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Two Theonellapeptolide Congeners from Marine Sponge *Theonella* sp.

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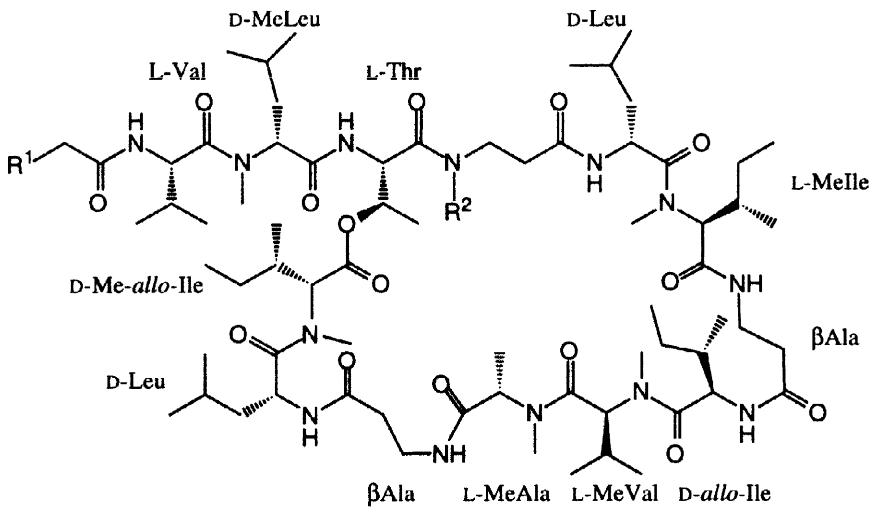
Abstract; Two new theonellapeptolide-related cyclic depsipeptides (**1** and **2**) have been isolated from an Okinawan marine sponge *Theonella* sp. and the structures were elucidated on the basis of the 2D NMR data, PSD analysis of MALDI-TOFMS, and chemical means. Compound **1** was a theonellapeptolide congener possessing a methylsulfinylacetyl group at the *N*-terminus, while **2** was another theonellapeptolide congener having an acetyl group at the *N*-terminus. Compounds **1** and **2** exhibited antimicrobial activity.

Marine sponges of the genus *Theonella* have been shown to be a rich source of unique cyclic peptides and depsipeptides with interesting biological activities.[1] In our continuing search for unique secondary metabolites from marine organisms, we have isolated a series of cyclic peptides, keramamides A ~ H and J ~ L from Okinawan marine sponges of genus *Theonella*.[2~7] Investigation of another *Theonella* sponge resulted in the isolation of two new theonellapeptolide[8~12] congeners (**1** and **2**). Here we describe the isolation and structure elucidation of **1** and **2**.



1 : $R^1 = SOCH_3, R^2 = H$ **5 :** $R^1 = OCH_3, R^2 = H$

2 : $R^1 = H, R^2 = CH_3$ **6 :** $R^1 = OCH_3, R^2 = CH_3$

3 : $R^1 = SCH_3, R^2 = H$

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The MeOH extract of the sponge *Theonella* sp. (SS-103) collected off Kerama Islands, Okinawa, was partitioned between EtOAc and water. The EtOAc-soluble materials were subjected to a silica gel ($CHCl_3$/MeOH, 7:3 ~ 1:1) and a Sephadex LH-20 (MeOH) columns, and HPLC on ODS (MeOH/H_2O/CF_3CO_2H, 93:7:0.1) to afford compounds **1** (0.0085 %, wet weight) and **2** (0.002 %) together with known-related peptides, theonellapeptolides Id[7,9] (**5**, 0.02 %) and Ie[8] (**6**, 0.004 %).

Compound **1** {$[\alpha]_D^{25}$ -45° (*c* 1.0, MeOH)} was obtained as a colorless amorphous solid, and showed the pseudomolecular ion peak at *m/z* 1459 $(M+Na)^+$ in the ESIMS, and the molecular formula, $C_{70}H_{125}N_{13}O_{16}S$, was established by the HRFABMS [*m/z* 1436.9180 $(M+H)^+$, Δ +1.3 mmu]. The IR spectrum was indicative of the presence of amide (ν_{max} 3320 and 1635 cm^{-1}) and ester carbonyl (ν_{max} 1735 cm^{-1}) groups. The ^1H NMR (Table 1) spectrum suggested **1** to be a peptide. Amino acid analysis of the hydrolysate of **1** revealed 1 mol each of threonine (Thr), valine (Val), and *allo*-isoleucine (*allo*-Ile), 2 mol of leucine (Leu), and three mol of β-alanine (βAla). Extensive analyses of ^1H and ^{13}C NMR data (Table 1) in CD_3OH including ^1H-^1H COSY, TOCSY, ^1H-^{13}C HSQC, ^1H-^{13}C HMBC, ^1H-^{15}N HSQC, and ^1H-^{15}N HMBC disclosed the presence of five *N*-methyl amino acid residues, 1 mol each of *N*-methylvaline (MeVal), *N*-methylalanine (MeAla), and *N*-methylleucine (MeLeu), and 2 mol of *N*-methylisoleucine (MeIle) in addition to eight amino acid residues as described above. Five *N*-methyl protons were assigned by the ^1H-^{15}N HMBC correlations to the amide nitrogen atoms.

