Theonellamides A-E, Cytotoxic Bicyclic Peptides, from a Marine Sponge *Theonella* sp.[1]

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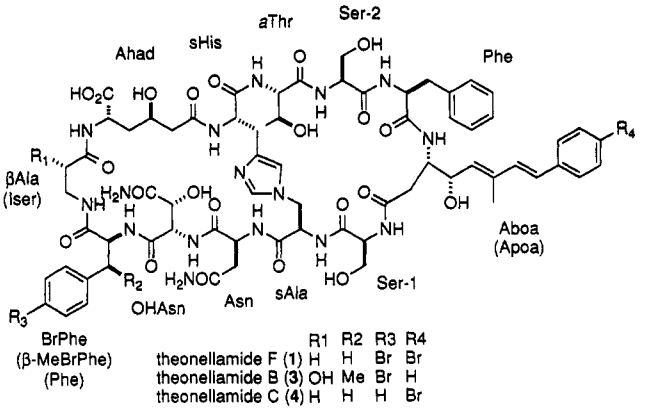
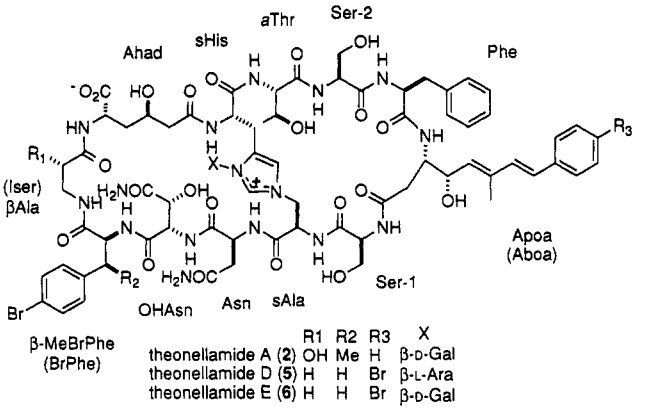
Five new cytotoxic peptides, related to theonellamide F (**1**), were isolated from a marine sponge *Theonella* sp. Theonellamide A (**2**) and B (**3**) differ from F (**1**) in three amino acid residues. Additionally, theonellamide A (**2**) bears a β-D-galactose linked to the free imidazole nitrogen. Theonellamide C (**4**) is debromo **1**. Theonellamide D (**5**) and E (**6**) are the β-L-arabinoside and β-D-galactoside of **1**. Structures **2-6** were assigned on the basis of spectral data and chromatographic analyses of degradation products.

Marine sponges of the order Lithistida which include the genera *Discodermia* and *Theonella* are prominent sources of bioactive secondary metabolites, especially peptides.[2] Similarity between metabolites of lithistid sponges and those isolated from the blue-green algae raised the question of the true producer of these metabolites.[3] In 1989, we reported isolation and structure of theonellamide F (**1**), a bicyclic peptide bridged by a histidinoalanine residue, as the major metabolite of a sponge, *Theonella* sp., collected off Hachijo-jima Island.[4] Further separation of the antifungal fraction of the sponge extract afforded five related peptides, theonellamides A-E. Their structure elucidation is the subject of this paper.

The *n*-BuOH-soluble portion of the aqueous *n*-PrOH extract was fractionated by chromatography on TSK G3000S yielding a crude antifungal fraction, which was subjected to silica gel chromatography to afford two active fractions. Reversed-phase HPLC of the less polar fraction yielded theonellamides B (**3**), C (**4**), and F (**1**), while the more polar fraction afforded theonellamides A (**2**), D (**5**), and E (**6**).[5] Compounds **2–6** were moderately cytotoxic against P388 murine leukemia cells with IC_{50} values of 5.0, 1.7, 2.5, 1.7, and 0.9 μg/mL, respectively.

Theonellamide A (**2**) was isolated as a white powder with a UV maximum at 288 nm, suggesting the presence of an ω-phenyl amino acid residue similar to (5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-(*p*-bromophenyl)-5,7-octadienoic acid (Aboa) found in **1**.[4] The isotope ion peaks in the FABMS indicated the presence of one bromine atom. The molecular formula of **2** was $C_{76}H_{99}BrN_{16}O_{28}$ on the basis of HRFABMS and NMR data.





Apoa = (5E, 7E)-3-amino-4-hydroxy-6-methyl-8-phenyl-5,7-octadienoic acid
Aboa = (5E,7E)-3-amino-4-hydroxy-6-methyl-8-(p-bromophenyl)-5,7-octadienoic acid
sAla = alanine portion of histidinoalanine
OHAsn = β-hydroxyasparagine
β-MeBrPhe = β-methyl-*p*-bromophenylalanine
BrPhe = *p*-bromophenylalanine
Iser = isoserine
Ahad =α-amino-γ-hydroxyadipic acid
sHis = histidine portion of histidinoalanine
*a*Thr = *allo*-threonine

Although theonellamide A (**2**) was soluble in D_2O/H_2O or in DMSO-d_6, the ^1H NMR signals in these solvents were broad. However, signals were sharper in mixtures of DMSO-d_6 and H_2O. Therefore, NMR spectra were measured in DMSO-d_6/H_2O (4:1, 2:1, or 1:1) at 308, 333, or 343 K.

