_3	30.00	2.94 s

* Recorded at 188 MHz.

** Recorded at 750 MHz. Coupling constants are in parenthesis.

determined by the advanced Marfey's method [6~8]. Clonostachysin A (0.3 mg) was hydrolyzed in 6 N HCl at 110°C for 1 hour, and the hydrolysate was derivatized with L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) for analysis by LC/MS. The mass chromatograms obtained by monitoring at *m*/*z* values for the protonated ions $[M+H]^+$ of the FDLA-derivatized amino acid constituents are shown in Figure 3. The expected peaks for the L-FDLA derivatives were detected in each chromatogram when monitored at the respective *m*/*z* values (Figure 3-a). Both of the diastereomers were detected, except for Gly (Figure 3-b), in the mass chromatograms of a mixture of the D- and L-FDLA derivatives of the hydrolysate. It has been reported that L-amino acid-L-FDLA derivatives are generally eluted faster than either the D-amino acid-L-FDLA derivatives or L-amino acid-D-FDLA derivatives under reversed-phase conditions with few exceptions [6, 7]. This rule has been shown to be applicable to the FDLA derivatives of N-methylated amino acid and O-methylated Tyr [9]. As shown in Figure 3, all of the constituent chiral amino acids in clonostacysin A were determined to be L-isomers, since no peaks due to the D-isomers could be detected. In addition, *N*-methyl-Ile was determined to be L-*N*-methyl-isoleucine, not L-*N*-methyl-*allo*-isoleucine, by comparing the HPLC retention time with those of authentic samples (data not shown). The absolute structure of clonostachysin A was therefore established as shown in Figure 1.

The structure of clonostachysin B was determined by the same procedure as that used for clonostachysin A. The molecular formula of clonostachysin B was determined to be $C_{54}H_{89}N_9O_{10}$, and the amino acid constituents were determined from detailed analyses of the NMR spectra. The difference between clonostachysins A and B was only in the substitution of MeVal[6] by MeIle[6]. In respect of the stereochemistry, all constituent amino acids in clonostachysin B were determined to be L-isomers, and two MeIles were determined to be L-*N*-methyl-isoleucines.

The antimicroalgal activity of clonostachysins A and B was evaluated by observing the motility of the tested microalgae, *Oscillatoria amphibia* (cyanobacteria), *Brachinonas submarina* (green alga), *Prorocentrum micans* (dinoflagellate) and *Skeletonema costatum* (diatom) [2]. Both clonostachysins A and B exhibited an inhibitory effect on *Prorocentrum micans* at 30 μM, but had no effect on other microalgae, even at 100 μM. Clonostachysins A and B exhibited no antibacterial activity toward *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* or *Salinivibrio costicola* at 100 μM. Clonostachysins A and B are thought to be specific inhibitors of dinoflagellates which are the most important contributors to the harmful algal bloom (red tide) [10, 11]. Clonostachysins A and B could prove to be useful tools for studying red tides.

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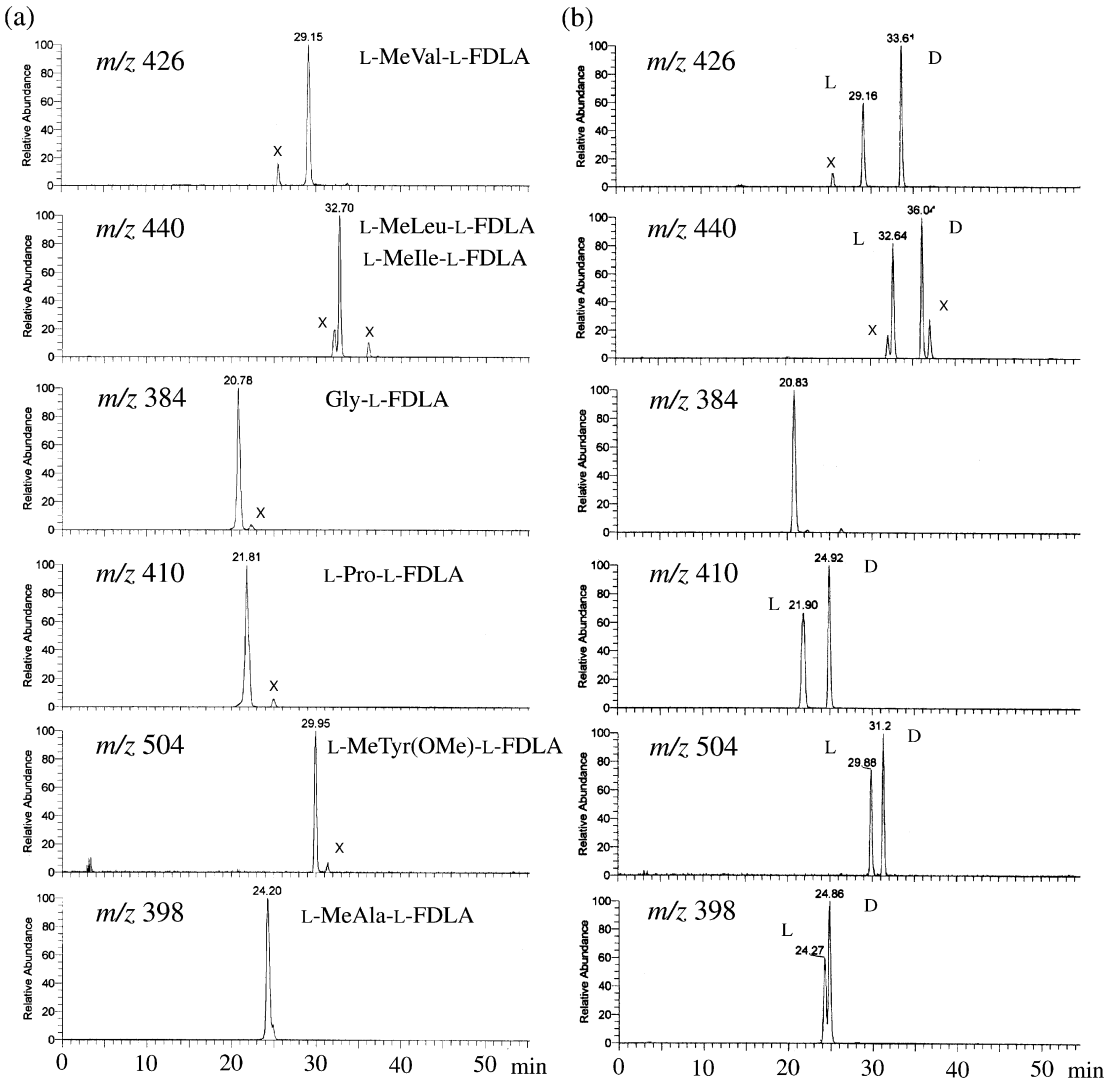

Fig. 3 Mass chromatograms of the L-FDLA derivatives (a) and the DL-FDLA derivatives (b) of a clonostachysin A hydrolysate by using ESI LC/MS. x: impurity.

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