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New Cyclic Peptides from the Indonesian Sponge *Theonella swinhoei*

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Dedicated to Professor Emeritus Paul J. Scheuer on the occasion of his 85th birthday, and to mark his 50th year of active research at University of Hawaii

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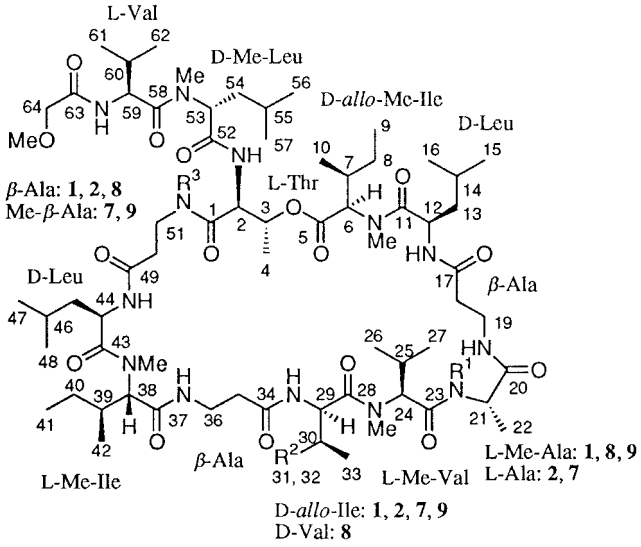
Abstract—Three new cyclic peptides, barangamides B, C, and D and a new depsipeptide, theonellapeptolide IIe as well as known theonellapeptolides Ia, Id, Ie, IId have been isolated from the sponge *Theonella swinhoei* collected in Baranglompo Island, Indonesia. The structures of barangamides were elucidated by interpretation of NMR data and application of Marfey's method. The structure of theonellapeptolide IIe, a mixture of several conformers showing complex NMR spectra, was determined by MS analysis of a ring-opened product and by chemical reaction. Some of the theonellapeptolides showed mild immunosuppressive activity, while barangamide A was inactive. © 2000 Elsevier Science Ltd. All rights reserved.

Numerous interesting compounds have been reported from the sponge *Theonella swinhoei* belonging to the Order Lithistida. Examples include swinholide A, a potent cytotoxic macrolide,[1] cyclotheonamide A, a cyclic pentapeptide possessing potent inhibitory activity against thrombin, trypsin and plasmin,[2,3] and other acyclic and cyclic polypeptides ranging from cyclic depsipeptide to large bicyclic peptides.[4]

Theonellapeptolides (Ia–e[5,6] and IId[7]), tridecapeptide lactones, have been reported from an Okinawan specimen of *T. swinhoei*. These peptides are characterized by the presence of high proportions of D-amino acids, *N*-methyl amino acids, and β-amino acids. Biological activity of these compounds includes cytotoxicity, ion-transport activity for Na^+ and K^+ ions,[6] and Na^+, K^+-ATPase inhibitory activity.[8] Other theonellapeptolides (III series) exhibiting cytotoxicity have also been reported from a New Zealand deep water sponge, *Lamellomorpha strongylata*, belonging to a different Order.[9]

In our screening for cytotoxicity of over 300 marine organisms collected in Indonesia, a lipophilic extract (MeOH) of the sponge *T. swinhoei*[10] was shown to be active against KB cells. We have recently reported the isolation of two known depsipeptides, theonellapeptolides Id (**1**) and IId (**2**), and a new cyclic peptide, barangamide A (**3**).[11] Further investigation of this sponge resulted in the isolation of three new cyclic peptides, barangamides B (**4**), C (**5**), and D (**6**) and four new depsipeptides, theonellapeptolides IIe (**7**), IV, V, and VI, as well as known theonellapeptolides Ia (**8**) and



1: Theonellapeptolide Id (R^1 = Me, R^2 = Et, R^3 = H)
2: Theonellapeptolide IId (R^1 = H, R^2 = Et, R^3 = H)
7: Theonellapeptolide IIe (R^1 = H, R^2 = Et, R^3 = Me)
8: Theonellapeptolide Ia (R^1 = Me, R^2 = Me, R^3 = H)
9: Theonellapeptolide Ie (R^1 = Me, R^2 = Et, R^3 = Me)

Keywords: cyclic peptide; depsipeptides; sponge; immunosuppressive activity.

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Table 1. NMR data (in $CDCl_3$) for theonellapeptolide Id (**1**) and barangamides A (**3**) and C (**5**)

No.		**1**		**3**[a]		**5**			
		^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	ROESY[d]	HMBC (8 Hz)[e]
L-Thr	1	168.2 (s)		174.5 (s)		174.5 (s)			H-2
	2	57.5 (d)	4.38 (dd, 9.8, 9.5)	52.7 (d)	4.83 (m)	52.7 (d)	4.82 (m)	**51-NH, H_2-51**	H_3-4
	3	69.1 (d)	5.16 (m)	64.6 (d)	4.05 (m)	64.7 (d)	4.07 (brq, 6.5)		H_3-4
	4	17.3 (q)	1.12 (d, 6.4)	21.9 (q)	1.31 (d, 6)	21.8 (q)	1.31 (d, 6.5)	H-3	
	2-NH		8.32 (brd, 8)		8.58 (d, 9.5)		8.62 (d, 9.5)	**H-6**	
	OH			-			5.92 (d, 6.5)		
D-*allo*-Me-Ile	5	170.4 (s)		169.3 (s)		169.6 (s)			**H-2**, 6, **NH-2**
	6	68.8 (d)[f]	5.15 (br)[f]	64.7 (d)	4.91 (d, 10.5)	64.8 (d)	4.90 (d, 11)	**2-NH**	
	7	33.9 (d)	2.43 (m)	32.4 (d)	2.27 (m)	32.4 (d)	2.26 (m)		
	8	28.4 (t)[f]	1.80 (m)	26.4 (t)	1.51 (dq, 15, 7.5)	26.4 (t)	1.52 (m)		
	8′		[f]		1.14 (m)		1.13 (m)		
	9	11.6 (q)	0.96 (t, 7)	11.4 (q)	0.99 (t, 7.5)	11.4 (q)	0.99 (t, 7)		
	10	14.5 (q)	0.77 (d, 7)	15.3 (q)	0.87 (d, 6.5)	15.3 (q)	0.87 (d, 6.5)	6-NMe	
	6-NMe	39.0 (q)[f]	3.24 (s)	29.0 (q)	2.65 (s)	29.0 (q)	2.64 (s)	H_3-10	
D-Leu	11	174.3 (s)		174.3 (s)		174.3 (s)			**6-NMe, H-6**
	12	48.0 (d)	5.02 (ddd, 12.2, 9.2, 2.4)	47.2 (d)	4.81 (brt, 6.5)	47.2 (d)	4.81 (m)		
	13	40.3 (t)	1.60 (brt, 12.9)	40.8 (t)	1.99 (m)	40.8 (t)	1.98 (m)		
	13′		1.26 (m)		1.29 (m)		1.30 (m)		
	14	24.8 (d)	1.71 (m)	24.5 (d)	1.80 (m)	24.5 (d)	1.78 (m)		H_3-15, 16
	15	23.5 (q)	0.96 (d, 7)	23.3 (q)	0.89 (d, 7)	23.4 (q)	0.89 (d, 6)		H_3-16
	16	20.9 (q)	0.98 (d, 7.3)	21.2 (q)	0.89 (d, 7)	21.2 (q)	0.89 (d, 6)		H_3-15
	12-NH		8.09 (d, 9.2)		9.09 (d, 6.5)		9.11 (d, 7)	**H_2-18**	
β-Ala	17	170.2 (s)		172.7 (s)		172.7 (s)			
	18	36.6 (t)	2.37 (m)	34.7 (t)	2.59 (dd, 15, 4)	34.7 (t)	2.59 (dd, 17.5, 4)	**12-NH**	
	18′		2.10 (m)		1.83 (m)		1.82 (m)		
	19	37.0 (t)	3.87 (m)	32.8 (t)	3.88 (m)	32.8 (t)	3.88 (brt, 12.5)		
	19′		3.10 (m)		3.13 (brt, 13)		3.12 (brt, 12.5)		
	19-NH		6.68 (dd, 7.6, 1.5)		7.77 (brd, 10)		7.79 (brd, 9)	**H-21**	
L-Ala (L-Me-Ala)	20	168.8 (s)		172.4 (s)		172.5 (s)			H_3-22
	21	55.6 (d)	4.92 (q, 7)	47.6 (d)	4.19 (dq, 9, 6.5)	47.6 (d)	4.18 (brquint, 7.5)	**19-NH**	H_3-22
	22	14.6 (q)	1.36 (d, 6.7)	15.8 (q)	0.74 (d, 6.5)	15.7 (q)	0.73 (d, 6.5)		
	21-NH (NMe)	28.6 (q)	2.76 (s)		8.61 (d, 9)		8.62 (d, 9.5)	**H-24**	
L-Me-Val	23	170.2 (s)		172.0 (s)		172.0 (s)			**21-NH**
	24	57.9 (d)	4.93 (d, 11)	62.2 (d)	4.84 (d, 10.5)	62.3 (d)	4.82 (d, 11.5)	**21-NH**	H_3-27
	25	28.1 (d)	2.37 (m)	27.7 (d)	2.16 (m)	27.7 (d)	2.18 (m)		
	26	19.8 (q)	0.91 (d, 6.4)	19.8 (q)	0.86 (d, 6.5)	19.9 (q)	0.86 (d, 6.5)		H_3-27
	27	19.4 (q)	0.87 (d, 6.4)	19.1 (q)	0.96 (d, 6)	19.1 (q)	0.96 (d, 6.5)		H_3-26
	24-NMe	31.4 (q)	3.32 (s)	31.3 (q)	3.30 (s)	31.5 (q)	3.29 (s)	**H-29**	
D-*allo*-Ile (D-Val)	28	176.3 (s)		173.6 (s)		173.4 (s)			**24-NMe**
	29	52.5 (d)	5.42 (dd, 9.5, 2.8)	53.3 (d)	4.90 (t, 10)	54.9 (d)	4.83 (t, 10.5)	**24-NMe**	H_3-32, 33
	30	37.0 (d)	1.75 (m)	37.6 (d)	1.94 (m)	31.5 (d)	2.10 (m)		H_3-32, 33
	31	26.6 (t)	1.44 (m)	25.6 (t)	1.37 (m)	-			
	31′		1.20 (m)		1.06 (m)				