Interpretation of the COSY, HOHAHA, NOESY, and HMQC spectra revealed the presence of one residue each

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(3) (a) Bewley, C. A.; Debitus, C.; Faulkner, D. J. *J. Am. Chem. Soc.***1994**, *116*, 7631–7636. (b) Faulkner, D. J.; He, H.; Unson, M. D.; Bewley, C. A. *Gazz. Chim. Ital.* **1993**, *123*, 301–307.

(4) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Wälchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582–2588.

(5) It was possible to prepare **1**, **2**, **5**, and **6** in high purity. Purification of **3** and **4** was extremely difficult because degradation products of **1** coeluted with **3** and **4** in ODS HPLC, which could not be separated even with recycling HPLC. Therefore, the purity of **3** and **4** for structure elucidation was approximately 90% and 85%, respectively. Theonellamides decomposed gradually as seen by new sets of disubstituted olefinic signals. We have not studied the structures of the degradation products further.

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of β-hydroxyasparagine (OHAsn), Asn, α-amino-γ-hydroxyadipic acid (Ahad), *a*Thr,[6] Phe, and histidinoalanine (Hisala),[7] and two residues of Ser, which were also found in theonellamide F (**1**). In addition to these units, one residue each of isoserine (Iser), β-methyl-*p*-bromophenylalanine (MeBrPhe), and (5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-phenyl-5,7-octadienoic acid (Apoa) were present (Table 1). The remaining six carbons, including an anomeric carbon, four oxymethines, and an oxymethylene, were reminiscent of a hexose unit. Although interpretation of ^{1}H NMR data for the sugar unit in a mixture of DMSO-d_6/H_2O (D_2O) was hampered by severe overlapping, this problem could be solved by measuring the NMR spectra in a mixture of DMSO-d_6/D_2O/pyridine-d_5 (2:2:1), which allowed assignment of a galactopyranose unit with an axial anomeric proton.[8]

The sequence of the amino acid residues and the position of glycosidation were determined by interpretation of the NOESY and HMBC spectra. In order to overcome degenerate amide and α-methine signals, the NMR spectra were measured at various temperatures in DMSO-d_6/H_2O mixtures of varied ratios. Thus, the amino acid sequences were deduced without exception.[9] HMBC cross peaks observed between H1 of the galactopyranose unit and both C2 and C4 of the imidazole ring indicated the attachment of galactose to the π-nitrogen on the imidazole ring. The side chain structures of the OHAsn and Asn residues were deduced from NOESY cross peaks between the amide protons and the pertinent β-proton(s). The α-carbonyl carbon of the Ahad residue must be a free carboxylic acid as observed in theonellamide F (**1**), which agreed with the chemical shift at 175.7 ppm, satisfying the molecular formula.

Amino acid analysis together with chiral GC analysis of the acid hydrolysate of theonellamide A (**2**) revealed the presence of *erythro*-β-hydroxyaspartic acid, L-Asp, (2*S*,4*R*)-Ahad, L-*a*Thr, two residues of L-Ser, and L-Phe. The stereochemistry of the galactose residue was also determined to be D by chiral GC analysis.[10] Stereochemical assignments of the OHAsp and Hisala residues were accomplished by application of Marfey's method.[11] Standard amino acid analysis indicated that OHAsp in **2** had either (2*S*,3*R*) or (2*R*,3*S*) stereochemistry. The retention time of the Marfey derivative of OHAsp in the acid hydrolysate of **2** coincided with that of the (2*S*,3*R*)-isomer but not with that of the (2*R*,3*S*)-isomer.[4] The stereochemistry of the Hisala residue in **2** was identical with

Table 1. ^{1}H and ^{13}C NMR Data for Theonellamide A (2)

