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Polytheonamides A and B, Highly Cytotoxic, Linear Polypeptides with Unprecedented Structural Features, from the Marine Sponge, *Theonella swinhoei*

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Abstract: Polytheonamides A and B are highly cytotoxic polypeptides with 48 amino acid residues isolated from the marine sponge, *Theonella swinhoei*. The structure of polytheonamide B was determined by spectral and chemical methods, especially extensive 2D NMR experiments, which resulted in the unprecedented polypeptide structure; the *N*-terminal glycine blocked with a 5,5-dimethyl-2-oxo-hexanoyl group, the presence of eight *tert*-leucine, three β-hydroxyvaline, six γ-*N*-methylasparagine, two γ-*N*-methyl-β-hydroxyasparagine, and β,β-dimethymethionine sulfoxide residues. More significantly, it has the sequence of alternating D- and L-amino acids. Polytheonamide A is an epimer of polytheonamide B differing only in the stereochemistry of the sulfoxide of the 44th residue.

Introduction

The marine sponge *Theonella swinhoei*, with a yellow interior and collected from Hachijo-jima Island, 300 km south of Tokyo, is extraordinarily rich in bioactive metabolites of unusual structures and potent activities, including serine protease inhibitory cyclotheonamides,[2] nazumamide A,[3] and pseudotheonamides,[4] cytotoxic onnamides,[5] theopederins,[6] and orbiculamide A,[7] and antifungal aurantosides.[8] In 1994, we reported the isolation and structure determination of the highly cytotoxic polypeptides, polytheonamides, from the same sponge.[9−11] During the stereochemical study of polytheonamide B, we realized that the previously proposed structures were incorrect. In this report, the revised complete structures of polytheonamides A and B including the sequence specific stereochemical assignments of amino acid residues are described.

Results

The sponge extract was subjected to solvent partitioning, gel filtration, and reversed-phase HPLC to afford polytheonamides A, B, and C (3.8, 5.2, and 1.0×10^{-4} % wet weight, respectively). Polytheonamides A, B, and C were cytotoxic against P388 murine leukemia cells with IC_{50} values of 78, 68, and 68 pg/mL, respectively.

The structure elucidation was commenced with polytheonamide B, the major polytheonamide. The electrospray ionization mass spectrum (ESIMS) exhibited ions at *m*/*z* 1700.9 $(M + 3Na)^{3+}$ and 2539.8 $(M + 2Na)^{2+}$, in agreement with the molecular weight of 5032, which was supported by the $(M + H)^+$ ion at *m*/*z* 5033 in the FABMS. The ^1H NMR spectrum of polytheonamide B (Figure 1A) exhibited numbers of signals in the α-methine and amide regions, whereas the ^{13}C NMR spectrum (Figure 1B) gave a cluster of amide signals, indicating its polypeptidic nature.

Component Amino Acids. Amino acid analysis of polytheonamide B showed the presence of Asp (7.9 residues), Thr (3.9), Ser (1.5), Glu (2.0), Gly (10.0), Ala (8.9), Val (4.3), and Ile (2.3).[12] In addition to these common amino acids, there were several unidentified peaks in the chromatogram: an acidic amino acid eluted faster than Asp; a neutral amino acid with a retention time close to that of Cys; and a neutral amino acid with a slightly longer retention time than that of Ile. The peaks corresponding to Thr, Ser, and Glu were broad, suggesting that they were overlapped with peaks of unassigned amino acids.

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(12) Thr and *a*Thr exhibited an identical retention time in the amino acid analysis. The molar equivalents are not precise due to peak overlapping with unusual amino acids. For the chromatogram of amino acid analysis, see Figure S14.

10.1021/ja045749e CCC: $30.25

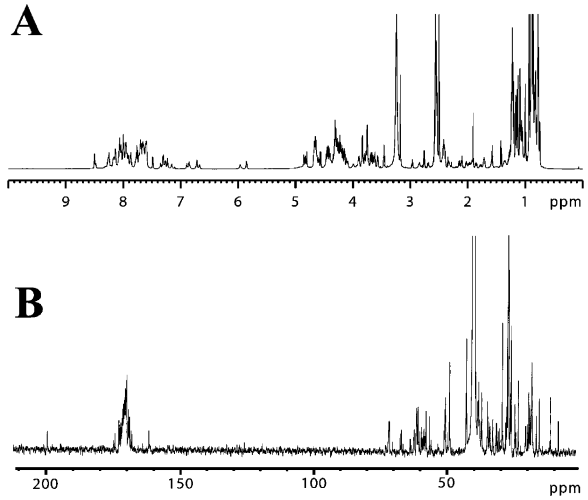

Figure 1. ^{1}H (A) and ^{13}C NMR spectra (B) of polytheonamide B in DMSO-d_6.