The amino acid sequence and the *N*-terminus of **1** were elucidated on the basis of HMBC data as well as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) data of the methanolysis product (**4**) of **1**. The amino acid seqence, Val–MeLeu–Thr–βAla^1–Leu^1–(MeIle)–βAla^2–*allo*-Ile–MeVal–MeAla–βAla^3–Leu^2–(MeIle), was suggested by HMBC correlations as shown in Figure 1. The 6th MeIle from the *N*-terminus and the MeIle at the *C*-terminus were revealed to be L-MeIle and D-Me-*allo*-Ile, respectively, by chiral HPLC analyses as described below. In the HMBC spectrum of **1**, the singlet methyl proton at δ_H 2.79 showed the correlation to the methylene carbon (δ_C 58.9), while the methylene protons (δ_H 3.86 and 3.67) showed cross-peaks to the amide carbonyl (δ_C 166.8). The chemical

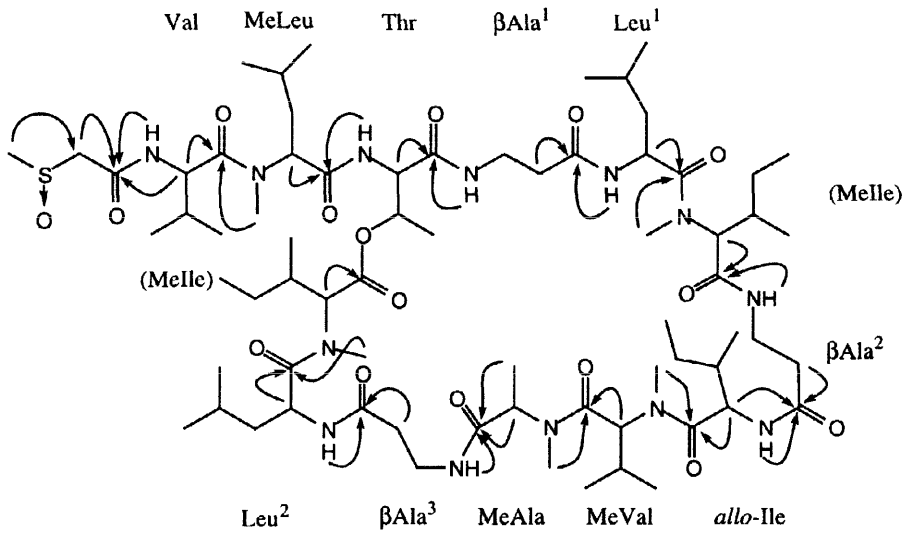

Figure 1. Selected HMBC Correlations of Compound **1**.

Table 1. ^{1}H, ^{13}C, and ^{15}N NMR Data of Compound **1** in CD_3OH.