		^{1}H[a]	^{13}C[b]
Ser-1	α	3.75 m[c]	56.4
	β	3.62 (2H,m)	60.8
	CONH	7.83 s	172.1
Apoa	α	2.11 (br d, 10), 2.37 (br t, 12.3)	37.1
	β	4.08 m	52.5
	γ	4.24 m	68.3
	δ	5.14 (d, 8.0)	132.5
	ϵ		135.8
	ϵ-Me	1.64 (3H, s)	13.1
	ζ	6.61 (d, 16.1)	133.6
	η	6.50 (d, 16.1)	128.1
	1		137.6
	2,6	7.41 (2H, d, 7.7)	126.6
	3,5	7.31 (2H, t, 7.7)	129.2
	4	7.20 (t, 7.7)	127.9
	CONH	7.77 (d, 9.5)	171.9
Phe	α	4.56 (q, 7.9)	54.4
	β	2.64 (dd, 6.4, 13.6), 2.81 (dd, 8.2, 13.5)	38.9
	1		137.0
	2,6	7.15 (2H, d, 7.3)	129.5
	3,5	7.23 (2H, t, 7.3)	128.7
	4	7.19 (t, 7.3)	127.0
	CONH	7.99 (d, 9.0)	171.0
Ser-2	α	4.44 m	56.2
	β	3.59 m, 3.64 m	61.6
	CONH	8.55 br	169.5
*a*Thr	α	4.23 m	58.5
	β	3.54 m	68.5
	γ	0.98 (3H, d, 5.8)	21.1
	OH	5.32 (d, 4.8)	
	CONH	7.67 (d, 8.0)	171.8
sHis	α	4.62 m	54.6
	β	2.98 (br d, 10.6), 3.27 m	25.9
	2	8.88 s	137.0
	4		131.7
	5	7.12 br s	123.7
	CONH	8.30 n.	170.7
Gal	1	5.02 (d, 8.8)	88.5
	2	3.61 m	69.7
	3	3.40 m	73.5
	4	3.81 s	69.5
	5	3.64 m	78.6
	6	3.65 m, 3.74 m	61.6
Ahad	α	3.88 m	52.5
	β	1.76 (2H, m)	38.9
	γ	3.65 m	65.4
	δ	1.89 (br d, 12.0), 2.17 (br t, 11.5)	44.2
	CO_2H		175.7
	CONH	7.55 (d, 7.1)	171.3
Iser	α	4.08 m	69.5
	β	2.88 m, 3.80 m	43.3
	CONH	7.35 m	172.0
β-MeBrPhe	α	4.43 (dd, 5.3, 8.1)	59.1
	β	3.23 m	39.7
	β-Me	1.07 (3H, d, 7.0)	17.3
	1		141.6
	2,6	7.01 (2H, d, 8.2)	130.5
	3,5	7.29 (2H, d, 8.2)	131.3
	4		120.0
	CONH	8.39 br	171.2
OHAsn	α	5.32 m	54.3
	β	3.88 m	72.1
	$CONH_2$	7.10 brs, 7.35 brs	174.2
	CONH	8.30 m	170.9
Asn	α	4.46 m	51.7
	β	2.27 (br d, 15), 2.56 (dd, 10.7, 15.5)	36.9
	$CONH_2$	6.84 br s, 7.38 br s	172.1
	CONH	7.75 br	170.4
sAla	α	5.07 br s	50.9
	β	4.20 m, 4.89 (br d, 13.5)	50.2
	CONH	8.30 m	169.3

[a] In DMSO-d_6/H_2O (4:1) at 308 K. [b] In DMSO-d_6/H_2O (4:1) at 333 K. [c] "m" indicates that the signal multiplicity is not determined due to overlapped signals.

(6) Differentiation of the *a*Thr residue and Thr residue was accomplished by chiral GC and Marfey analyses.

(7) H2 proton of the imidazole ring completely exchanged with deuterium after the peptide was dissolved in a mixture of DMSO-d_6 and D_2O for 1 day. The rate of exchange was faster in the glycosylated peptides. H2 was clearly observed in **3** and **4** after standing in DMSO-d_6/D_2O for weeks.

(8) ^{1}H NMR data of the galactose residue in **2** in DMSO-d_6/D_2O/pyridine-d_5 (2:2:1) at 333 K: δ 5.31 (d, J = 9.2 Hz; H1), 4.02 (d, 3.4; H4), 4.01 (dd, 6.4, 12.1; H6b), 3.92 (dd, 9.2, 9.7; H2), 3.87 (m; H5), 3.83 (m; H6a), 3.69 (dd, 3.4, 9.7; H3). A ROESY cross peak was observed between H1 and H5.

(9) Sequential NOESY and HMBC cross peaks across all amide bonds were observed.

(10) For the analysis of galactose and Ahad residues, the peptide was hydrolyzed with 6 N HCl at 110 °C for 3 h, which cleaved most amide bonds yielding peaks of identical intensities as those obtained under standard hydrolytic conditions (6 N HCl, 110 °C, 16 h) in chiral GC analysis (König, W. A.; Benecke, I.; Bretting, H., *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 693–694). When hydrolyzed for 16 h, galactose was no longer detected, while Ahad was racemized.[4]

(11) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 91–596.

that in **1** by Marfey analysis.[12] A positive Cotton effect[13] and a negative specific rotation value[14] of isoserine isolated from the acid hydrolysate indicated the 2*S*-stereochemistry. The CD spectrum of β-methyl-*p*-bromophenylalanine which showed a positive Cotton effect at 227 nm revealed 2*S*-stereochemistry.[13] β-Methyl-*p*-bromophenylalanine was hydrogenolyzed to β-methylphenylalanine, whose four stereoisomers are reported in the literature.[15] ^{1}H NMR coupling constant values of α- and β-protons allowed stereochemical assignment of C-3 with respect to C-2 and led to 2*S*, 3*S* stereochemistry. In order to determine the stereochemistry of the Apoa residue, theonellamide A was hydrogenated, followed by acid hydrolysis, and the resulting acid hydrolysate was analyzed by chiral GC, which resulted in the same stereochemistry for Aboa as in the case of theonellamide F.[4]

The molecular formula of theonellamide B (**3**) was smaller than **2** by $C_6H_{10}O_5$, suggesting absence of the galactose unit. Interpretation of the NMR data allowed the assignment of a likely gross structure (Tables 2 and 3). Theonellamide B was confirmed as degalactosyltheonellamide A (**1**) by chiral GC and Marfey analyses of the acid hydrolysate of **3** and (**2**) by chiral GC analysis of the acid hydrolysate of the hydrogenation product of **3**.