To identify the uncommon amino acids, the acid hydrolysate was directly analyzed by 2D NMR; the hydrolysate exhibited the much simpler NMR spectra than those of the intact peptide, which was due to the convergence of signals. In addition to the common amino acids mentioned above, *a*Thr,[13] *threo*-β-hydroxyaspartic acid (OHAsp),[14] β-methylglutamic acid (βMeGlu),[15] *t*-Leu,[16] β-hydroxyvaline (OHVal),[17] and β-methylisoleucine (βMeIle)[18] could be assigned. Since βMeIle was not thoroughly assigned due to the heavily crowded aliphatic signals, this amino acid was isolated and identified from the hydrolysate.[19] Intense signals assignable to methylamine were also observed.[20]

Structures of the *N*-Terminus and 44th Amino Acid Residues. Previously, the *N*-terminal-blocking group and the 44th residue were assigned as carbamoyl and γ-hydroxy-*t*-leucine, respectively, on the basis of NMR data.[10,11] In addition, polytheonamide B was assigned as a tripropylammonium salt in order to account for the MS data and due to the NMR signals for tripropylamine. Subsequently, tripropylamine was found to be a contaminant from the *n*-PrOH used for HPLC. In the meantime, we realized the presence of a functional group reducible with $NaBH_4$ which prompted the reinvestigation of the total structure. Inspection of the 2D NMR spectra indicated a pair of mutually coupled methylenes and a ketone resonance. On reduction with $NaBH_4$, polytheonamide B afforded a 2:1 mixture of dihydropolytheonamides B1 and B2, both of which exhibited an oxymethine signal. The COSY spectrum of dihydropolytheonamide B1 displayed signals assignable to a partial structure $-C_\delta H_2-C_\gamma H_2-C_\beta H(OH)-$ [δ_H 3.80 (H_β), 1.60, 1.40 ($H_{\gamma,\gamma'}$), 1.29, 1.17 ($H_{\delta,\delta'}$); δ_C 71.5 (C_β), 29.6 (C_γ), 39.0 (C_δ)]. Although the methyl region of the HMBC spectrum of polytheonamide B was highly crowded, cross-peaks were observed from a singlet methyl (δ_H 0.87) to a methylene carbon at δ 36.6 (C_δ) and to a nonprotonated carbon at δ 29.6, thus suggesting the linkage of C_δ to a *tert*-butyl group. HMBC cross-peaks were also observed between the amide and methylene protons of the *N*-terminal Gly residue and the C_α carbon (δ 161.2) of the blocking group. Although no NOESY cross-peaks between Gly1 and the blocking group were detected, probably due to an unfavorable correlation time for this portion of the molecule, the ROESY cross-peak between the amide proton (Gly1) and the oxygenated methine proton in dihydropolytheonamide B1 was observed, thereby disclosing that the *N*-terminal Gly of polytheonamide B was blocked with a 5,5-dimethyl-2-oxo-hexanoyl group.

Since the *N*-terminal structure was revised and tripropylamine was not contained in the molecule, the 44th residue, which had previously been assigned as γ-OH-*t*-Leu, had to be revised. Interpretation of the HMBC data showed a cross-peak between a singlet methyl (δ 2.52) and the methylene carbon at δ 63.3, which was originally assigned to an oxygenated methylene. However, these chemical shift values were more consistent with two carbons which were separated by a sulfoxide group, which was also in accordance with MS data. In fact, the elemental analysis of polytheonamide B demonstrated the presence of one sulfur atom. Therefore, the 44th residue was β,β-dimethylmethionine sulfoxide [Me_2Met(O)]. This amino acid and 5,5-dimethyl-2-oxo-hexanoic acid were not detected in the acid hydrolysate of polytheonamide B.