Table 1 (*continued*)

No.		**1**		**3**[a]		**5**			
		^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	ROESY[d]	HMBC (8 Hz)[e]
	32	12.0 (q)	0.98 (t, 7.3)	11.1 (q)	0.88 (t, 7)	19.1 (q)	0.87 (d, 6.5)		H_3-33
	33	13.9 (q)	0.72 (d, 7)	14.5 (q)	0.91 (d, 6.5)	18.5 (q)	0.94 (d, 7)		H_3-32
	29-NH		8.22 (brs)		8.07 (d, 10)		8.12 (d, 10)	**H_2-35**	
β-Ala	34	171.1 (s)		170.4 (s)		171.3 (s)			**29-NH**
	35	35.2 (t)	2.42 (m)	34.3 (t)	2.35 (m)	34.1 (t)	2.38 (m)	**29-NH**, 36-NH	
	35′		2.22 (m)		2.35 (m)		2.38 (m)	**29-NH**, 36-NH	
	36	35.0 (t)	4.18 (m)	36.5 (t)	3.74 (m)	36.7 (t)	3.73 (m)		
	36′		3.10 (m)		3.44 (m)		3.43 (m)		
	36-NH		6.87 (dd, 7.6, 3.1)		6.36 (brd, 7.5)		6.37 (dd, 7.5, 2.5)	**H-38, 38-NMe**	
L-Me-Ile	37	169.7 (s)		169.2 (s)		169.4 (s)			**36-NH**, H-38
	38	60.5 (d)	4.98 (d, 11)	60.6 (d)	4.97 (d, 10)	60.6 (d)	4.96 (d, 10)	**36-NH**	
	39	32.3 (d)	2.10 (m)	31.9 (d)	2.20 (m)	31.8 (d)	2.20 (m)		H-38, H_3-42
	40	24.8 (t)	1.29 (m)	25.1 (t)	1.37 (m)	25.0 (t)	1.37 (m)	38-NMe	H-38, H_3-41
	40′		0.97 (m)		1.06 (m)		1.03 (m)	38-NMe	
	41	10.1 (q)	0.85 (t, 7.3)	9.7 (q)	0.85 (t, 7.5)	9.7 (q)	0.84 (t, 7)		
	42	15.6 (q)	0.95 (d, 6.5)	16.8 (q)	1.11 (d, 6)	16.7 (q)	1.10 (d, 6.5)		H-38
	38-NMe	31.1 (q)	3.18 (s)	31.7 (q)	2.98 (s)	31.7 (q)	2.97 (s)	H_2-40, **H-44**	
D-Leu	43	175.0 (s)		175.7 (s)		175.8 (s)			**38-NMe, H-38**
	44	48.2 (d)	5.09 (ddd, 11.6, 8.9, 2.8)	49.1 (d)	4.44 (dt, 10, 5)	49.1 (d)	4.44 (dt, 9.5, 5)	**38-NMe**	
	45	40.2 (t)	1.61 (m)	39.5 (t)	1.84 (m)	39.5 (t)	1.83 (m)		H_3-47, 48
	45′		1.24 (brt, 12.9)		1.45 (ddd, 13, 8.5, 5)		1.44 (m)		
	46	24.9 (d)	1.78 (m)	24.3 (d)	1.91 (m)	24.3 (d)	1.91 (m)		H_3-47, 48
	47	23.2 (q)	0.90 (d, 6.4)	23.4 (q)	1.03 (d, 6.5)	23.4 (q)	1.03 (d, 6.5)		H_3-48
	48	21.2 (q)	0.97 (d, 6.4)	21.8 (q)	0.99 (d, 6.5)	21.8 (q)	0.98 (d, 6.5)		H_3-47
	44-NH		8.03 (brs)		8.88 (d, 5)		8.88 (d, 5)	**H_2-50**	
β-Ala	49	171.2 (s)		171.8 (s)		171.8 (s)			**NH-44**
	50	36.9 (t)	2.27 (m)	34.0 (t)	2.31 (m)	34.0 (t)	2.32 (m)		
	50′		2.13 (m)		1.96 (m)		1.97 (m)		
	51	36.6 (t)	3.70 (m)	39.2 (t)	3.51 (m)	39.1 (t)	3.51 (m)	**H-2**	
	51′		3.28 (m)		3.27 (m)		3.27 (m)	**H-2**	
	51-NH		6.53 (brt, 5.6)		9.14 (d, 6)		9.14 (d, 6.5)		
D-Me-Leu	52	173.0 (s)							
	53	55.4 (d)	5.16 (m)						
	54	37.9 (t)	1.92 (brt, 11.9)						
	54′		1.40 (m)						
	55	25.1 (d)	1.36 (m)						
	56	23.3 (q)	0.93 (d, 6.7)						
	57	20.6 (q)	0.80 (d, 6.4)						
	53-NMe	31.6 (q)	3.19 (s)						
L-Val	58	173.7 (s)							
	59	53.6 (d)	4.98 (dd, 8.9, 5.2)						
	60	31.5 (d)	2.03 (dsept, 5.2, 6.7)						
	61	19.6 (q)	0.99 (d, 6.7)						

Table 1 (*continued*)

No.		**1**		**3**[a]		**5**			
		^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	^{13}C NMR (mult.)[b]	^{1}H NMR (mult., *J* /Hz)[c]	ROESY[d]	HMBC (8 Hz)[e]
	62	16.9 (q)	0.86 (d, 6.7)						
	59-NH		7.19 (d, 8.9)						
MeOAc	63	169.1 (s)							
	64	72.0 (t)	3.96 (d, 15)						
	64′		3.87 (d, 15)						
	64-OMe	59.1 (q)	3.38 (s)						

[a] Reference 11.
[b] Taken at 125 MHz (δ_{CDCl3} 77.0).
[c] Taken at 500 MHz (δ_{TMS} 0).
[d] Taken on phase-sensitive mode. Inter-residual correlation is shown in bold face.
[e] Inter-residual correlation is shown in bold face.
[f] Broadening signals.

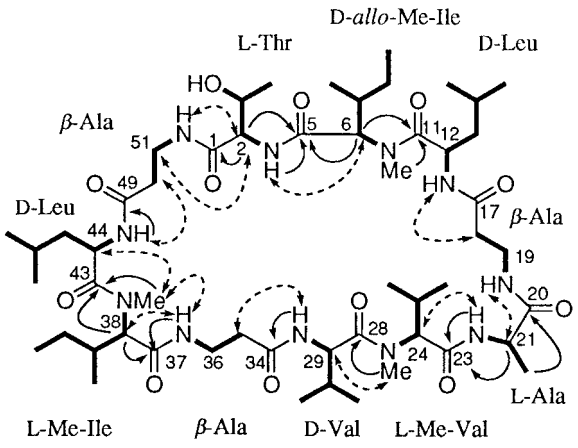
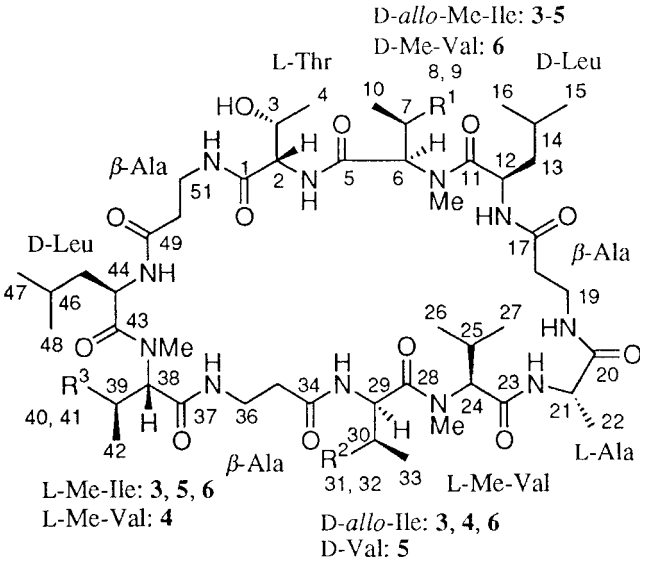


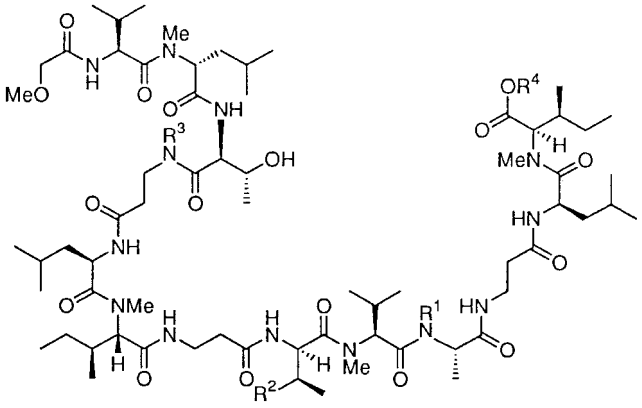
Figure 1. HOHAHA (bold line), ROESY (dashed arrow) and HMBC correlations (arrow) for barangamide C (**5**).