Theonellamide C (**4**) had the molecular formula $C_{69}H_{88}BrN_{16}O_{22}$. Amino acid analysis of the acid hydrolysate suggested that **4** was closely related to theonellamide F, the only difference being the presence of Phe instead of BrPhe in **1**. Interpretation of NMR data (Tables 2 and 3) suggested that **4** indeed was debromotheonellamide F, which was confirmed by stereochemical assignments of the amino acid residues as in case of **3**.

Theonellamides D (**5**) and E (**6**) could be separated by reversed-phase HPLC in a recycling mode (five to eight times). Amino acid analyses of the acid hydrolysates of **5** and **6** exhibited essentially identical chromatograms, which were also identical with that of theonellamide F. FABMS and NMR data (Tables 2 and 3) indicated that **5** and **6** were glycosylated derivatives of **1** at the π-nitrogen of the imidazole ring.[7] Coupling constant analysis[16] and ^{13}C NMR data[17] revealed the presence of arabopyranose and galactopyranose units in **5** and **6**, respectively. Both anomeric protons were axial. Absolute stereochemistry of the amino acid residues in **5** and **6** was assigned as in case of **3**, while the stereochemistry of the monosaccharide units was determined by chiral GC analyses of the acid hydrolysate.[18]

Recently, a closely related peptide, theonegramide, was reported from the Philippine lithistid sponge, *Theonella swinhoei*.[19] Cytotoxic macrodiolides, bistheonellides,[20] and antithrombin cyclic peptides, cyclotheonamides,[21] were also contained in our *Theonella* sponge. However, an Okinawan *Theonella* sponge that contained bistheonellides elaborated no theonellamides.[22] Variability in secondary metabolites may indicate the presence of different producing microorganisms in *Theonella* sponges.

Experimental Section[23]

Isolation of Theonellamides. The antifungal fraction eluted from a column of TSK G3000S was subjected to silica gel column chromatography as described previously.[4] The less polar active fraction (1.4 g) was fractionated by ODS-HPLC [*n*-PrOH/50 mM KH_2PO_4 in H_2O (32:68)] to yield **1** and a mixture of **3** and **4**. The mixture was further subjected to ODS-HPLC [MeCN/100 mM $NaClO_4$ in H_2O (38:62)] with recycling to afford **3** (19 mg) and **4** (32 mg). The more polar active fraction (2 g) was fractionated by ODS-HPLC [*n*-PrOH/50 mM KH_2PO_4 in H_2O (28:72)] to yield **2** (200 mg) and a mixture of **5** and **6**. The mixture was subjected to ODS-HPLC [MeCN/100 mM $NaClO_4$ in H_2O (38:62)] with recycling to yield **5** (14 mg) and **6** (30 mg).

Theonellamide A (2): white powder; $[\alpha]^{23}_D = +23°$ [*c* 0.1, *n*-PrOH/H_2O (2:1)]; UV [*n*-PrOH/H_2O (2:1)] 288 nm (ε 12 000); HRFABMS *m*/*z* 1763.6068 (M + H)$^+$, $C_{76}H_{100}{}^{79}BrN_{16}O_{28}$ (Δ −0.8 mmu); ^{1}H NMR see Table 1; ^{13}C NMR see Table 1.

Theonellamide B (3): white powder; $[\alpha]^{23}_D = +6.6°$ [*c* 0.1, *n*-PrOH/H_2O (2:1)]; UV [*n*-PrOH/H_2O (2:1)] 290 nm (ε 9500); HRFABMS *m*/*z* 1601.5604 (M + H)$^+$, $C_{70}H_{90}{}^{79}BrN_{16}O_{23}$ (Δ +5.6 mmu); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3.

Theonellamide C (4): white powder; $[\alpha]^{23}_D = 0.0°$ [*c* 0.1, *n*-PrOH/H_2O (2:1)]; UV [*n*-PrOH/H_2O (2:1)] 292 nm (ε 18 000); HRFABMS *m*/*z* 1571.5442 (M + H)$^+$, $C_{69}H_{88}{}^{79}BrN_{16}O_{22}$ (Δ +6.3 mmu); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3.

Theonellamide D (5): white powder; $[\alpha]^{23}_D = +16°$ [*c* 0.1, *n*-PrOH/H_2O (2:1)]; UV [*n*-PrOH/H_2O (2:1)] 290 nm (ε 2 0000); HRFABMS *m*/*z* 1781.4841 (M + H)$^+$, $C_{74}H_{95}{}^{79}Br_2N_{16}O_{26}$ (Δ −12.9 mmu); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3.

Theonellamide E (6): white powder; $[\alpha]^{23}_D = +20°$ [*c* 0.1, *n*-PrOH/H_2O (2:1)]; UV [*n*-PrOH/H_2O (2:1)] 292 nm (ε 27 000);