positn.		δ_H	δ_C	δ_N [a]	positn.		δ_H	δ_C	δ_N [a]
Methylsulfinylacetyl					βAla[2]				
CH_3	2.79 s		39.2 q		NH	7.34			120.8
CH_2	3.86	3.67	58.9 t		βCH_2	4.26	3.12	36.5 t	
CO			166.8 s		αCH_2	2.60	2.28	36.5 t	
L-Val					CO			173.6 s	
NH	8.38			126.8	D-*allo*-Ile				
α-CH	4.63		57.7 d		NH	8.63			116.3
β-CH	2.12		32.7 d		α-CH	5.34		54.7 d	
CH_3	1.04		19.3 q		β-CH	1.81		39.2 d	
CH_3	0.98		20.3 q		γ-CH_2	1.20	1.45	28.7 t	
CO			176.1 s		γ-CH_3	0.76		15.6 q	
D-MeLeu					δ-CH_3	1.00		13.0 q	
NCH_3	3.31 s		33.2 q	114.7	CO			176.6 s	
α-CH	5.24		57.5 d		L-MeVal				
β-CH_2	2.03	1.41	39.6 t		NCH_3	3.32 s		32.1 q	114.2
γ-CH	1.52		27.2 d		α-CH	5.05		59.5 d	
CH_3	0.95		24.5 q		β-CH	2.39		30.0 d	
CH_3	0.83		22.1 q		CH_3	0.93		20.7 q	
CO			175.3 s		CH_3	0.87		20.2 q	
L-Thr					CO			173.2 s	
NH	8.74			118.5	L-MeAla				
α-CH	4.38		58.6 d		NCH_3	2.76 s		30.3 q	120.9
β-CH	5.19		70.5 d		α-CH	5.20		58.7 d	
CH_3	1.11		19.1 q		CH_3	1.48 d		15.9 q	
CO			171.2 s		CO			172.6 s	
βAla[1]					βAla[3]				
NH	7.34			116.1	NH	7.73			114.0
β-CH_2	3.85	3.12	37.9 t		β-CH_2	3.69	3.26 br	38.5 t	
α-CH_2	2.39	2.28	39.2 t		α-CH_2	2.29	2.21	39.2 t	
CO			175.0 s		CO			174.4 s	
D-Leu[1]					D-Leu[2]				
NH	8.58			126.8	NH	8.53			125.9
α-CH	5.06		50.2 d		α-CH	5.05		50.2 d	
β-CH_2	1.71	1.30	42.0 t		β-CH_2	1.79	1.40	41.2 t	
γ-CH	1.80		26.5 d		γ-CH	1.81		26.4 d	
CH_3	1.04		21.7 q		CH_3	0.87		21.8 q	
CH_3	1.03		24.8 q		CH_3	0.91		24.4 q	
CO			176.0 s		CO			176.6 s	
L-MeIle					D-Me-*allo*-Ile				
NCH_3	3.34 s		40.1 q	115.8	NCH_3	3.24 s		32.0 q	113.5
α-CH	3.36		70.8 d		α-CH	5.06		62.6 d	
β-CH	2.48		36.2 d		β-CH	2.11		34.0 d	
γ-CH_2	1.93	1.04	26.5 t		γ-CH_2	1.38	1.08	26.5 t	
γ-CH_3	0.81		15.9 q		γ-CH_3	0.98		17.0 q	
δ-CH_3	0.99		13.1 q		δ-CH_3	0.88		11.0 q	
CO			173.3 s		CO			172.3 s	

[a]These chemical shifts were based on the HSQC and HMBC correlations.

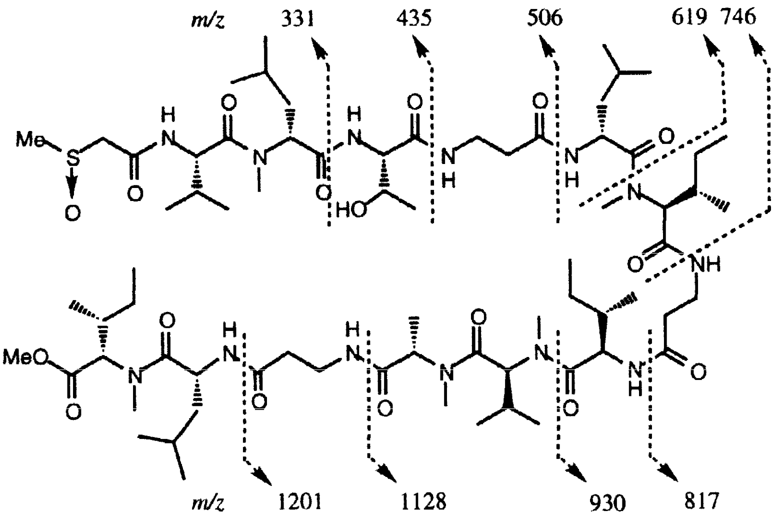

Figure 2. PSD Analysis of Methanolysis Product (**4**) of Compound **1** Using MALDI-TOFMS [precursor ion, *m/z* 1490.80 $(M+Na)^+$].

shifts of the methylene and methyl (δ_C 39.2) carbons indicated that these carbons were attached to a sulfoxide group.[13] Treatment of **1** with thioglycolic acid afforded a reductive product (**3**), of which the molecular formula, $C_{70}H_{125}N_{13}O_{15}S$, corresponded to the deoxy form of **1**. The methyl and methylene signals adjacent to the sulfur atom of **3** were shifted to higher field (CH_3, δ_H 2.20, δ_C 16.4; CH_2, δ_H 3.73 and 3.84, δ_C 37.2), suggesting that **3** possessed a methysulfanylacetyl (CH_3SCH_2CO) group at the *N*-terminus. The HMBC correlation from the NH proton (δ_H 8.38) of Val to the amide carbonyl suggested that the *N*-terminus of Val was connected to the methylsulfinylacetyl [$CH_3S(O)CH_2CO$] group. The relatively low-field β-proton (δ_H 5.19) of Thr was indicative of connectivity between Thr and the *C*-terminal MeIle (D-Me-*allo*-Ile) through an ester linkage. Post-source decay (PSD) analysis[14,15] using MALDI-TOFMS was carried out to provide further evidence of the amino acid sequence of **1**. The PSD spectrum of the methanolysis product (**4**) [precursor ion, *m/z* 1490.80 $(M+Na)^+$] of **1** showed fragment ions corroborating the amino acid sequence as shown in Figure 2. Thus the amino acid sequence of **1** was assigned.