Ie (**9**). Since these compounds have some common structural features with those of well known immunosuppressive drugs, the cyclosporins, we are interested to see whether they have immunomodulatory activity and, if so, their structure–activity relationship. In this paper we now report the isolation and structure elucidation of **4**–**7** and a preliminary result on the screening of immunosuppressive activity of **1**–**3** and **8**.

The sponge (600 g dry weight) collected at Baranglompo Island, Indonesia by SCUBA was dried in the sun, and extracted successively with heptane, ethyl acetate, acetone, and methanol. The concentrated acetone extract (14.6 g) was subjected to separation on silica gel (CH_2Cl_2–MeOH) and ODS (MeOH) and by HPLC on ODS (MeCN–MeOH–H_2O, MeOH–H_2O, MeCN–MeOH–H_2O–TFA) to afford barangamides A (**3**, 11 mg), B (**4**, 1.3 mg), C (**5**, 0.8 mg), and D (**6**, 1.8 mg) and theonellapeptolides IIe (**7**, 12 mg), V (7 mg), and VI (5 mg) along with known theonellapeptolides Id (**1**), IId (**2**), and Ia (**8**). The ethyl acetate extract



3: Barangamide A (R^1 = Et, R^2 = Et, R^3 = Et)
4: Barangamide B (R^1 = Et, R^2 = Et, R^3 = Me)
5: Barangamide C (R^1 = Et, R^2 = Me, R^3 = Et)
6: Barangamide D (R^1 = Me, R^2 = Et, R^3 = Et)



1a: R^1 = Me, R^2 = Et, R^3 = H, R^4 = Me
1b: R^1 = Me, R^2 = Et, R^3 = H, R^4 = H
2a: R^1 = H, R^2 = Et, R^3 = H, R^4 = Me
2b: R^1 = H, R^2 = Et, R^3 = H, R^4 = H
7a: R^1 = H, R^2 = Et, R^3 = Me, R^4 = Me
7b: R^1 = H, R^2 = Et, R^3 = Me, R^4 = H
8a: R^1 = Me, R^2 = Me, R^3 = H, R^4 = Me
8b: R^1 = Me, R^2 = Me, R^3 = H, R^4 = H
9a: R^1 = Me, R^2 = Et, R^3 = Me, R^4 = Me
9b: R^1 = Me, R^2 = Et, R^3 = Me, R^4 = H

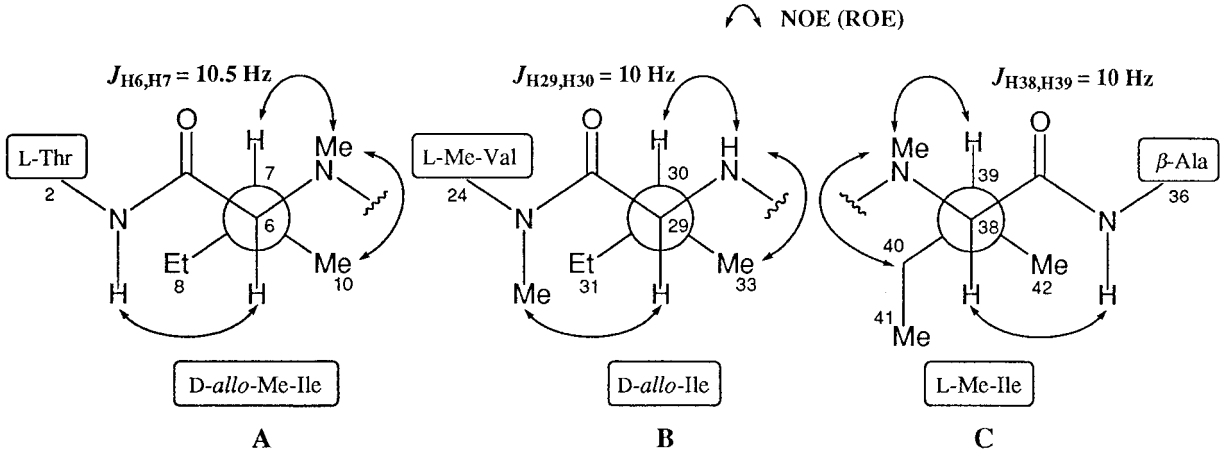


Figure 2. ROESY and NOEDS correlations and assignment of isomeric isoleucine units in **3** and **5**.

Table 2. NMR data (in $CDCl_3$) for barangamides B (**4**) and D (**6**)

No.		**4**				**6**			
		^{13}C NMR (mult.)[a]	^{1}H NMR (mult., *J* /Hz)[b]	ROESY[c]	HMBC (8 Hz)[d]	^{13}C NMR (mult.)[a]	^{1}H NMR (mult., *J* /Hz)[b]	ROESY[c]	HMBC (8 Hz)d
L-Thr	1	174.6 (s)			H-2	174.5 (s)			H-2
	2	52.6 (d)	4.82 (m)	H-3, **H_2-51**	H_3-4	52.6 (d)	4.82 (m)	**H_2-51**, 2-NH, H-3	H_3-4
	3	64.7 (d)	4.06 (brq, 6.5)	H-4	H_3-4	64.8 (d)	4.06 (m)	**H_2-51**	H_3-4
	4	21.9 (q)	1.31 (d, 6.5)	H-3		21.8 (q)	1.30 (d, 6)	H-3	
	2-NH		8.62 (d, 9.5)	**H-6**			8.60 (d, 9.5)	H-2, **H-6**	
	OH								
D-*allo*-Me-Ile (D-Me-Val)	5	169.4 (s)			**H-2**	169.2 (s)			**2-NH**, H-6
	6	64.4 (d)	4.89 (d, 9.5)	**2-NH**	H_3-10	66.1 (d)	4.83 (m)	**2-NH**, 6-NMe	H_3-9, 10, 6-NMe
	7	32.4 (d)	2.26 (m)		H_3-10	26.3 (d)	2.45 (m)		H_3-9
	8	26.4 (t)	1.50 (m)			-			
	8′		1.13 (m)						
	9	11.4 (q)	0.99 (t, 7)			19.5 (q)	1.03 (d, 6.5)		H_3-10
	10	15.3 (q)	0.87 (d, 6.5)	6-NMe		19.1 (q)	0.91 (d, 6.5)		H_3-9
	6-NMe	29.0 (q)	2.64 (s)	H_3-10		29.1 (q)	2.65 (s)	H-6, **H-12**	H-6
D-Leu	11	174.4 (s)			**6-NMe**	174.4 (s)			**6-NMe, H-6**
	12	47.3 (d)	4.82 (brt, 10.5)			47.3 (d)	4.82 (overlapping)	**6-NMe**	
	13	40.8 (t)	1.99 (m)		H_3-15, 16	40.8 (t)	1.98 (m)		H_3-15, 16
	13′		1.30 (m)				1.30 (m)		
	14	24.5 (d)	1.80 (m)		H_3-16	24.5 (d)	1.78 (m)		H_3-15, 16
	15	23.4 (q)	0.90 (overlapping)		H_3-15	23.4 (q)	0.89 (overlapping)		H_3-16
	16	21.2 (q)	0.90 (overlapping)			21.2 (q)	0.89 (overlapping)		H_3-15
	12-NH		9.07 (brd, 7)	**H_2-18**			9.12 (d, 6.5)		
β-Ala	17	172.7 (s)			**12-NH**	172.8 (s)			**12-NH**
	18	34.7 (t)	2.57 (m)	**12-NH**		34.7 (t)	2.60 (m)		
	18′		1.78 (m)	**12-NH**			1.82 (m)		
	19	32.9 (t)	3.87 (m)			32.9 (t)	3.88 (brt, 9.5)	**H-21**	
	19′		3.12 (brt, 12)				3.12 (brt, 12.5)	**H-21**	
	19-NH		7.79 (brd, 9.5)	H_2-19, **H-21**			7.77 (brd, 10)	H_2-19, **H-21**	
L-Ala	20	172.5 (s)			H-21, H_3-22	172.4 (s)			**H-21, H_3-22**
	21	47.6 (d)	4.19 (dq, 9.5, 6.5)	**19-NH**, 21-NH	H_3-22	47.6 (d)	4.18 (m)	**19-NH**, H-22	H_3-22
	22	15.8 (q)	0.73 (d, 6.5)	H-21		15.8 (q)	0.73 (d, 6.5)	H-21	
	21-NH		8.61 (d, 9.5)	H-21, **H-24**			8.61 (d, 8.5)	**H-24**	
L-Me-Val	23	172.0 (s)			**NH-21**, H-24	172.0 (s)			**21-NH**, H-24
	24	62.2 (d)	4.84 (d, 10.5)	**21-NH**	H_3-26, 27	62.2 (d)	4.84 (overlapping)	**21-NH**	24-NMe, H_3-26, 27
	25	27.7 (d)	2.17 (m)		H_3-26, 27	27.7 (d)	2.17 (m)		H-24, H_3-26, 27
	26	19.8 (q)	0.86 (d, 6.5)		H_3-27	19.8 (q)	0.87 (overlapping)		H-24
	27	19.1 (q)	0.97 (d, 7)		H_3-26	19.1 (q)	0.96 (d, 6.5)		
	24-NMe	31.4 (q)	3.29 (s)	**H-29**		31.4 (q)	3.29 (s)	**H-29**	H-24
D-*allo*-Ile	28	173.5 (s)			**24-NMe**	173.6 (s)			**24-NMe, H-24**, 29
	29	53.4 (d)	4.90 (t, 9.5)	**24-NMe**, 29-NH		53.3 (d)	4.91 (brd, 10)	**24-NMe**	
	30	37.6 (d)	1.95 (m)			37.6 (d)	1.95 (m)		H-29, H_3-32
	31	25.6 (t)	1.37 (m)		H_3-33	25.6 (q)	1.35 (m)		H_3-32, 33
	31′		1.08 (m)				1.04 (m)		H-29
	32	11.2 (q)	0.88 (overlapping)		H_3-33	14.5 (q)	0.91 (d, 6.5)		
	33	14.5 (q)	0.91 (d, 6.5)		H_3-32	11.2 (q)	0.89 (overlapping)		
	29-NH		8.13 (d, 10.5)	**H_2-35**, H-29			8.12 (d, 10)	**H_2-35**, H_3-33	
β-Ala	34	170.9 (s)			**29-NH, H-29**	170.6 (s)			**29-NH, H-29**