Sequencing of Amino Acids. To sequence the amino acids in polytheonamide B using NMR spectroscopy solvents were required which gave sharp and well-dispersed ^{1}H NMR signals, and high-quality NOESY spectra; $CDCl_3/CD_3OH$ (1:1) and DMSO-d_6 satisfied these criteria. In $CDCl_3/CD_3OH$ (1:1), the NOESY cross-peaks were observed between adjacent residues and between sequentially distant residues. However, this feature also complicated the sequence analysis. By contrast, polytheonamide B appeared to adopt a random-coil conformation in DMSO-d_6, in which NOESY cross-peaks were observed only between adjacent residues, thereby facilitating sequential signal assignments. The number of amino acid residues was established by interpretation of the HOHAHA spectrum as follows: OHAsx (2), Asx (8), Thr/*a*Thr (1 each), Ser (1), Glx (1), βMeGlx (1), Ala (7), Val (3), and Ile (2). The residues that contained a quaternary carbon in the side chain were analyzed on the basis of intra-residual NOESY cross-peaks which were more intense than inter-residual cross-peaks, allowing the determination of eight *t*-Leu, one βMeIle, three OHVal, and one Me_2Met(O) unit (Figure 2). Sequence analysis of the peptide chain in DMSO-d_6 was unexceptional once the types and numbers of components were established (Figure 3, Table 1).

There were eight doublet *N*-methyl resonances attributable to *N*-methylation in the side-chain amide groups. All of the

(13) In addition to an A_3MX system for Thr (δ 1.21, 3.82, and 4.28), there was another A_3MX system with similar chemical shift values (δ 1.15, 3.94, and, 4.25), which could be assigned as *a*Thr.

(14) An AB system at δ 4.75 and 4.28 indicated the presence of *threo*-β-hydroxyAsp. [(a) Kato, T.; Hinoo, H.; Terui, Y.; Kikuchi, J.; Shoji, J. *J. Antibiot.* **1988**, *41*, 719−725. (b) Tymiak, A. A.; McCormick, T. J.; Unger. S. E. *J. Org. Chem.* **1989**, *54*, 1149−1157. (c) ElDin, A. L. M. S.; Kyslik, P.; Stephan, D.; Abdallah, M. A. *Tetrahedron*, **1997**, *53*, 12539−12552.]

(15) A methine proton at δ 2.50 exhibited COSY cross-peaks with an α-methine signal at δ 3.98, a methylene at δ 2.39 and 2.53, and a methyl at δ 0.95. (Debono, M.; Barnhart, M.; Carrell, C. B.; Hoffmann, J. A.; Occolowitz, J. L.; Abbott, B. J.; Fukuda, D. S. *J. Antibiot.* **1987**, *40*, 761−777.)

(16) There was a large methyl singlet at δ 0.96, which exhibited HMBC correlations with carbons at δ 26.0, 33.0, and 62.5. The α-methine proton attached to a carbon at δ 62.5 resonated at δ 3.32, as a singlet.

(17) The HMBC spectra revealed that both singlet methyls at δ 1.17 and 1.32 were correlated with carbons at δ 62.9 and 70.6. (Ikai, K.; Takesako, K.; Shiomi, K.; Moriguchi, M.; Umeda, Y.; Yamamoto, J.; Kato, I. *J. Antibiot.* **1991**, *44*, 925−933.)

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(19) ^{1}H NMR data for the isolated βMeIle: δ 0.78 (3H, t), 0.88 (3H, s), 0.89 (3H, s), 1.32 (2H, q), and 3.40 (1H, s).

(20) ^{1}H NMR data for the isolated $MeNH_2$: δ 2.44 (3H, s).