Chiral HPLC analyses (SUMICHIRAL OA-5000) of the acid hydrolysate of **1** were carried out to determine the absolute configuration of each amino acid residue. As a result, Val, MeLeu, Thr, *allo*-Ile, MeVal, and MeAla were found to be L-, D-, L-, D-, L-, and L-forms, respectively, and two Leu residues were both D-configurations. On the other hand, two MeIle residues were detected as L-MeIle and D-Me-*allo*-Ile. In order to confirm the position of D-Me-*allo*-Ile, compound **1** was treated with lithium borohydride, and then hydrolysis followed by chiral HPLC analyses of the product to result in no detection of D-Me-*allo*-Ile, suggesting the presence of the D-Me-*allo*-Ile residue at the *C*-teminus of **1**. Therefore compound **1** was concluded to be theonellapeptolide Id[7,9] (**5**) congener possessing a methylsulfinylacetyl group at the *N*-terminus.

HRFABMS data [*m/z* 1388.9490 $(M+H)^+$, Δ -0.6 mmu] of **2** {$[\alpha]_D^{24}$ -56° (*c* 1.0, MeOH)} indicated the molecular formula, $C_{70}H_{125}N_{13}O_{15}$. Amino acid analysis of the hydrolysate of **2** showed the existence of 1 mol each of Thr, Val, and *allo*-Ile, and 2 mol each of Leu and βAla. The exsistence of six *N*-methyl amino acid residues consisting of 1 mol each of MeVal, MeAla, MeLeu,and *N*-methyl-β-alanine (MeβAla),

Table 2. ^{1}H and ^{13}C NMR Data of Compound **2** in CD_3OH.

positn.	δ_H		δ_C	positn.	δ_H		δ_C
Acetyl				βAla[1]			
CH$_3$	2.02 s		22.9 q	NH	7.47 d		
CO			173.7 s	βCH$_2$	4.32	3.12	36.7 t
L-Val				αCH$_2$	2.63	2.21	36.6 t
NH	8.29 d			CO			173.5 s
α-CH	4.65		58.1 d	D-*allo*-Ile			
β-CH	2.08		32.2 d	NH	8.72 d		
CH$_3$	0.97		20.3 q	α-CH	5.36		54.9 d
CH$_3$	1.04		19.7 q	β-CH	1.85		39.3 d
CO			176.6 s	γ-CH$_2$	1.45	1.20	28.8 t
D-MeLeu				γ-CH$_3$	0.76 d		15.5 q
NCH$_3$	3.39 s		32.9 q	δ-CH$_3$	1.01		13.2 q
α-CH	5.12		57.1 d	CO			176.7 s
β-CH$_2$	2.12	1.31	39.6 t	L-MeVal			
γ-CH	1.51		27.0 d	NCH$_3$	3.31 s		32.5 q
CH$_3$	0.98		24.7 q	α-CH	5.03		59.7 d
CH$_3$	0.81 d		22.4 q	β-CH	2.40		30.3 d
CO			174.2 s	CH$_3$	0.92		20.9 q
L-Thr				CH$_3$	0.89		20.5 q
NH	9.47 d			CO			173.0 s
α-CH	4.72 t		54.8 d	L-MeAla			
β-CH	5.28		71.7 d	NCH$_3$	2.76 s		30.8 q
CH$_3$	1.06		21.8 q	α-CH	5.20		58.7 d
CO			170.7 s	CH$_3$	1.48 d		16.1 q
MeβAla				CO			172.3 s
NCH$_3$	2.79 s		36.2 q	βAla[2]			
β-CH$_2$	4.64	2.78	46.5 t	NH	7.74 brd		
α-CH$_2$	2.49	2.22	35.9 t	β-CH$_2$	3.72	3.24	38.5 t
CO			174.7 s	α-CH$_2$	2.29	2.20	39.5 t
D-Leu[1]				CO			174.5 s
NH	8.59 d			D-Leu[2]			
α-CH	5.06		50.4 d	NH	8.51 d		
β-CH$_2$	1.77	1.39	41.5 t	α-CH	5.03		50.3 d
γ-CH	1.83		26.6 d	β-CH$_2$	1.74	1.25	40.3 t
CH$_3$	1.04		25.0 q	γ-CH	1.86		26.6 d
CH$_3$	1.05		19.4 q	CH$_3$	0.92		24.5 q
CO			176.2 s	CH$_3$	0.94		21.9
L-MeIle				CO			176.9 s
NCH$_3$	3.35 s		40.6 q	D-Me-*allo*-Ile			
α-CH	3.17 d		71.4 d	NCH$_3$	3.23 s		32.1 q
β-CH	2.51		36.5 d	α-CH	5.09		62.3 d
γ-CH$_2$	1.96	1.00	30.8 t	β-CH	2.15		32.9 d
γ-CH$_3$	0.84 d		15.7 q	γ-CH$_2$	1.39	1.09	26.6 t
δ-CH$_3$	0.99		13.1 q	γ-CH$_3$	0.97		17.1 q
CO			172.7 s	δ-CH$_3$	0.87 t		11.0 q
				CO			172.4 s