Table 2 (*continued*)

No.		4				6			
		^{13}C NMR (mult.)[a]	^{1}H NMR (mult., *J* /Hz)[b]	ROESY[c]	HMBC (8 Hz)[d]	^{13}C NMR (mult.)[a]	^{1}H NMR (mult., *J* /Hz)[b]	ROESY[c]	HMBC (8 Hz)d
	35	34.1 (t)	2.38 (m)	**29-NH**		34.2 (t)	2.35 (m)	**29-NH**, 36-NH	
	35′		2.38 (m)	**29-NH**, 36-NH			2.35 (m)	**29-NH**, 36-NH	
	36	36.5 (t)	3.76 (m)			36.6 (t)	3.73 (dq, 7.5, 3)		
	36′		3.41 (m)				3.44 (m)		
	36-NH		6.49 (dd, 7.5, 2)	**38-NMe, H-38**			6.38 (dd, 7.5, 3)	**H-38**, H_2-35	
L-Me-Ile (L-Me-Val)	37	169.3 (s)				169.3 (s)			**36-NH, H-38**
	38	61.6 (d)	4.90 (overlapping)	**36-NH**	H_3-41, 42	60.5 (d)	4.97 (d, 9.5)	**36-NH**, 38-NMe	NMe-38, H_3-42
	39	26.6 (d)	2.31 (m)		H_3-41, 42	31.9 (d)	2.21 (m)		H-38, H_3-42
	40	-				25.1 (t)	1.35 (m)	**38-NMe**	H_3-42
	40′						1.04 (m)	**38-NMe**	
	41	21.2 (q)	1.16 (d, 6.5)		H_3-42	9.7 (q)	0.85 (t, 6.5)		
	42	19.0 (q)	0.82 (d, 6.5)	38-NMe	H_3-41	16.8 (q)	1.11 (d, 6.5)		H-38
	38-NMe	31.8 (q)	2.98 (s)	36-NH, H_3-42, **H-44**		31.8 (q)	2.97 (s)	H-38,**44**, H_2-40	
D-Leu	43	175.7 (s)			**38-NMe, H-38**	175.7 (s)			**38-NMe, H-38**, 44
	44	49.0 (d)	4.44 (dt, 10, 5)	**38-NMe**, 44-NH		49.1 (d)	4.44 (brdt, 9.5, 5)	**38-NMe**, 44-NH	
	45	39.6 (t)	1.84 (m)		H_3-47, 48	39.5 (t)	1.84 (m)		H_3-47
	45′		1.48 (m)				1.45 (m)		
	46	24.3 (d)	1.91 (m)		H_3-47, 48	24.3 (d)	1.94 (m)		H_3-47
	47	23.4 (q)	1.03 (d, 7)		H_3-48	23.4 (q)	1.03 (d, 6.5)		H_3-48
	48	22.0 (q)	0.99 (d, 7)		H_3-47	21.8 (q)	0.98 (d, 6.5)		H_3-47
	44-NH		8.90 (d, 5.5)	H-44, **H_2-50**			8.88 (d, 5)	H-44, **H_2-50**	
β-Ala	49	171.8 (s)			**NH-44**	171.8 (s)			**44-NH**
	50	34.1 (t)	2.30 (m)	**44-NH**		34.0 (t)	2.32 (m)	**44-NH**	
	50′		1.97 (m)	**44-NH**			1.97 (m)	**44-NH**	
	51	39.2 (t)	3.51 (m)	**H-2**		39.2 (t)	3.51 (m)	**H-2, 3**	
	51′		3.27 (m)	**H-2**			3.27 (m)	**H-2, 3**	
	51-NH		9.16 (brd, 5)				9.13 (d, 6.5)		

[a] Taken at 125 MHz (δ_{CDCl3} 77.0).
[b] Taken at 500 MHz (δ_{TMS} 0).
[c] Taken on phase-sensitive mode. Inter-residual correlation is shown in **bold face**.
[d] Inter-residual correlation is shown in **bold face**.

(5.9 g) was separated by a silica gel (CH_2Cl_2–MeOH) column followed by HPLC on ODS (MeCN–MeOH–H_2O, MeCN–MeOH–H_2O–TFA) to afford theonellapeptolides IV (9 mg), V (24 mg), and VI (5.5 mg) along with **1**–**9**. Swinholide A (11.3 mg)[1] was also isolated from the ethyl acetate extract as a cytotoxic principle.

The molecular formula of barangamide C (**5**) was determined as $C_{53}H_{95}N_{11}O_{12}$ by high-resolution FABMS (MH^+ 1078.7230, Δ −1.0 mmu), which was 14 mass units (CH_2) smaller than that of barangamide A (**3**). The ^1H and ^{13}C NMR spectra of **5**, which were quite similar to those of **3**, contained signals for eight NH (δ_H 6.3–9.2), three NMe (δ_H 2.64, 2.97, 3.29) and eleven C=O (δ_C 169–176) (Table 1). Cyclic nature was suggested by negative ninhydrin reaction. NMR analyses indicated the presence of eleven amino acid residues, β-Ala (×3), Ala, Me-Ile (×2), Leu (×2), Thr, Val, and Me-Val, the same composition found in **3** except for one residue, i.e. Val (*allo*-Ile in **3**). One of the Me-Ile was the *allo*-form as shown by comparison of the ^1H NMR data of the acid hydrolysate of **5** with those of authentic samples. Interpretation of 2D NMR including HOHAHA, HMBC and NOEDS data (Table 1) led to the planar structure of **5** (Fig. 1). Application of the Marfey's method[12] enabled us to determine the chirality of the residues as L-Ala, L-Me-Ile, D-*allo*-Me-Ile, D-Leu (×2), L-Thr, D-Val and L-Me-Val.

As observed in **3**, **5** also had two isomeric Me-Ile units (C5–10, C37–42). In general, it is difficult to distinguish diastereomers by NMR. We have successfully determined the positions of the two isomeric Me-Ile units in **3** by examination of coupling constants and NOE data (Fig. 2).[11] The essence of this method is as follows. Authentic samples of isoleucine and *allo*-isoleucine showed small vicinal coupling constants (4 Hz) for H-2 and H-3, indicating no rotation barrier around C2–C3 axis. On the other hand **3** showed large values for the corresponding coupling constants ($J_{H6,H7}$=10.5 Hz and $J_{H38,H39}$=10 Hz) for the Me-Ile units, suggesting restricted rotation and *anti* relationship of these protons. In ROESY and NOEDS experiments, the 6-NMe showed significant NOE with H_3-10 and no NOE with H_2-8 and H_3-9, suggesting that this unit (C5–10) is the *allo*-Me-Ile (**A**). Similar NOE relationship was observed for the *allo*-Ile unit (C28–33) as shown in **B**. The other unit (C37–42) was assigned as Me-Ile by NOE between 38-NMe and H_2-40 and by no NOE between 38-NMe and H_3-42 (**C**). The validity of this method was confirmed by reaching the same conclusion regarding the position of D-*allo*-Ile and D-*allo*-Me-Ile units in **1**, the structure of which has been determined by a more elaborate method.[5] The two isomeric Me-Ile units, (C5–10, C37–42) of **5** were assigned in the same manner (Fig. 2) to complete the total structure as shown. Barangamide C (**5**) differs from **3** only in the substitution of a D-Val for a D-*allo*-Ile. Barangamide C (**5**) is a cyclic undecapeptide possessing the same amino acid sequence with the ring portion of the known theonellapeptolide Ia (**8**), which was also isolated in this study. Similar relationship is found between barangamide A (**3**) and theonellapeptolide Id (**1**).

Both barangamides B (**4**) and D (**6**) had the same molecular formula, $C_{53}H_{95}N_{11}O_{12}$, as determined by their high-resolution FABMS data. The ^1H and ^{13}C NMR spectra of **4** and **6** were similar to those of **5** (Table 2). Since only small amounts of samples were isolated, structure elucidation of **4** and **6** was mainly carried out by the analysis of HOHAHA and ROESY data. Interpretation of HOHAHA spectrum indicated the structures of the component amino acids (Fig. 3), which were confirmed by comparison of ^1H NMR spectrum of their acid hydrolysates with authentic samples. The absolute configuration of each amino acid was determined by the Marfey analysis. Detailed analysis of ROESY and some HMBC data led to the structures of **4** and **6** as shown in Fig. 3. Barangamides B (**4**) and D (**6**) are closely related to theonellapeptolides Ib and Ic, respectively.