(12) We previously deduced the stereochemistry of Hisala residue in **1** as L for the histidine portion and D for the alanine portion (LD-isomer) by chemical methods. When the acid hydrolysate was prepared under standard conditions (6 N HCl, 110 °C, 16 h), we obtained a 1:1 mixture of the LD- and the LL-isomers.[4] Under milder hydrolysis condition (6 N HCl, 107 °C, 8 h), the ratio of LD-and LL-isomers was 3:1.[4] In the mild acid hydrolysate (6 N HCl, 110 °C, 3 h) of **2**, we detected the LD-and LL-isomers in a ratio of 5:1 by HPLC analysis of the Marfey derivative, suggesting the stereochemistry of the Hisala residue in **2** to be identical with that in **1**. However, there was still a possibility of an overlap of the DL- and DD-isomer peaks with the LL-isomer peak in the Marfey analysis. In order to exclude this possibility, the following experiment was carried out. The LD- and LL-isomers were derivatized with Marfey's reagent prepared from D-Ala (D-Marfey's reagent), which introduced opposite chiral centers in the molecule. The LD-isomer and the LL-isomer derivatized with D-Marfey's reagent are enantiomeric to, and therefore chromatographically equivalent to, the DL-isomer and the DD-isomer derivatized with the conventional Marfey's reagent. All four peaks were well separated, allowing us to determine unambiguously that the major Hisala residue liberated from **2** by mild acid hydrolysis was the LD-isomer.

(13) (a) Iizuka, E.; Yang, J. T. *Biochemistry* **1964**, *3*, 1519–1524. (b) Fowden, L.; Scope, P. M.; Thomas, P. N. *J. Chem. Soc. C* **1971**, 833–840.

(14) Andruszkiewicz, R.; Czerwinski, A.; Grybowska, J. *Synthesis* **1983**, 31.

(15) Kataoka, Y.; Seto, Y.; Yamamoto, M.; Yamada, T.; Kuwata, S.; Watanabe, H. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1081–1084. Due to the paucity of material, we were unable to determine the specific rotation. The magnitude of the coupling constant of our material ($J_{\alpha,\beta}$ = 7.6 Hz) agreed well with the reported value for the *erythro* isomer (7.5 Hz) rather than the *threo* isomer (4.8 Hz).

(16) ^{1}H NMR data in DMSO-d_6/D_2O/pyridine-d_5 (2:2:1) for the arabinose residue in **5** (343 K): δ 5.15 (d, *J* = 9.0 Hz; H1), 4.05 (dd, 1.0, 12.1; H5eq), 3.95 (br s; H4), 3.89 (t, 9; H2), 3.82 (m; H5ax), 3.64 (dd, 1, 9; H3). ^{1}H NMR data in DMSO-d_6/D_2O/pyridine-d_5 (2:2:1) for the galactose residue in **6** (343 K): δ 5.22 (d, *J* = 9.0 Hz; H1), 3.97 (d, 3.1; H4), 3.92(d, 6.3, 11.5; H6b), 3.85 (m; H2), 3.80 (m; H5), 3.77 (d, 11.5; H6a), 3.63 (dd, 3.1, 9.3; H3).

(17) Bock, K.; Pedersen, C. *Adv. Carbohyd. Chem. Biochem.* **1983**, *41*, 27–66.

(18) Arabinose in **5** was very labile under acidic conditions compared with galactose. Arainose was not detected after acid hydrolysis with 6 N HCl, 110 °C, 3 h; however, L-arabinose was detected in chiral GC analysis after methanolysis (10% HCl/MeOH, 100 °C, 1 h) followed by treatment with TFAA/CH_2Cl_2 (100 °C, 10 min).

(19) Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1994**, *59*, 4849–4852. We thank Professor Faulkner for a prepublication copy.

(20) Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Sakai, R.; Higa, T.; Kashman, Y. *Tetrahedron. Lett.* **1987**, *28*, 6225–6228.

(21) Fusetani, N.; Matsunaga, S.; Matsumoto, T.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053–7054. We isolated cyclotheonamides from a morphologically different *Theonella* sp., which was later identified as *T. swinhoei* collected from the same area on the Hachijo-jima Island. We also isolated cyclotheonamides from the *Theonella* sponge that contained theonellamides (unpublished data).

(22) Higa, T. Personal communication.

(23) For general procedures, see ref 4.

Table 2. ^{1}H NMR data for 3-6 [DMSO-d_6/H_2O (4:1), 308 K]