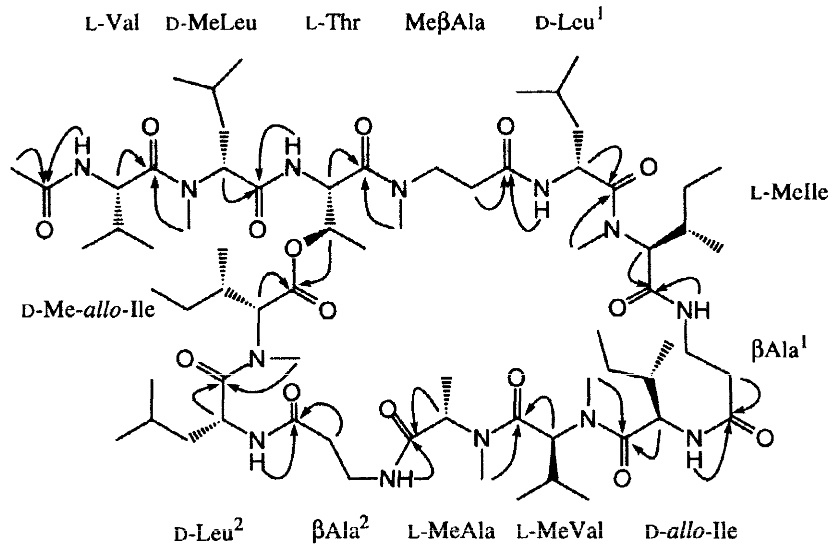

Figure 3. Selected HMBC Correlations of Compound **2**.

and two mol of MeIle (MeIle and Me-*allo*-Ile), were elucidated on the basis of extensive analyses of ^{1}H and ^{13}C NMR data including 2D NMR data. The singlet methyl resonance (δ_H 2.02) in the ^{1}H NMR spectrum was suggestive of the presence of an acetyl group. The HMBC correlations (Figure 3) disclosed the amino acid sequence of Val–MeLeu–Thr–MeβAla–Leu¹–(MeIle)–βAla¹–*allo*-Ile–MeVal–MeAla–βAla²–Leu²–(Me-*allo*-Ile). The ester linkage between the hydroxyl group of Thr and the carbonyl group of Me-*allo*-Ile were deduced from the HMBC correlation from βH–Thr to CO–Me-*allo*-Ile. The HMBC spectrum showed the correlations from the acetyl methyl protons and the amide proton of Val (δ_H 8.29) to the amide carbonyl carbon (δ_C 173.7), indicating that the acetyl group was attached to the amino group of Val residue in the *N*-terminus. The absolute configulation of each amino acid residue was determined by chiral HPLC analyses under the same condition as described above. Compound **2** was treated with lithium borohydride, and then hydrolysis followed by chiral HPLC analyses of the product to result in no detection of D-Me-*allo*-Ile, suggesting the presence of the D-Me-*allo*-Ile residue at the *C*-teminus of **2**. Thus compound **2** was determined to be the theonellapeptolide Ie⁸ (**6**) congener having an acetyl group at the *N*-terminus.

Compounds **1** and **2** are new congeners of theonellapeptolides, and possess a methylsulfinylacetyl and an acetyl group, respectively, at each *N*-terminus, although all the *N*-termini of known theonelapeptolides are a methoxyacetyl group.[7-12] As compound **1**,[16] natural products having a sulfur-containing acyl group are very rare.[17] Compounds **1** and **2** showed antimicrobial activity against some Gram-positive bacteria such as *Staphylococcus aureus* (MIC 8.0, and >16 µg/mL, respectively), *Micrococcus luteus* (MIC 8.0, and 8.0 µg/mL, respectively), *Bacillus subtilis* (MIC 8.0 and 16 µg/mL, respectively), and *Mycobacterium smegmatis* (MIC 16 and 66 µg/mL, respectively) and against fungi such as *Trichophyton mentagrophytes* (MIC 4.0, and 8.0 µg/mL, respectively) and *Aspergillus niger* (MIC >66 and 8.0 µg/mL, respectively) (Table 3). On the other hand, compound **3** exhibited weaker inhibitory activities than those of **1**, indicating that the sulfoxide group in **1** was important for the activity. Compounds **1** and **2** were cytotoxic against murine leukemia L1210 cells in *vitro* (IC_{50} values: 9.0 and 7.5 µg/mL, respectively).