We have isolated four known [Id (**1**), IId (**2**), Ia (**8**), Ie (**9**)] and four new theonellapeptolides [IIe (**7**), IV, V, VI] in this study. Each of these peptides existed as a mixture of several conformers in such NMR solvents as $CDCl_3$, CD_3OD, acetone-d_6, CD_3CN, C_6D_6, DMSO-d_6, pyridine-d_5, and showed broad and complex signals. In $CDCl_3$, **1** and **8** existed as a single major conformer, while in other solvents their spectra were complex due to the existence of conformers. For example, **1** exhibited five NMe signals in

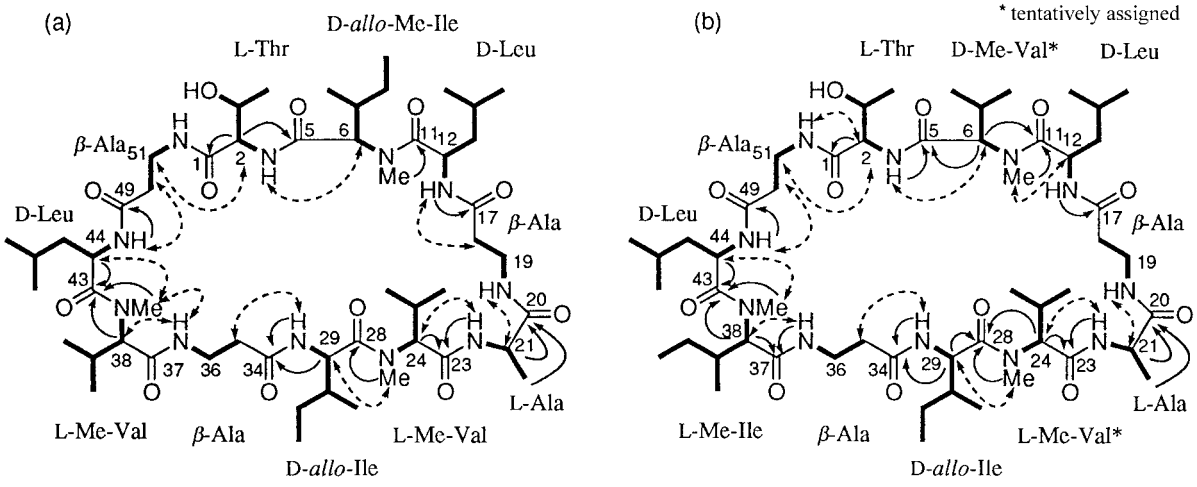


Figure 3. HOHAHA (bold line), HMBC (→), and ROESY correlations (↔) for barangamides B (**4**, a) and D (**6**, b).

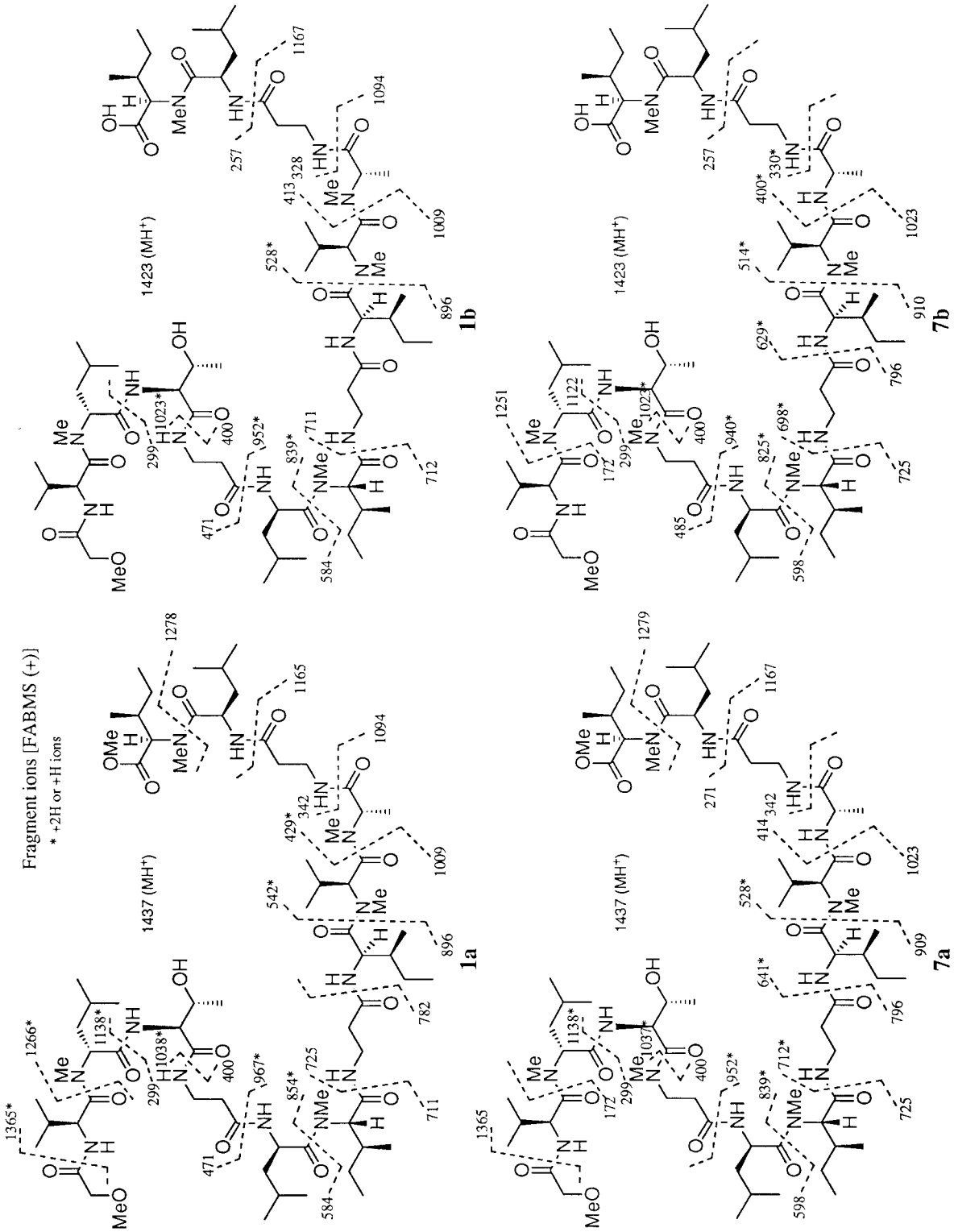


Figure 4. MS analysis of the ring opened products of theonellapeptolides Id (**1**) and IIe (**7**).

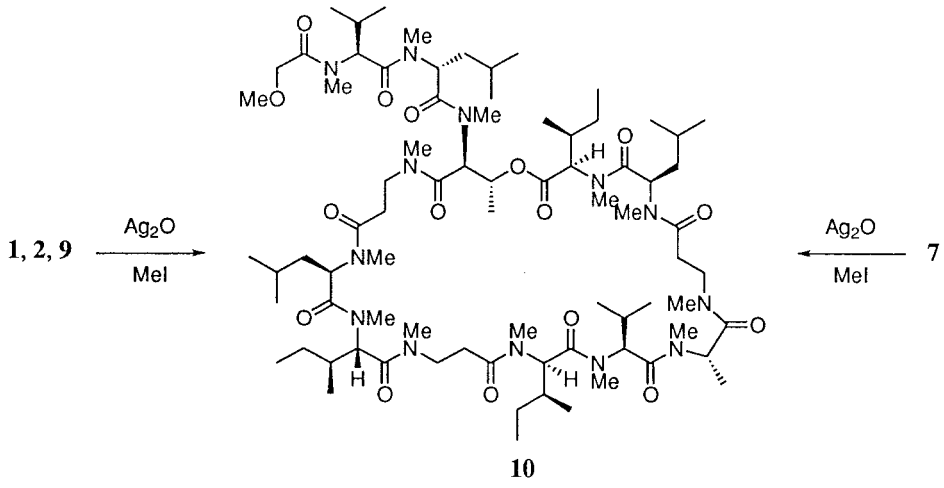


Figure 5. Methylation of theonellapeptolides (**1**, **2**, **7**, and **9**) to **10**.