		3	4	5	6
Ser-1	α	4.01 m[e]	4.00 m	3.74 m	3.75 m
	β	3.55 m	3.55 m	3.62 m	3.62 m
	β′	3.62 m	3.62 m	3.62 m	3.62 m
	NH	7.71 s	7.72 s	7.80 s	7.81 s
Aboa[a]	α	2.12 (br d, 9.6)	2.10 (br d, 10.3)	2.09 (br d, 10.3)	2.10 (br d, 9.9)
	α′	2.35 (br t, 13.4)	2.34 m	2.37 m	2.38 m
	β	4.09 m	4.04 m	4.08 m	4.09 m
	γ	4.22 m	4.22 m	4.23 m	4.23 m
	δ	5.16 m	5.17 m	5.19 (d, 8.4)	5.18 (d, 7.9)
	ε-Me	1.64 (3H, s)	1.62 (3H, s)	1.63 (3H, s)	1.62 (3H, s)
	ζ	6.61 (d, 16.1)	6.63 (d, 16.2)	6.64 (d, 16.2)	6.63 (d, 16.1)
	η	6.50 (d, 16.1)	6.47 (d, 16.2)	6.47 (d, 16.2)	6.47 (d, 16.1)
	2,6	7.41 (2H, d, 7.8)	7.37 (2H, d, 8.6)	7.38 (2H, d, 8.5)	7.37 (2H, d, 8.5)
	3,5	7.31 (2H, t, 7.8)	7.49 (2H, d 8.6)	7.48 (2H, d 8.5)	7.48 (2H, d 8.5)
	4	7.19 m			
	NH	7.67 (d, 9.9)	7.67 m	7.77 (d, 9.3)	7.78 (d, 9.3)
Phe	α	4.54 m	4.53 m	4.56 m	4.54 m
	β	2.65 (dd, 6.3, 13.2)	2.63 m	2.63 (dd, 6.5, 13.5)	2.64 (dd, 6.3, 13.4)
	β′	2.82 m	2.83 m	2.81 m	2.80 m
	2,6	7.15 (2H, d, 7.3)	7.14 (2H, m)	7.15 (2H, d, 7.3)	7.14 (2H, d, 7.5)
	3,5	7.22 (2H, t, 7.3)	7.22 (2H, t, 7.3)	7.23 (2H, t, 7.3)	7.23 (2H, t, 7.5)
	4	7.19 (t, 7.3)	7.18 (t, 7.3)	7.18 (t, 7.3)	7.18 (t, 7.5)
	NH	7.93 (d, 7.2)	7.93 (d, 9.7)	8.06 (d, 8.2)	7.99 (d, 8.5)
Ser-2	α	4.39 m	4.39 m	4.51 m	4.45 m
	β	3.62 m	3.62 m	3.55 m	3.59 m
	β′	3.67 m	3.66 m	3.67 m	3.63 m
	NH	8.38 br	8.38 (d, 7.9)	8.62 br s	8.54 br s
*a*Thr	α	4.20 m	4.15 (t, 9.2)	4.22 m	4.21 m
	β	3.63 m	3.55 m	3.47 m	3.49 m
	γ	1.01 (3H, d, 5.8)	0.87 (3H, d, 5.8)	0.89 (3H, d, 5.7)	0.87 (3H, d, 5.7)
	OH	5.33 (d, 5.0)	5.25 br s	5.37 br s	5.28 (d, 4.4)
	NH	7.63 m	7.63 (d, 9.1)	7.50 m	7.67 (d, 8.3)
sHis[b]	α	4.41 m	4.36 m	4.53 m	4.58 m
	β	2.60 (br d, 11.3)	2.59 m	2.90 m	3.00 m
	β′	2.79 m	2.78 m	3.21 (br t, 12.5)	3.29 m
	2	7.12 m	7.19 m	8.83 br s	8.88 br s
	5	6.65 br s	6.69 br s	7.10 br s	7.12 m
	NH	8.11 br s	8.20 br	8.10 br	8.39 br s
Ahad	α	3.91 m	4.08 m	3.96 m	3.97 m
	β	1.77 (2H, m)	1.85 (2H, m)	1.82 (2H, m)	1.82 (2H, m)
	γ	3.74 m	3.92 m	3.82 m	3.85 m
	d	1.93 (br d, 11.0)	1.89 (br d, 11.9)	1.86 m	1.88 (br d, 11.7)
	δ′	2.19 m	2.18 (br t, 11.9)	2.23 m	2.23 (br t, 10.8)
	NH	7.55 (d, 6.1)	7.69 m	7.30 m	7.36 m
β-Ala[c]	α	4.06 m	2.30 m	2.35 m	2.35 m
	α′		2.42 m	2.42 m	2.42 m
	β	2.89 m	2.95 m	2.95 m	2.88 m
	β′	3.74 m	3.59 m	3.64 m	3.64 m
	NH	7.42 m	7.53 br s	7.55 br s	7.60 br s
BrPhe[d]	α	4.43 m	4.22 m	4.24 m	4.25 m
	β	3.20 m	2.80 m	2.83 m	2.85 m
	β′		2.89 m	2.83 m	2.85 m
	2,6	7.02 (2H, d, 8.3)	7.06 (2H, d, 7.2)	7.02 (2H, d, 8.2)	7.03 (2H, d, 8.4)
	3,5	7.30 (2H, d, 8.3)	7.14 (2H, m)	7.28 (2H, d, 8.2)	7.28 (2H, d, 8.4)
	4		7.11 m		
	NH	8.38 br	8.81 br	8.82 m	8.81 br s
OHAsn	α	5.20 m	5.17 m	5.23 m	5.28 m
	β	3.90 m	3.94 m	3.92 m	3.94 m
	NH_2	7.10 brs, 7.28 brs	7.22 m, 7.39 m	7.09 br s, 7.52 br s	7.18 m, 7.51 m
	NH	8.25 (d, 8.5)	8.26 (d, 9.4)	8.30 (d, 8.4)	8.35 br s
Asn	α	4.54 m	4.55 m	4.47 m	4.49 m
	β	2.27 (br d, 15)	2.26 (br d, 14.8)	2.28 (br d, 15.8)	2.32 m
	β′	2.55 m	2.54 (dd, 10.2, 15.6)	2.54 (dd, 11.4, 15.8)	2.55 (dd, 10.6, 16.0)
	NH_2	6.80 br s, 7.33 m	6.81 br s, 7.32 br s	6.82 br s, 7.35 br s	6.83 br s, 7.34 br s
	NH	7.62 br	7.56 br	7.71 br s	7.72 br s
sAla	α	4.79 m	4.80 (br d, 10.2)	5.12 br s	5.05 br s
	β	3.95 m	3.95 m	4.20 m	4.20 m
	β′	4.53 m	4.56 m	4.85 (br d, 13.2)	4.88 (br d, 13.3)
	NH	8.18 br	8.18 br	8.22 br	8.30 br s