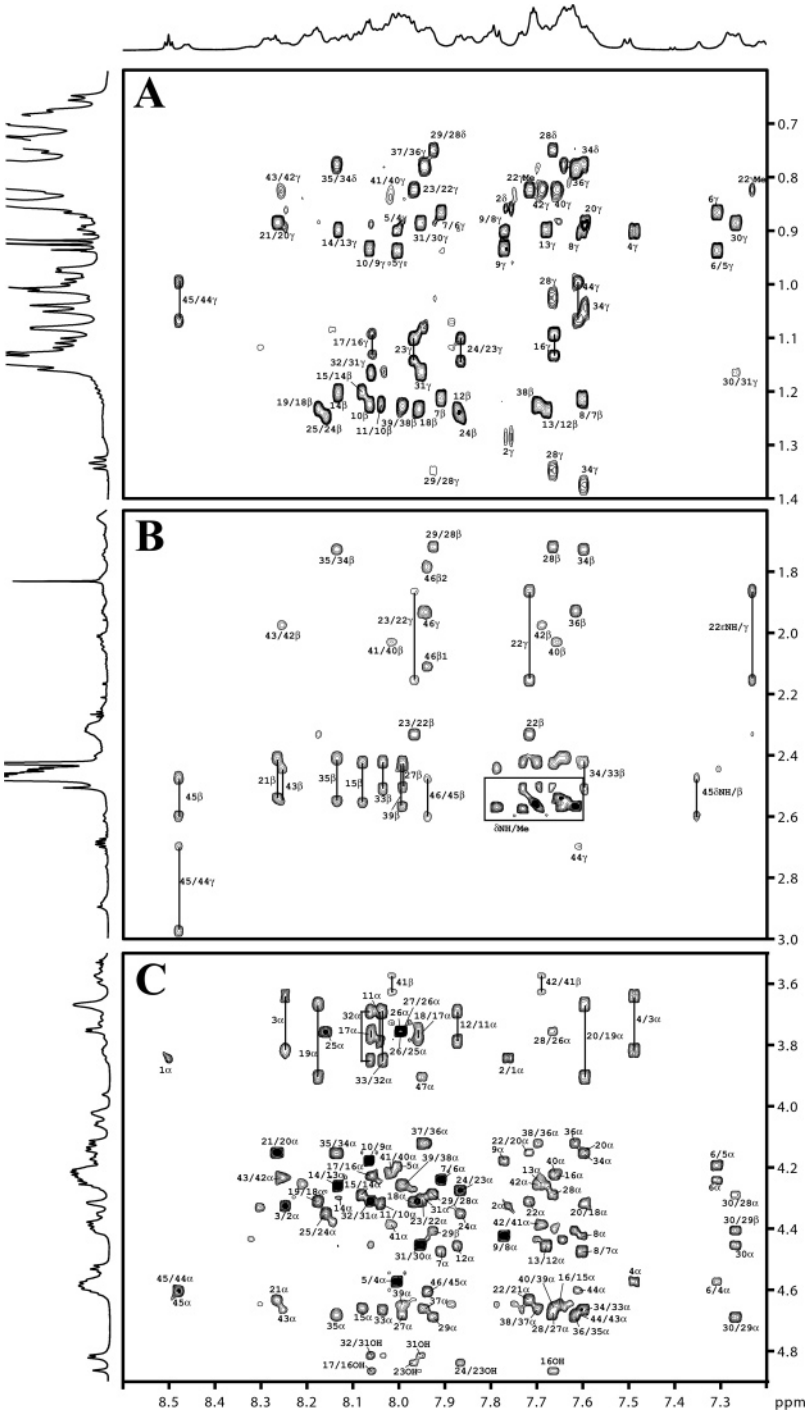

Figure 2. Amide-aliphatic proton (A and B) and amide-α-proton (C) regions of the NOESY spectrum of polytheonamide B in DMSO-d_6 at 300K. Assigned cross-peaks are indicated in the spectrum.

carbonyl groups in the side chain were linked to either amide or *N*-methylamide moieties. Sequence specific assignment of these groups was accomplished by the interpretation of NOESY data, which disclosed that residues 15, 21, 27, 29, 33, 35, 37, and 39 were *N*-methylated in the side chain.

Stereochemistry of Amino Acids. The stereochemistry of the amino acid residues in polytheonamide B was determined by GC analysis on a chiral stationary phase[22] and HPLC analysis of FDAA derivatives (Marfey analysis).[23] Thr, Ile, Glu, Val, and *β*MeIle were found to be in the L-form, while OHAsp, Ser, and *a*Thr were in the D-form. A 2*S*,3*S*-stereochemistry was assigned for *β*MeGlu by GC analysis.[24] The remaining amino acids were mixtures of both forms: Ala (D/L = 2:5), *t*-Leu (4:4), Asp (7:1), and OHVal (2:1). Consequently, it was necessary to assign the stereochemistry of these residues in a sequence-specific manner in order to complete the structure elucidation of polytheonamide B. To solve this issue, attempts were made to digest polytheonamide B with a variety of proteases; this was not successful. In contrast, mild acid hydrolysis worked