Table 3. Antimicrobial Activities of Compounds **1**, **2**, and **3**

	MIC (μg/mL)									
Compd.	*C. alb.*	*T.m.*	*P.var.*	*A.nig.*	*C.neo.*	*S.aur.*	*M.lut.*	*B.sub.*	*E.col.*	*M.sm.*
1	>66	4.0	>66	>66	>66	8.0	8.0	8.0	>66	16
2	>66	8.0	>66	8.0	>66	>16	8.0	16	>66	66
3	>66	>66	>66	>66	>66	>66	33.0	66	>66	>66

fungi: *Candida albicans*, *Trichophyton memtagrophytes*, *Paecilomyces variotii*, *Aspergillus niger*, *Cryptococcus neoformans*; bacteria: *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli*, *Mycobacterium smegmatis*

Experimental

NMR Experiments. ^{1}H and 2D NMR spectra were recorded on a 600 MHz spectrometer, while ^{13}C NMR spectra were measured on a 500 MHz spectrometer. ^{1}H-^{1}H COSY, TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC spectra in CD_3OH were measured using standard pulse sequence with *Z*-axis PFG. For HSQC and HMBC, a total of 256 increments of 1*K* data points were collected. For ^{1}H-^{13}C HMBC, 50 ms delay time was used for long range C–H coupling. ^{1}H-^{15}N HSQC and ^{1}H-^{15}N HMBC experiments were measured using 70 mM solution in CD_3OH and CD_3OD, respectively. 95 % formamide in $CDCl_3$ was used for external reference (δ_N 112.4) of ^{15}N NMR. The spectral width in F_1 of ^{1}H-^{15}N HSQC and HMBC was 100 ~ 150 ppm. For ^{1}H-^{15}N HSQC, 5.55 ms delay time was used for one-bond N–H coupling, while ^{1}H-^{15}N HMBC was measured using 60 ms delay for long range N–H coupling.

PSD Experiments. PSD mass spectrum was recorded on a single-stage reflectron MALDI-TOF mass spectrometer (Voyager Pro, PerSeptive Biosystems, Inc.). α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used as the matrix. The precursor ions were selected with a time ion selector wit mass window of approximately 15 mass units. The accelerating voltage of nitrogen laser beam was set 17 kV. 128 shots were averaged for each mass range acquired before generating the PSD spectrum under the control of the Voyager and Grams software. External calibration was performed with angiotensin I with monoisotopic *m/z* mass for $(M+H)^+$ of 1296.685 Da.

Extraction and Isolation. The sponge *Theonella* sp. (SS-103) was collected off Kerama Islands, Okinawa, and kept frozen until used. The sponge (1.2 kg, wet weight) was extracted with methanol (1.5 L and 1 L). The methanolic extract (75.1 g) was partitioned between 1 M NaCl aq. (300 mL) and ethyl acetate (300 mL x 3). The EtOAc soluble material (13.24 g) was subjected to a silica gel ($CHCl_3$/MeOH, 70:30 → 50:50) and a Sephadex LH-20 columns (MeOH) followed by C_{18} HPLC [Mightysil RP-18, Kanto Chemical Co., Inc., 4.6 x 250 mm; eluent, MeOH/H_2O/CF_3CO_2H (93:7:0.1); flow rate, 2.5 mL/min; UV detection at 220 nm] to afford compounds **1** (0.0085 %, wet weight, t_R 12 min) and **2** (0.002 %, t_R 16 min)

Compound 1. Colorless amorphous solid; $[\alpha]_D^{25}$ -45° (*c* 1.0, MeOH); IR (film) ν_{max} 3320, 1730, and 1630 cm^{-1}; ^1H and ^{13}C NMR (Table 1); ESIMS *m/z* 1459 $(M+Na)^+$; FABMS *m/z* 1437 $(M+H)^+$; HRFABMS *m/z* 1436.9180 $(M+H)^+$, calcd for $C_{70}H_{126}N_{13}O_{16}S$, 1436.9167.

Compound 2. Colorless amorphous solid; $[\alpha]_D^{24}$ -56° (*c* 1.0, MeOH); IR (film) ν_{max} 3325, 1735, and 1635 cm^{-1}; ^1H and ^{13}C NMR (Table 2); ESIMS *m/z* 1411 $(M+Na)^+$; FABMS *m/z* 1389 $(M+H)^+$; HRFABMS *m/z* 1388.9490 $(M+H)^+$, calcd for $C_{70}H_{126}N_{13}O_{15}$, 1388.9496.