$CDCl_3$ and more than 7 NMe signals in DMSO-d_6. The structure of **1** could be confirmed by the complete assignment of NMR signals in $CDCl_3$ (Table 1), while the NMR analyses of other theonellapeptolides were quite difficult. This difficulty was overcome by analysis of MS fragment ions of the ring-opened products. The ring open products **1a** and **1b** were prepared from **1** by treating with NaOMe and KOH, respectively. The negative FABMS showed weak fragment ions, whereas the positive FAB, APCI, and/or ESI MS of **1a** and **1b** showed expected fragment ions (amide-bond cleavage), which were clearly assigned by comparison of MS data of **1a** and **1b** (Fig. 4). The amino acid sequences of other known theonellapeptolides (**2**, **8**, **9**) were also deduced from the detailed analyses of the fragment ions of the ring-opened products (**2a**,**b**, **8a**,**b**, **9a,b**). Thus, the amino acid sequences of theonellapeptolides can be deduced from the MS analyses of methanolysis and hydrolysis products. Detailed analysis of MS data for **7a** and **7b**, derived from the new theonellapeptolide **7**, suggested the sequence as shown in Fig. 4. The suggested structure of **7** was an analogue having an L-Ala instead of L-Me-Ala in the known theonellapeptolide Ie (**9**). The relationship between **7** and **9** is the same as that found for theonellapeptolides Id (**1**) and IId (**2**), thus we named **7** theonellapeptolide IIe. In the analysis of fragment ions of **7a** and **7b**, it was impossible to distinguish the amino acids having the same molecular weights, such as Ala and β-Ala; Leu, *allo*-Ile, and Me-Val; Me-Leu, Me-Ile, and Me-*allo*-Ile. Therefore, validity of the suggested structure should be confirmed by other means. Methylation of **1**, **2**, **7**, and **9** with MeI and Ag_2O gave the same compound **10**, $C_{78}H_{141}N_{13}O_{16}$, whose structure was confirmed by FABMS (MH^+ *m*/*z* 1517), Marfey's analysis, and ^1H NMR analysis of complete acid hydrolysate (Fig. 5). Thus, the structure of theonellapeptolide IIe (**7**) was unambiguously established as shown. The structures of other new theonellapeptolides IV, V, VI are now under investigation and will be reported elsewhere.

Theonellapeptolides and barangamides are tridecapeptide

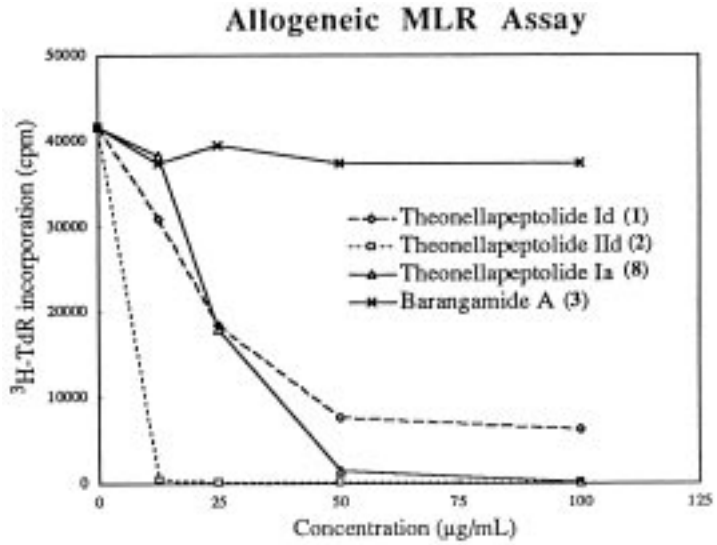


Figure 6. Result of mixed lymphocyte reaction assay for theonellapeptolides Id (**1**), IId (**2**), Ia (**8**), and barangamide A (**3**). This figure was supplied by Dr Y. Koezuka, Kirin Brewery Co. Ltd.

lactones and cyclic undecapeptides, respectively, possessing a high proportion of *N*-methylated amino acids and no aromatic amino acids. Similar structural features are found in the cyclosporins, cyclic undecapeptides, some of which are widely used as immunosuppressants after organ transplant surgery.[13] In order to test immunomodulatory activity of these compounds we submitted samples of theonellapeptolides (**1**, **2**, **8**) and barangamide A (**3**) for mixed lymphocyte reaction (MLR) assay. The results are shown in Fig. 6. Barangamide A (**3**) showed no activity even at the highest concentration of 100 μg/mL, while all theonellapeptolides (**1**, **2**, **8**) were active. Among the three compounds, **2** showed the strongest immunosuppressive activity. It is interesting to note that small variation in the structures of these compounds significantly affect their biological activity. In a preliminary assay, **3** and **7** had no cytotoxicity against L1210 tumor cells even at 10 μg/mL, while theonellapeptolides Ia–Ie have been reported to be cytotoxic (IC_{50} 1.3–2.4 μg/mL) against the same cell line.[6] The observed immunosuppressive activity of theonellapeptolides may largely originate from their cytotoxic effect. Further chemical and biological studies are under way.

Experimental

General experimental procedures

The ^1H and ^{13}C NMR spectra were recorded on a JEOL α-500 spectrometer. The APCI, ESI, and FAB mass spectra were measured on a JMS-700. The IR spectra and the optical rotations were measured using a JASCO FT/IR-300 and a JASCO DIP-1000, respectively. High-performance liquid chromatography (HPLC) was performed on a Hitachi L-6000 pump equipped with a Hitachi RI monitor (655A-30) and a Hitachi L-4000 UV detector, using COSMOSIL packed ODS HPLC column (5C18-AR-II, 10×250 mm). Kieselgel 60 (70–230 mesh, MERCK) and Cosmosil 75C18-OPN (Nacalai Tesque) were used for column chromatography. All solvents used were reagent grade.

Extraction and isolation

A sample of *T. swinhoei* was collected by hand using SCUBA in Baranglompo Island, Indonesia, in August, 1997, and sun dried prior to transportation to Okinawa. The sample (600 g dry weight) was extracted successively with heptane, ethyl acetate, acetone, and methanol. The extracts were concentrated under reduced pressure to afford 1.5, 5.9, 14.6 and 51.6 g of materials, respectively. The ethyl acetate and acetone extracts showed strong and moderate cytotoxicity against L1210 cell line, respectively, while the heptane and methanol extracts showed no activity at 10 μg/mL. The acetone extract was chromatographed on silica gel eluting with CH_2Cl_2 and increasing amount of MeOH (1–100%) to give eight fractions. The 5th fraction was further chromatographed on ODS using MeOH as eluent. The fractions 1~3 were combined and subjected to further separation by reversed phase HPLC on ODS using MeCN–MeOH–H_2O (16:3:1) to yield eight fractions. Final purification of the peptides was carried out by repeated HPLC on ODS using MeOH–H_2O (88:12), MeOH–MeCN–H_2O (8:2:0.8), and/or MeOH–MeCN–H_2O (8:1:1) with 0.02% TFA as eluents. The 2nd fraction yielded barangamides A (**3**), B (**4**), C (**5**), and D (**6**) on repeated purification on ODS HPLC eluting with MeOH–H_2O (88:12) and MeOH–MeCN–H_2O (3.5:3:3.5) with 0.1% TFA. The last fraction gave theonellapeptolides Id (**1**) and Ia (**8**). Theonellapeptolides IId (**2**) and V were isolated from the 5th fraction. The 7th fraction yielded theonellapeptolides VI and IIe (**7**). Similarly, ethyl acetate extract was first chromatographed on silica gel eluting with CH_2Cl_2 and MeOH (0–10%) to give 16 fractions. The 13th fraction yielded **1**–**8** on further purification by HPLC. The 14th and 15th fractions were purified by ODS HPLC using MeOH–MeCN–H_2O (8:2:0.8) to yield **1**, **2**, and **7**–**9**. The 10th fraction was rechromatographed on silica gel using CH_2Cl_2 and MeOH (0–10%) to give 8 fractions. Swinholide A was isolated from the 4th and 5th fractions by PTLC on silica gel (CH_2Cl_2–MeOH, 12:1). The 6th fraction yielded theonellapeptolides IId (**2**), IIe (**7**), IV, V, and VI by repeated reversed phase HPLC using MeOH–MeCN–H_2O (8:1:1) with 0.1% TFA. Theonellapeptolides Id (**1**) and Ia (**8**) were also obtained from the 7th and 8th fractions by repeated ODS HPLC, using MeOH–MeCN–H_2O (8:1:1) as eluent. The yields of **1**–**9** were 2000, 85, 22, 1.3, 0.8, 1.8, 12, 300, and 32 mg, respectively.

Swinholide A. ^1H and ^{13}C NMR ($CDCl_3$) were identical with those reported.[1]

Theonellapeptolide Id (1). A white crystalline solid; IR (film) ν_{max} 3315, 2962, 1735, 1652, 1627, 1542 cm^{-1}; LRFABMS, positive ion, *m/z* 1405 $[M+H]^+$, negative ion, 1403 $[M-H]^-$; ^1H and ^{13}C NMR ($CDCl_3$) are listed in Table 1 (complete NMR data have not been described in the literature[5]). It was identified by the ^1H NMR comparison with an authentic sample.

Theonellapeptolide IId (2). A white crystalline solid; IR (film) ν_{max} 3311, 2962, 1735, 1633, 1538 cm^{-1}; LRFABMS, negative ion, *m/z* 1389 $[M-H]^-$. It was identified by the ^1H NMR comparison with an authentic sample.

Barangamide A (3). A glassy solid (22 mg); $[\alpha]_D^{25}=-38.4°$ (c=0.3, MeOH),[11] $[\alpha]_D^{28}=+44.6°$ (c=0.3, $CHCl_3$).

Barangamide B (4). A glassy solid (1.3 mg); $[\alpha]_D^{29}=+76.5°$ (c=0.04, $CHCl_3$); IR (film) ν_{max} 3264, 2962, 1658, 1623 cm^{-1}; LRFABMS, negative ion, *m/z* 1076.7 $[M-H]^-$, positive ion, *m/z* 1078.7 $[M+H]^+$; HRFABMS *m/z* 1078.7238 $[M+H]^+$, calcd for $C_{53}H_{96}N_{11}O_{12}$ 1078.7240 (Δ −0.2 mmu); ^1H and ^{13}C NMR ($CDCl_3$) are listed in Table 2.