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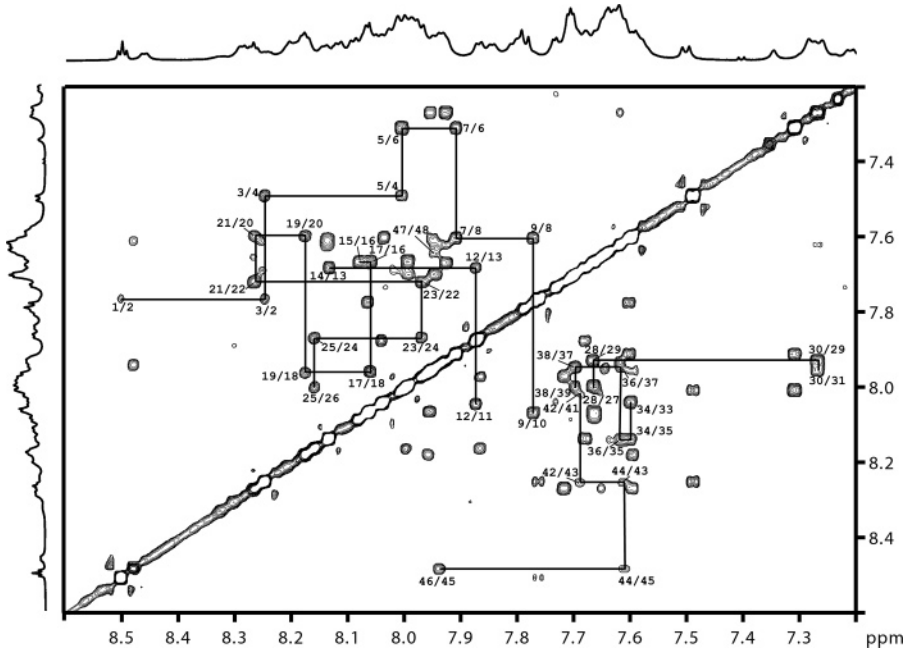

Figure 3. Amide-amide proton region of the NOESY spectrum of polytheonamide B in DMSO-d_6 at 300K. Sequential NOEs are indicated in the spectrum.

well; polytheonamide B was treated with 4N HCl in EtOH at 70 °C for 30 min to afford a mixture of fragment peptides which was separated by reversed-phase HPLC to afford 120 fractions. Each fraction was analyzed by tandem-FABMS. In this way, the purities of the fractions could be checked and at the same time the amino acid sequence of the fragment peptides obtained.[25] The results of the analysis are summarized in Table 2.

Eleven fractions were chosen for stereochemical study of the component amino acids. All of these fractions were directly subjected to stereochemical study, except for fraction 46 which was dansylated and separated into fractions 46A and 46B. The chiralities of amino acid residues were determined as follows: (1) The stereochemistry of the amino acids in the acid hydrolysate of the dansylated derivative was analyzed by either Marfey's method or by chiral GCMS analysis; (2) the chirality of the *N*-terminal residue of a dansyl derivative was determined by HPLC on a chiral stationary phase; (3) by one cycle of Edman degradation, the *N*-terminal residue of a peptide was removed and the second residue from the *N*-terminus was analyzed as a dansyl derivative.[26]

t-Leu4 was determined to be L by the *N*-terminal analysis of dansylated fraction 100, whereas *t*-Leu5 was D from the *N*-terminal analysis of fraction 90 after dansylation followed by one cycle of Edman degradation. Marfey analysis of the dansylated fraction 72 led to L-*t*-Leu6 and D-Ala7, while the *N*-terminal analysis of dansylated fraction 65 disclosed D-*t*-Leu8. L-*t*-Leu9 and D-Ala10 were assinged by the Marfey analysis of dansylated fraction 52. Similarly, Marfey analysis of dansylated fractions 23 and 39 resulted in L-Ala12, D-*t*-Leu13, L-Ala14, D-Asp15, and L-Ala18, whereas GCMS analysis of dansylated fraction 39 identified L-OHVal16. Thus, both OHVals at positions 23 and 31 were D. L-*t*-Leu20, D-βMeGlu21, L-Ala24, D-Asp27, L-*t*-Leu30, D-Asp33, D-Asp35, L-Ala38, and D-Asp39 were derived from the Marfey analysis of dansylated fractions 46A, 46B, and 36.