[a] Apoa residue in **3**. [b] π-Nitrogen of the imidazole ring in **5** and **6** was substituted by arabinose and galactose, respectively. Arabinose residue in **5**: δ 4.95 (d, 8.4; H1), 3.87 (m; H5a), 3.77 (br s; H4), 3.73 (m; H5b), 3.70 (m; H2), 3.40 (m; H3). Galactose residue in **6**: 5.02 (d, 8.8; H1), 3.81 (br s; H4), 3.74 (m, H6a), 3.66 (m; H6b), 3.64 (m; H5), 3.62 (m; H2), 3.39 (m; H3). [c] Iser residue in **3**. [d] β-MeBrPhe residue in **3** and Phe residue in **4**. β-Me signal at δ 1.09 (3H, d, 7.1) in **3**. [e] "m" indicates that the signal multiplicity is not determined due to overlapped signals.

HRFABMS m/z 1811.4941 $(M + H)^+$, $C_{75}H_{97}{}^{79}Br_2N_{16}O_{27}$ (Δ −13.4 mmu); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3.

Chiral GC Analysis of Theonellamides. A 100 μg portion of each peptide was dissolved in 6 N HCl (0.2 mL) and

Table 3. ^{13}C NMR Data for 3-6 [DMSO-d_6/H_2O (4:1), 333 K][a]

		3	4	5	6			3	4	5	6
Ser-1	α	55.8	55.7	56.4	56.4	*a*Thr	γ	21.0	21.1	21.2	21.1
	β	61.0	61.0	60.7	60.7	sHis[c]	α	55.0	56.5	54.6	54.6
Aboa[b]	α	37.2	37.3	37.1	37.1		β	31.1	30.8	26.0	25.8
	β	52.4	52.5	52.5	52.5		2	137.6	137.4	137.0	137.0
	γ	68.3	68.4	68.3	68.3		4	136.6	136.5	131.6	131.5
	δ	132.5	133.2	133.2	133.2		5	118.5	118.7	123.5	123.6
	ϵ	135.8	135.6	135.7	135.6	Ahad	α	52.2	52.5	53.0	53.0
	ϵ-Me	13.1	13.0	13.1	13.0		β	38.7	38.0	38.4	38.6
	ζ	133.6	134.5	134.5	134.5		γ	65.6	65.4	65.5	65.5
	η	128.1	126.8	126.8	126.7		d	44.0	44.0	44.5	44.4
	1	137.6	136.5	136.9	136.9	β-Ala[d]	α	69.7	34.8	34.8	34.9
	2,6	126.6	128.6	128.6	128.6		β	43.1	36.4	36.7	36.6
	3,5	129.2	132.0	132.0	132.0	BrPhe[e]	α	58.9	56.0	55.4	55.4
	4	127.9	120.6	120.1	120.1		β	39.5	37.3	36.7	36.8
Phe	α	54.5	54.6	54.5	54.5		1	141.7	136.9	137.0	137.0
	β	38.7	38.7	38.9	38.9		2,6	130.5	129.4	131.6	131.6
	1	137.2	137.8	137.0	137.1		3,5	131.2	128.7	131.5	131.5
	2,6	129.5	129.5	129.5	129.5		4	120.0	126.8	120.6	120.6
	3,5	128.7	128.7	128.7	128.7	OHAsn	α	54.5	54.4	54.2	54.2
	4	127.0	127.0	127.0	127.0		β	71.8	72.0	72.5	72.5
Ser-2	α	56.4	55.3	56.3	56.2	Asn	α	51.0	51.1	51.5	51.5
	β	61.3	61.5	61.7	61.6		β	37.4	37.1	36.8	36.7
*a*Thr	α	58.2	58.3	58.3	58.4	sAla	α	52.1	52.0	50.9	50.9
	β	68.3	68.6	68.6	68.4		β	47.5	47.6	50.0	50.1

[a] Amide carbons were not assigned. Their chemical shifts are as follows: **2** δ 175.6, 174.0, 172.1, 172.0, 171.8, 171.7 (2C), 171.4, 171.3, 171.1, 171.0, 170.7, 170.5, 170.0, 169.6; **3** δ 174.6, 174.0, 172.1, 172.0, 171.8 (3C), 171.6, 171.5, 171.0, 170.8 (2C), 170.2, 170.0, 169.6; **4** δ 175.8, 174.1, 172.4, 172.3, 172.1, 171.9, 171.8, 171.7 , 171.2, 170.9, 170.4, 170.2, 170.1, 169.5, 169.2; **5** δ 175.8, 174.1, 172.2 (2C), 172.1, 172.0, 171.8, 171.7 , 171.1, 171.0, 170.4, 170.3 (2C), 169.5, 169.3. [b] Apoa residue in **3**. [c] π-Nitrogen of the imidazole ring in **5** and **6** was substituted by arabinose and galactose unit, respectively. Arabinose residue in **5**: δ 88.4 (C1), 73.0 (C3), 69.4 (C2), 69.0 (C5), 68.3 (C4) ; galactose residue in **6**: δ 88.5 (C1), 78.5 (C5), 73.5 (C3), 69.5 (C2), 69.2 (C4), 61.6 (C6). [d] Iser residue in **3**. [e] β-MeBrPhe residue in **3** and Phe residue in **4**. β-Me signal in **3** resonated at 17.1 ppm.