Barangamide C (5). A glassy solid (0.8 mg); $[\alpha]_D^{26}=+57.8°$ (c=0.07, $CHCl_3$); IR (film) ν_{max} 3266, 2962, 1658, 1625 cm^{-1}; LRFABMS, negative ion, *m/z* 1076.7 $[M-H]^-$, positive ion, *m/z* 1078.7 $[M+H]^+$; HRFABMS *m/z* 1078.7230 $[M+H]^+$, calcd for $C_{53}H_{96}N_{11}O_{12}$ 1078.7240 (Δ −1.0 mmu); ^1H and ^{13}C NMR ($CDCl_3$) are listed in Table 1.

Barangamide D (6). A glassy solid (1.8 mg); $[\alpha]_D^{29}=+84.6°$ (c=0.1, $CHCl_3$); IR (film) ν_{max} 3266, 2962, 1658, 1625 cm^{-1}; LRFABMS, negative ion, m/z 1076.7 $[M-H]^-$, positive ion, m/z 1078.7 $[M+H]^+$; HRFABMS m/z 1078.7244 $[M+H]^+$, calcd for $C_{53}H_{96}N_{11}O_{12}$ 1078.7240 (Δ +0.4 mmu); ^1H and ^{13}C NMR ($CDCl_3$) are listed in Table 2.

Theonellapeptolide IIe (7). A white solid (11.9 mg); $[\alpha]_D^{30}=-13.4°$ (c=0.6, $CHCl_3$); IR (film) ν_{max} 3326, 2962, 1751, 1633, 1538 cm^{-1}; LRFABMS, positive ion, m/z 1405 $[M+H]^+$; HRFABMS m/z 1404.9473 $[M+H]^+$, calcd for $C_{70}H_{126}N_{13}O_{16}$ 1404.9446 (Δ +2.7 mmu); ^1H NMR (500 MHz, $CDCl_3$) δ 7.90 (br), 7.70 (br), 7.53 (br), 7.16 (br), 6.52 (br), 6.14 (br), 3.96 (d, J=15 Hz), 3.90 (d, J=15 Hz), 3.43 (s), 3.42 (s), 3.18 (s), 3.12 (s), 3.06 (s), 3.04 (s), 3.02 (s) 2.88 (s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.0, 173.9, 173.7, 173.1, 172.8, 172.5, 172.4, 170.6 (×2), 170.4, 170.2, 169.5, 169.3, 167.9, 71.7, 70.5, 64.5, 62.4, 59.9, 54.8, 53.6, 53.5, 52.1, 48.7, 47.7, 45.2, 42.6, 41.4, 37.4, 36.3, 36.0, 35.8, 34.6, 34.4, 33.2, 31.3, 33.1, 30.8, 29.5, 26.4, 24.9, 24.8, 24.6, 23.3, 23.2, 22.0, 21.8, 21.4, 20.0, 19.5, 18.7, 17.4, 17.2, 15.6, 15.2, 14.5, 14.3, 11.6, 11.4, 10.7, 9.8.

Theonellapeptolide Ia (8). White crystalline solid; IR (film) ν_{max} 3326, 2962, 1733, 1681, 1633, 1538 cm^{-1}; LRFABMS, negative ion, 1389 $[M-H]^-$. It was identified by ^1H NMR comparison with an authentic sample.

Theonellapeptolide Ie (9). A white solid (32.0 mg); IR (film) ν_{max} 3315, 2962, 1733, 1633, 1540 cm^{-1}; LRFABMS, positive ion, m/z 1419 $[M+H]^+$. It was identified by comparison of the NMR data with those of an authentic sample.

Preparation of *N*-methyl amino acids

Except for commercially available D-Me-alanine, L-Me-alanine, D/L-Me-valine, standard *N*-methyl amino acids were prepared by Retro-Aza Diels–Alder reaction[14] as follows: To a solution of L-valine (100 mg, 0.85 mM) in 1.5 mL of H_2O was added 70 μL (0.86 mM) of 37% aqueous formaldehyde and 160 μL of cyclopentadiene (1.95 mM) and the mixture was stirred vigorously under N_2 at room temperature for 16 h. After washing with CH_2Cl_2, the solution was neutralized with 0.1 mL of 2 M $NaHCO_3$ and concentrated under reduced pressure. The residue was extracted with CH_2Cl_2 (2 mL×3), dried over $MgSO_4$, and purified by column chromatography on silica gel eluting with CH_2Cl_2–MeOH (85:15) to furnish a diastereomeric mixture of 2-azanorbornenes (43 mg), which was used directly in the next reaction. To a solution of the above adduct in 1.0 mL $CHCl_3$ was added 1.0 mL of trifluoroacetic acid and triethylsilane (160 μL, 0.99 mM). The resulting homogeneous reaction mixture was stirred at ambient temperature under N_2 for 6 h. After removal of the solvent by N_2 stream, the yellow residue was dissolved in CH_2Cl_2 (1.5 mL), treated with 1.0 mL of 10% HCl and washed with CH_2Cl_2/EtOAc. The aqueous layer was filtered and dried to furnish *N*-methyl-L-valine (23.5 mg).

Similarly, *N*-methyl-D-leucine, *N*-methyl-D-isoleucine, *N*-methyl-L-isoleucine, *N*-methyl-D-*allo*isoleucine, *N*-methyl-L-*allo*isoleucine and *N*-methyl-β-alanine were prepared from respective amino acids.

L-Me-Val. ^1H NMR (D_2O) δ 3.69 (1H, d, J=4.5 Hz), 2.62 (3H, s), 2.20 (1H, dsept, J=4.5, 7 Hz), 0.94 (3H, d, J=7 Hz), 0.89 (3H, d, J=7 Hz); ^{13}C NMR (D_2O) δ 170.9 (s), 67.4 (d), 32.9 (q), 29.2 (d), 18.1 (q), 17.0 (q).

D-Me-Leu. ^1H NMR (D_2O) δ 3.80 (1H), 2.60 (3H, s), 1.70 (1H, m), 1.65 (2H, m), 0.82 (6H).

L-*allo*-Me-Ile. ^1H NMR (D_2O) δ 3.50 (1H, d, J=4 Hz), 2.60 (3H, s), 1.90 (1H, m), 1.36 (1H, dq, J=14, 7 Hz), 1.18 (1H, dq, J=14, 7 Hz), 0.88 (3H, d, J=7 Hz), 0.82 (3H, t, J=7 Hz).

L-Me-Ile. ^1H NMR (D_2O) δ 3.77 (1H, d, J=3.5 Hz), 2.62 (3H, s), 1.92 (1H, m), 1.41 (1H, dq, J=14, 7 Hz), 1.25 (1H, dq, J=14, 7 Hz), 0.86 (3H, d, J=6.5 Hz), 0.81 (3H, t, J=7 Hz).

Complete acid hydrolysis of theonellapeptolides and barangamides

Each sample (0.02–0.5 mg) in 6N HCl (0.5 mL) was heated in a glass tube sealed under N_2 at 110°C for 24 h. After the reaction mixture was cooled to ambient temperature, it was concentrated to dryness. ^1H NMR spectrum of each hydrolysate was recorded. Amino acid residues for each compound were identified by ^1H NMR (D_2O) comparison with that of each authentic sample as shown below.

1. β-Ala (×3), Me-Ala, *allo*-Ile, Me-Ile, *allo*-Me-Ile, Leu (×2), Me-Leu, Thr, Val, Me-Val.

2. β-Ala (×3), Ala, *allo*-Ile, Me-Ile, *allo*-Me-Ile, Leu (×2), Me-Leu, Thr, Val, Me-Val.

3. β-Ala (×3), Ala, *allo*-Ile, Me-Ile, *allo*-Me-Ile, Leu (×2), Thr, Me-Val.

4. β-Ala (×3), Ala, *allo*-Ile, *allo*-Me-Ile, Leu (×2), Thr, Me-Val (×2).

5. β-Ala (×3), Ala, Me-Ile, *allo*-Me-Ile, Leu (×2), Thr, Val, Me-Val

6. β-Ala (×3), Ala, *allo*-Ile, Me-Ile, Leu (×2), Thr, Me-Val (×2)

7. β-Ala (×2), Me-β-Ala, Ala, *allo*-Ile, Me-Ile, *allo*-Me-Ile, Leu (×2), Me-Leu, Thr, Val, Me-Val

8. β-Ala (×3), Me-Ala, Me-Ile, *allo*-Me-Ile, Leu (×2), Me-Leu, Thr, Val (×2), Me-Val

9. β-Ala (×2), Me-β-Ala, Me-Ala, *allo*-Ile, Me-Ile, *allo*-Me-Ile, Leu (×2), Me-Leu, Thr, Val, Me-Val

Marfey analysis

Each of the commercially available D- and L-amino acids (0.3 mg) in 10 μL of water was treated with 50 μL of FDAA (Marfey's reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) solution in acetone (10 mg/mL) in the presence of aqueous $NaHCO_3$ solution (20 μL, 1 M), and the mixture was heated at 40°C for 1 h. After the reaction mixture was cooled to room temperature, 10 μL of 2 M HCl solution was added for neutralization and concentrated to dryness. Acetone solution of the residue was filtered and analyzed by HPLC. The aliquot (0.02–0.2 mg) of the each acid hydrolysate described above was treated with FDAA and analyzed by HPLC.