Although fragments containing any of eight residues from the *C*-terminus could not be obtained, fortunately *des*-Thr48-dihydropolytheonamide B2 could be isolated during the purification of dihydropolytheonamide B2.[27] GC analysis of its acid hydrolysate indicated the absence of L-Thr, thereby accommodating L-Thr at the *C*-terminus. The chirality of the amino acids thus far obtained showed a sequence of alternating D and L amino acids. If this rule is applicable to the whole peptide sequence, then both Asn43 and Asn45 should be in the D-form, although the amino acid analysis of polytheonamide B indicated one of the two Asn residues to be L. The possibility of racemization in the Asx residues during acidic hydrolysis was explored.[28] To address this issue, the hydrolysate obtained from the hydrolysis of polytheonamide B with 6N DCl in D_2O was analyzed by chiral GCMS. As a result, the L-Asp fraction exhibited ion peaks higher than those of D-Asp by 1 amu, indicating that L-Asp was the product of inversion by acid.[29] Thus, all of the Asx residues, including residues 43 and 45, in polytheonamide B were originally D.

Since β,β-dimethylmethionine sulfoxide was decomposed during acid hydrolysis, its conversion to an acid-resistant derivative was required in order to determine the stereochemistry. Advantage was taken of the structural resemblance between this residue and methionine. In Met-containing peptides, the *C*-terminal amide bond of Met is cleaved with CNBr, with concomitant conversion to homoserine lactone.[30] Therefore, it was anticipated that the β,β-dimethylmethionine sulfoxide unit in polytheonamide B could be converted to β,β-dimethyl-

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(29) Because incorporation of deuterium was observed for both α- and β-positions, EI−MS peaks were observed as a cluster of isotope ions. The pattern of peaks for L-Asp shifted to the higher mass region by one amu.
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Table 1. ^{1}H NMR Chemical Shift of Polytheonamide B in DMSO-d_6 at 313K[a]

residue	NH	C_αH	C_βH	others
Gly1	8.50	3.83		
βMeIle2	7.76	4.33		$C_\gamma H_3$0.85; $C_\gamma H_3$0.89; $C_\gamma H_2$1.30; $C_\delta H_3$0.78
Gly3	8.25	3.63, 3.82		
t-Leu4	7.49	4.58		$C_\gamma H_3$0.90
t-Leu5	8.00	4.20		$C_\gamma H_3$0.94
t-Leu6	7.31	4.24		$C_\gamma H_3$0.86
Ala7	7.91	4.49	1.21	
t-Leu8	7.60	4.42		$C_\gamma H_3$0.90
t-Leu9	7.77	4.19		$C_\gamma H_3$0.93
Ala10	8.07	4.32	1.23	
Gly11	8.04	3.70, 3.80		
Ala12	7.88	4.46	1.23	
t-Leu13	7.68	4.24		$C_\gamma H_3$0.90
Ala14	8.13	4.31	1.20	
Asm15	8.08	4.65	2.42, 2.55	
OHVal16	7.67	4.22	1.09, 1.13	
Gly17	8.06	3.78		
Ala18	7.96	4.31	1.23	
Gly19	8.18	3.66, 3.92		
t-Leu20	7.60	4.16		$C_\gamma H_3$0.88
Asm21	8.27	4.63	2.41, 2.53	
βMeGln22	7.72	4.32	2.33	$C_\gamma H_3$0.82; $C_\gamma H_2$1.85, 2.17; NH_2 6.73, 7.25
OHVal23	7.97	4.28		$C_\gamma H_3$1.11; $C_\gamma H_3$1.14
Ala24	7.87	4.35	1.24	
Gly25	8.16	3.78		
Gly26	7.99	3.78		
Asm27	7.99	4.68	2.43, 2.50	
Ile28	7.67	4.30	1.72	$C_\gamma H_3$1.02; $C_\gamma H_2$1.35; $C_\delta H_3$0.75
OHAsm29	7.93	4.68	4.41	
t-Leu30	7.27	4.46		$C_\gamma H_3$0.88
OHVal31	7.95	4.31		$C_\gamma H_3$1.16; $C_\gamma H_3$1.16
Gly32	8.07	3.71, 3.85		
Asm33	8.03	4.65	2.42, 2.52	
Ile34	7.60	4.16	1.73	$C_\gamma H_3$1.05; $C_\gamma H_2$1.37; $C_\delta H_3$0.78
Asm35	8.14	4.68	2.41, 2.55	
Val36	7.62	4.13	1.94	$C_\gamma H_3$0.78; $C_\gamma H_3$0.78
OHAsm37	7.95	4.65	4.43	
Ala38	7.69	4.25	1.23	
Asm39	7.99	4.65	2.43, 2.58	
Val40	7.66	4.22	2.03	$C_\gamma H_3$0.83; $C_\gamma H_3$0.83
Ser41	8.01	4.40	3.58, 3.62	
Val42	7.68	4.23	1.99	$C_\gamma H_3$0.83; $C_\gamma H_3$0.83
Asn43	8.25	4.66	2.42, 2.55	NH_2 6.88, 7.29
Me_2Met(O)44	7.61	4.60		$C_\gamma H_3$0.99; $C_\gamma H_3$1.07; $C_\gamma H_2$2.70, 2.98; $C_\epsilon H_3$2.52
Asn45	8.47	4.61	2.47, 2.60	NH_2 6.90, 7.36
Gln46	7.93	4.29	2.12	$C_\gamma H_2$1.79, 1.93; NH_2 6.69, 7.16
*a*Thr47	7.96	4.31	3.89	$C_\gamma H_3$1.09
Thr48	7.63	4.18	4.07	$C_\gamma H_3$1.05