Reduction of 1 with Thioglycolic Acid. To a solution of compound **1** (1.0 mg) in H_2O (90 μL) was added thioglycolic acid (60 μL), and the mixture was stirred at 50 °C for 24 h. The reaction mixture was passed through Sep-Pak® C_{18} cartridge ($H_2O \rightarrow$ MeOH). The fraction eluted with MeOH was evapolated *in vacuo* to give compound **3** (1.0 mg) as a colorless amorphous solid; $[\alpha]_D^{22}$ -54° (*c* 0.75, MeOH); IR (film) ν_{max} 3430, 1735, and 1635 cm^{-1}; ^1H NMR (CD_3OD) δ [methylsulfanylacetyl] 2.20 (3H, s, CH_3), 3.73 (1H, m, CH_2), 3.84 (1H, m, CH_2), [L-Val] 8.19 (1H, d, J = 8.5 Hz, NH), 4.83 (1H, m, αH), 2.15 (1H, m, βH), 0.97 (3H, m, CH_3), 1.05 (3H, m, CH_3), [D-MeLeu] 3.31 (3H, s, NCH_3), 5.26 (1H, m, αH), 1.40 (1H, m, βH), 2.07 (1H, m, βH), 1.53 (1H, m, γH), 0.89 (3H, m, CH_3), 1.01 (3H, m, CH_3), [L-Thr] 8.71 (1H, d, J = 10.0 Hz, NH), 4.38 (1H, m, αH), 5.21 (1H, m, βH), 1.12 (3H, m, CH_3), [βAla¹] 7.34 (1H, m, NH), 2.23 (1H, m, αH), 2.28 (1H, m, αH), 3.11 (1H, m, βH), 3.84 (1H, m, βH), [D-Leu¹] 8.56 (1H, d, J = 7.5 Hz, NH), 5.05 (1H, m, αH), 1.30 (1H, m, βH), 1.74 (1H, m, βH), 1.80 (1H, m, γH), 1.05 (3H, m, CH_3), 1.07 (3H, m, CH_3), [L-MeIle] 3.35 (3H, s, NCH_3), 3.36 (1H, m, αH), 1.82 (1H, m, βH), 1.04 (1H, m, γH), 1.87 (1H, m, γH), 0.86 (3H, d, J = 6.7 Hz, γCH_3), 1.01 (3H, m, δCH_3), [βAla²] 7.35 (1H, m, NH), 2.30 (1H, m, αH), 2.61 1H, m, αH), 3.09 (1H, m, βH), 4.28 (1H, m, βH), [D-*allo*-Ile] 8.62 (1H, d, J = 9.7 Hz, NH), 5.36 (1H, m, αH), 2.50 (1H, m, βH), 1.23 (1H, m, γH), 1.46 (1H, m, γH), 0.79 (3H, d, J = 6.7 Hz, γCH_3), 0.97 (3H, m, δCH_3), [L-MeVal] 3.27 (3H, s, NCH_3), 5.07 (1H, m, αH), 2.41 (1H, m, βH), 0.91 (3H, m, CH_3), 0.92 (3H, m, CH_3), [L-MeAla] 2.77 (3H, s, NCH_3), 5.20 (1H, m, αH), 1.49 (3H, d, J = 5.8 Hz, CH_3), [βAla³] 7.73 (1H, dd, J = 3.6 and 8.0 Hz, NH), 2.23 (1H, m, αH), 2.28 (1H, m, αH), 3.28 (1H, m, βH), 3.75 (1H, m, βH), [D-Leu²] 8.55 (1H, d, J = 8.6 Hz, NH), 5.08 (1H, m, αH), 1.40 (1H, m, βH), 1.83 (1H, m, βH), 1.82 (1H, m, γH), 0.89 (3H, d J = 6.5 Hz, CH_3), 0.95 (3H, m, CH_3), [D-Me-*allo*-Ile] 3.24 (3H, s, NCH_3), 5.09 (1H, m, αH), 2.14 (1H, m, βH), 1.06 (1H, m, γH), 1.41 (1H, m, γH), 1.01 (3H, m, γCH_3), and 0.91 (3H, m, δCH_3); ^{13}C NMR (CD_3OD) δ [methylsulfanyl] 16.4 (q, CH_3), 37.2 (t, CH_2), 170.5 (s, CO), [L-Val] 59.6 (d, αC), 31.4 (d, βC), 20.1 (q, CH_3), 18.4 (q, CH_3), 175.5 (s, CO), [D-MeLeu] 33.4 (q, NCH_3), 56.6 (d, αC), 38.0 (t, βC), 26.5 (d, γC), 21.5 (q, CH_3), 23.8 (q, CH_3), 175.4 (s, CO), [L-Thr] 57.9 (d, αC), 69.9 (d, βC), 18.3 (q, CH_3), 171.5 (s, CO), [βAla¹] 37.9 (t, αC), 37.7 (t, βC), 174.7 (s, CO), [D-Leu¹] 49.6 (d, αC), 40.6 (t, βC), 26.1 (d, γC), 24.2 (q, CH_3), 21.0 (q, CH_3), 175.5 (q, CO), [L-MeIle] 39.1 (q, NCH_3), 71.0 (d, αC), 39.1 (d, βC), 26.1 (t, γC), 14.7 (q, γCH_3), 12.3 (q, δCH_3), 172.9 (s, CO), [βAla²] 35.7 (t, αC), 35.7 (t, βC), 173.7 (s, CO), [D-*allo*-Ile] 54.0 (d, αC), 35.7 (d, βC), 28.0 (t, γC), 14.6 (q, γCH_3), 12.3 (q, γCH_3), 176.0 (s, CO), [L-MeVal] 32.2 (q, NCH_3), 58.9 (d, αC), 30.0 (d, βC), 19.6 (q, CH_3), 19.9 (q, CH_3), 172.7 (s, CO), [L-MeAla] 29.4 (q, NCH_3), 58.1 (d, αC), 14.9 (q, CH_3), 172.6 (s, CO), [βAla³] 37.7 (t, αC), 37.2 (t, βC), 174.1 (s, CO), [D-Leu²] 49.6 (d, αC), 39.1 (t, βC), 25.8 (d, γC), 21.1 (q, CH_3), 23.7 (q, CH_3), 176.0 (s, CO), [D-Me-*allo*-Ile] 31.4 (q, NCH_3), 62.0 (d, αC), 33.4 (d, βC), 26.1 (t, γC), 15.3 (q, γCH_3), 10.5 (q, γCH_3), and 171.8 (s,