HPLC analysis was carried out by using a reverse phase column (COSMOSIL, $5C_{18}$-AR-II, 10×250 mm) at ambient temperature, eluting with 60% mixed buffer in MeCN (flow rate: 2 mL/min) and detecting by UV detector at 340 nm. The mixed buffer was prepared by mixing aqueous solution of sodium borate, potassium chloride, citric acid, and tris(hydroxymethyl)aminomethane (each 25 mM) and 0.05 M HCl in 3:2 ratio (pH 2–3). Individual amino acid peak was identified by retention time as well as coinjection with standard amino acid derivatives. The retention times of the FDAA standards were 8.15 min for L-Thr, 9.10 min for D-Thr, 8.15 min for L-*allo*-Thr, 8.40 min for D-*allo*-Thr, 9.25 min for β-Ala, 9.58 min for Me-β-Ala, 10.15 min for L-Ala, 12.15 min for D-Ala, 10.42 min for L-Me-Ala, 12.40 min for D-Me-Ala, 13.45 min for L-Val, 18.00 min for D-Val, 17.20 min for L-Me-Val, and 20.30 min for D-Me-Val, 18.10 min for L-Ile, 25.25 min for D-Ile, 17.48 min for L-*allo*-Ile, 25.00 min for D-*allo*-Ile, 22.25 min for L-Me-Ile, 28.48 min for D-Me-Ile, 21.55 min for L-*allo*-Me-Ile, 28.25 min for D-*allo*-Me-Ile, 18.45 min for L-Leu, 26.20 min for D-Leu, 21.20 min for L-Me-Leu, and 29.00 min for D-Me-Leu.

The observed amino acids and their retention times (min) for FDAA derivatives of acid hydrolysate of **1**–**9** are as follows:

1. 8.15 (L-Thr), 9.26 (β-Ala), 10.45 (L-Me-Ala), 13.40 (L-Val), 17.18 (L-Me-Val), 22.28 (L-Me-Ile), 25.05 (D-*allo*-Ile), 26.25 (D-Leu), 28.30 (D-*allo*-Me-Ile).

2. 8.15 (L-Thr), 9.27 (β-Ala), 10.15 (L-Ala), 13.42 (L-Val), 17.15 (L-Me-Val), 22.25 (L-Me-Ile), 25.00 (D-*allo*-Ile), 26.26 (D-Leu), 28.32 (D-*allo*-Me-Ile).

3. 8.15 (L-Thr), 9.25 (β-Ala), 10.18 (L-Ala), 17.15 (L-Me-Val), 22.20 (L-Me-Ile), 25.02 (D-*allo*-Ile), 26.22 (D-Leu), 28.28 (D-*allo*-Me-Ile).

4. 8.17 (L-Thr), 9.25 (β-Ala), 10.16 (L-Ala), 17.18 (L-Me-Val), 25.01 (D-*allo*-Ile), 26.22 (D-Leu), 28.25 (D-*allo*-Me-Ile).

5. 8.15 (L-Thr), 9.24 (β-Ala), 10.15 (L-Ala), 17.22 (L-Me-Val), 18.10 (D-Val), 22.24 (L-Me-Ile), 26.20 (D-Leu), 28.28 (D-*allo*-Me-Ile).

6. 8.17 (L-Thr), 9.26 (β-Ala), 10.15 (L-Ala), 17.15 (L-Me-Val), 18.03 (D-Me-Val), 22.30 (L-Me-Ile), 25.05 (D-*allo*-Ile), 26.21 (D-Leu).

7. 8.15 (L-Thr), 9.27 (β-Ala), 9.55 (Me-β-Ala), 10.15 (L-Ala), 13.48 (L-Val), 17.18 (L-Me-Val), 22.23 (L-Me-Ile), 25.01 (D-*allo*-Ile), 26.18 (D-Leu), 28.28 (D-*allo*-Me-Ile).

8. 8.18 (L-Thr), 9.25 (β-Ala), 10.42 (L-Me-Ala), 13.42 (L-Val), 17.18 (L-Me-Val), 18.05 (D-Val), 22.31 (L-Me-Ile), 26.21 (D-Leu), 28.25 (D-*allo*-Me-Ile).

9. 8.15 (L-Thr), 9.25 (β-Ala), 9.52 (Me-β-Ala), 10.12 (L-Me-Ala), 13.48 (L-Val), 17.25 (L-Me-Val), 22.29 (L-Me-Ile), 25.03 (D-*allo*-Ile), 26.15 (D-Leu), 28.22 (D-*allo*-Me-Ile).

D-Me-Leu was not detected in this analysis, but it was confirmed from the ^{1}H NMR of complete acid hydrolysate of individual peptolide. FDVA [N^{α}-(2,4-dinitro-5-fluorophenyl)-D-valinamide] instead of FDAA was used as the derivatization agent, which also enabled us to separate L-Thr and L-*allo*-Thr with the above mentioned solvent system.

Methanolysis of theonellapeptolides

To a solution of each sample (1–5 mg) in 1 mL of dry MeOH was added 28% NaOMe (50 μL), and the solution was stirred at room temperature for 6 h. The reaction mixture was poured into ice water (1 mL) and extracted with EtOAc (1 mL×3). The ethyl acetate extract was washed with brine, dried over anhydrous $MgSO_4$, and concentrated. The product was purified by ODS HPLC with MeOH-MeCN-H_2O (7.8:1:1.2).

1a. LRFABMS, negative ion, *m/z* 1435 $[M-H]^-$, 1391 $[M-C_2H_4O]^-$, positive ion, *m/z* 1437 $[M+H]^+$, fragment ions are shown in Fig. 4.

2a. LRFABMS, negative ion, *m/z* 1421 $[M-H]^-$, 1377 $[M-C_2H_4O]^-$, positive ion, *m/z* 1445 $[M+Na]^+$, 1423 $[M+H]^+$, 1377, 1351, 1252, 1125, 1024, 1009, 953, 896, 840, 782, 712, 711, 637, 584, 528, 471, 413, 400, 342, 299, 172, 158.

7a. LRFABMS, positive ion, *m/z* 1437 $[M+H]^+$, fragment ions are shown in Fig. 4.

8a. LRFABMS, negative ion, *m/z* 1421 $[M-H]^-$, and 1377 $[M-C_2H_4O]^-$, positive ion, *m/z* 1445 $[M+Na]^+$, 1423 $[M+H]^+$, 1351, 1264, 1252, 1151, 1125, 1080, 1024, 995, 953, 882, 840, 782, 712, 711, 641, 584, 542, 471, 429, 400, 340, 299.

9a. LRFABMS *m/z* 1451 $[M+H]^+$, 1379, 1280, 1180, 1107, 1052, 1022, 967, 853, 796, 726, 725, 654, 598, 541, 484, 428, 344, 299, 172, 158.

Partial hydrolysis of theonellapeptolides

To a solution of each sample (1–5 mg) in 1 mL of dioxane was added 50 μL of 2 M KOH solution in water, and the

solution was stirred at room temperature for 3 h. The reaction mixture was poured into ice water and acidified with 1N HCl, and extracted with EtOAc (1 mL×2). The combined EtOAc extract was washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified by ODS PTLC with MeOH-H_2O (9:1).

1b. LRFABMS, positive ion, *m/z* 1423 $[M+H]^+$, fragment ions are shown in Fig. 4.

2b. LRFABMS, positive ion, *m/z* 1409 $[M+H]^+$, 1237, 1110, 1009, 1008, 937, 895, 825, 711, 698, 628, 584, 514, 471, 400, 399, 330, 299, 257, 172, 144, 73.

7b. LRFABMS, positive ion, *m/z* 1423 $[M+H]^+$, fragment ions are shown in Fig. 4.

8b. LRFABMS, negative ion, *m/z* 1408 $[M]^-$, 1389, 1153, 1110, 1079, 1061, 1009, 892, 792, 411 positive ion, *m/z* 1431 $[M+Na]^+$, 1409 $[M+H]^+$, 1391, 1166, 1079, 1061, 1009, 994, 976, 938, 882, 825, 785, 711, 697, 625, 584, 528, 467, 413, 400, 299, 328.

9b. LRFABMS, negative ion, *m/z* 1436 $[M]^-$, 1390, 1194, 1035, 908, 837, 795 positive ion, *m/z* 1437 $[M+H]^+$, 1196, 1107, 1037, 1023, 953, 910, 839, 797, 725, 711, 640, 526, 413, 400, 299, 328, 257, 172, 73.

Methylation of 1, 2, 7, and 9.[15] To a solution of each sample (ca. 2 mg) in 300 μL of dry DMF was added 36 mg (155.2 μM) of Ag_2O and 25 μL (405 μM) of MeI, and the mixture was heated at 50°C (oil bath) for 4 h. The reaction mixture was diluted with DMF (500 μL) and filtered off. To the filtrate CH_2Cl_2 (2.5 mL) was added, whereupon a white precipitate appeared, which was removed by washing with KCN solution (5%, 2 mL). Then the CH_2Cl_2 layer was washed with water several times, dried over Na_2SO_4 and concentrated under N_2 stream. The solid product (**10**) was purified by ODS HPLC (MeOH–MeCN–H_2O, 8:1:1 with 0.01% TFA).

10. LRFABMS, positive ion, *m/z* 1517 $[M+H]^+$. The same amino acid residues, Me-β-Ala (×3), Me-Ala, Me-Ile, *allo*-Me-Ile (×2), Me-Leu (×3), Me-Thr, Me-Val (×2), for **10** were identified by ^1H NMR (D_2O) analysis of acid hydrolysate of each sample of **10** prepared from **1**, **2**, **7**, and **9**. The absolute configuration of each amino acid was confirmed by Marfey analysis as L-Me-Ala, L-Me-Ile, D-*allo*-Me-Ile (×2), and L-Me-Val (×2).

Mixed lymphocyte reaction (MLR) assay

The assay was carried out by Dr Y. Koezuka and his coworkers, Kirin Brewery Co. Ltd.

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