heated at 110 °C for 3 h. The reaction mixture was evaporated in a stream of nitrogen, dissolved in 10% HCl in MeOH, and heated at 100 °C for 30 min. The product was evaporated, dissolved in trifluoroacetic anhydride (50 μL) and CH_2Cl_2 (50 μL), reacted at 100 °C for 10 min, and evaporated in a stream of nitrogen. The residue was dissolved in CH_2Cl_2 and analyzed on a Chirasil-L-Val (Chrompack) column. The oven temperature was maintained for 3 min at 60 °C and raised to 200 °C at 4 °C/min. Retention times for amino acid residues (min): D-Ser (12.38), D-*a*Thr (12.80), L-Ser (13.38), L-*a*Thr (13.98), D-Asp (15.28), L-Asp (15.67), (2*R*)-Iser (16.07), (2*S*)-Iser (16.30), D-Gal (18.54), L-Gal (18.96), D-Phe (22.06), L-Phe (22.93), D-BrPhe (31.28), and L-BrPhe (31.88). Retention times of GC peaks in the acid hydrolysate of theonellamide A (min): 13.38, 14.02, 15.76, 16.33, 16.95 (OHAsp), 18.46, 23.08, 27.12 (Ahad), 28.52 (Ahad), and 30.94.(L-β-MeBrPhe).

Hydrogenation of Theonellamides. A 100 μg portion of each peptide was dissolved in *n*-PrOH/H_2O (2:1, 0.2 mL) and hydrogenated at 1 atm in the presence of 10% Pd/C (2 mg). The solution was filtered and the filtrate evaporated to dryness.

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of a 10 μg portion of the peptide was added 5 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (L-FDAA) (10 mg/mL) and 10 μL of 1 M $NaHCO_3$, and the mixture was kept at 50 °C for 1 h. To the reaction mixture was added 5 μL of 2 N HCl and 20 μL of MeOH and analyzed by ODS-HPLC: column 4.6 × 250 mm; mobile phase, gradient elution from MeCN/25 m KH_2PO_4 in H_2O (1:9) to MeCN/H_2O/TFA (50: 50:0.05) in 20 min; UV (340 nm). D-FDAA was prepared as reported,[12] and the amino acids were derivatized as in the case of L-FDAA. Retention times of OHAsp: (2*S*,3*R*)-isomer, 12.8 min; (2*R*,3*S*)-isomer, 13.2 min. Retention times of Hisala: τ-L-histidino-D-alanine (LD-isomer), 13.7 min; LL-isomer, 14.8 min; DL-isomer, 14.1 min; DD-isomer, 13.3 min.

Isolation of Iseserine and β-Methyl-*p*-bromophenylalanine. Theonellamide A (75 mg) was dissolved in 10 mL of 6 N HCl and kept at 110 °C for 16 h. The reaction mixture was evaporated and applied to a column of Hitachi custom 2624 (1 × 100 cm) and eluted with 0.5 N triethylammonium acetate buffer (pH 5.1) to give Iser (4.6 mg) and MeBrPhe (8 mg). Iseserine: $[\alpha]^{23}_D$ −15° (*c* 0.2, H_2O); CD (1N HCl) $[\theta]^{212}$ = +1030°; ^1H NMR (D_2O) δ 4.16 (dd, *J* = 4.1, 8.2 Hz), 3.28 (dd, 4.1, 13.1), 3.07 (dd, 8.2, 13.1). β-Methyl-*p*-bromophenylalanine: UV (H_2O) 262 nm (ϵ 1600); CD (1 N HCl) $[\theta]^{227}$ = +3100°, $[\theta]^{205}$ = −3100°; ^1H NMR (1 N DCl in D_2O) δ 7.57 (2H, d, *J* = 8.5 Hz), 7.27 (2H, d, 8.5), 4.20 (d, 7.0), 3.43 (quint, 7.1), 1.39 (3H, d, 7.2).

β-Methyl-phenylalanine. β-Methyl-*p*-bromophenylalanine (6 mg) was hydrogenated in H_2O/EtOH (8:2) in the presence of 10% Pd/C (7 mg). The solution was centrifuged, filtered, and separated on a column of Hitachi custom 2612 gel (1 × 30 cm) with 0.5 N triethylammonium acetate buffer (pH 5.1) followed by ODS HPLC with H_2O to give β-methylphenylalanine (0.6 mg). β-Methylphenylalanine: ^1H NMR (D_2O) δ 7.12 (2H, t, 7.8), 6.82 (3H, m), 3.79 (d, 7.6), 3.39 (quint, 7.4), 1.29 (3H, d, 7.2).

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Supplementary Material Available: ^1H NMR, ^{13}C NMR, HMQC spectra, and expansions of the amide region of HOHAHA and NOESY spectra of theonellamides A-E (**1**-**5**) and expansion of the carbonyl region of the HMBC spectrum of **1** (38 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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