CO); FABMS m/z 1421 $(M+H)^+$; HRFABMS m/z 1420.9920 $(M+H)^+$, calcd for $C_{70}H_{126}N_{13}O_{15}S$, 1420.9916.

Methanolysis of Compound 1. Compound **1** (5.0 mg) was treated with 6 % NaOMe in MeOH (250 μL) at room temperature for 3 h. The reaction mixture was neutralized with 0.5 N HCl aq. followed by extraction with EtOAc (1 mL x 3). The organic phase was washed with H_2O and evaporated *in vacuo* to afford compound **4** (5.0 mg) as a colorless amorphous solid; ESIMS m/z 1491 $(M+Na)^+$.

Amino Acid Analysis by Chiral HPLC. Compound **1** or **2** (each 100 μg) was dissolved in 6N HCl (100 μL) and heated in a sealed tube at 110 °C for 24 h. Chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 column [Sumitomo Chemical Industry; 4 x 150 mm; 40 °C, detection at 254 nm]. Retention times (min) of authentic amino acids were as follows: L-MeAla (55.0) and D-MeAla (57.8) [eluent: H_2O containing 0.5 mM $CuSO_4$, flow rate 0.2 mL/min]; L-Thr (7.6) and D-Thr (8.8) [eluent: H_2O containing 0.5 mM $Cu(OAc)_2$, flow rate 1.0 mL/min]; βAla (3.8) and MeβAla (4.8) [eluent: H_2O containing 1 mM $CuSO_4$, flow rate 1 mL/min]; L-Val (10.4), D-Val (15.6), L-MeVal (13.6), and D-MeVal (22.8) [eluent: MeOH/H_2O (10:90) containing 1.0 mM $CuSO_4$, flow rate 1.0 mL/min]; L-Me-*allo*-Ile (11.6) and D-Me-*allo*-Ile (18.8) [eluent: MeOH/H_2O (15:85) containing 2.0 mM $CuSO_4$, flow rate 1.0 mL/min]; L-Leu (11.6), D-Leu (15.6), L-MeLeu (14.8), D-MeLeu (20.0), L-*allo*-Ile (12.4), D-*allo*-Ile (18.0), L-MeIle (9.1), and D-MeIle (14.0) [eluent: MeOH/H_2O (20:80) containing 2.0 mM $CuSO_4$, flow rate 1.0 mL/min]. Retention times of the hydrolysate of **1** were as follows: L-MeAla (55.0), L-Thr (7.6), βAla (3.8), L-Val (10.4), L-MeVal (13.6), D-Me-*allo*-Ile (18.8), D-Leu (15.6), D-MeLeu (20.0), D-*allo*-Ile (18.0), and L-MeIle (9.1). Retention times of the hydrolysate of **2** were as follows: L-MeAla (55.0), L-Thr (7.6), βAla (3.8), MeβAla (4.8), L-Val (10.4), L-MeVal (13.6), D-Me-*allo*-Ile (18.8), D-Leu (15.6), D-MeLeu (20.0), D-*allo*-Ile (18.0), and L-MeIle (9.1).

Determination of the Position of D-Me-*allo*-Ile. Compound **1** (0.5 mg) in ether (100 μL) was treated with $LiBH_4$ (2 mg) at room temperature for 2 h. The reaction mixture was partitioned between CH_2Cl_2 (2 mL x 3) and 0.5 N H_2SO_4 (1 mL), and the organic phase was washed with H_2O, dried over $MgSO_4$, and evaporated *in vacuo*. The reductive product was hydrolyzed with 12 N HCl at 120 °C for 24 h. Compound **2** (0.3 mg) was converted into the reductive product under the same procedure as described above. Each hydrolysate was subjected to chiral HPLC analyses under the same condition as described above. D-Me-*allo*-Ile was not detected for each hydrolysate.

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