[a] *N*-methyl amides in the side chain: NH/CH_3, 7.62/2.58, 7.63/2.55, 7.65/2.54, 7.66/2.52, 7.71/2.52, 7.72/2.52, 7.73/2.58, 7.79/2.58.

homoserine lactone, if this residue was reduced to a sulfide. β,β-Dimethylhomoserine lactone was shown to be stable to acid using a synthetic sample.[31] Polytheonamide B was treated with Me_2S in the presence of NH_4I[32] to afford a deoxygenated derivative which was then subjected to reaction with CNBr to afford the expected truncated derivative (Scheme 1). GC analysis of the product showed the presence of L-β,β-dimethylhomoserine lactone, thus completing the sequence-specific, stereochemical assignments of polytheonamide B. Therefore, the odd number residues are in the D form, whereas the even number amino acids in the L form.

The gross structure of polytheonamide A was assigned to be identical with that of polytheonamide B by interpretation of the 2D NMR data in DMSO-d_6 (Table 3). The ^{1}H NMR spectra of the two peptides were almost superimposable, except for minor chemical shift differences observed in the signals for residues 41−48, among which the most notable differences were found in the residue 44. The Marfey and chiral GC analyses of polytheonamides A and B provided identical results. Therefore, there were two possibilities: (1) polytheonamides A and B are positional isomers in which stereochemistries of at least one pair of amino acid residues were exchanged; (2) polytheonamides A and B are diastereomeric at the sulfoxide in residue 44. An accidental finding solved this problem. When the deoxygenation products of polytheonamides A and B were dissolved in *n*-PrOH/H_2O (1:1) and the mixture left to stand at room temperature for a week, they underwent air oxidation to furnish a pair of HPLC peaks corresponding to polytheonamides A and B in a 1:1 ratio. Furthermore, on oxidation with Oxone[33] polytheonamides A and B afforded sulfones which were indistinguishable by HPLC, FABMS, and ^{1}H NMR analysis. Therefore, it was concluded that the two peptides are isomeric at the sulfoxide moiety.

Discussion

In this paper, it was shown that polytheonamides A and B, two highly cytotoxic constituents of the marine sponge *T. swinhoei*, are polypeptides with unprecedented structural features. The structure of the *N*-terminal blocking group is unusual among natural products. Although the 5,5-dimethyl-2-oxoheptanoyl group was reported in synthetic compounds,[34] this is the first report of this component in a natural product. *t*-Leu is a rare amino acid among nonribosomal peptides of microbial or marine origin; this amino acid has so far been reported as a component in bottromycins,[35] discodermin A[36] and related peptides,[18,37] and hemiasterlins.[38] The presence of eight *t*-Leu residues in one peptide is unprecedented. Other nonproteinogenic amino acid residues in polytheonamides, such as βMeIle, OHVal, βMeGlx, and Me_2Met(O), are also methylated variants of proteinogenic amino acids, as is *t*-Leu, a β-methylated variant of valine. Me_2Met(O) is a new amino acid. Another novel feature of the component amino acids is the presence of eight residues of γ-*N*-methylasparagine. This amino acid is a component of the phycobiliproteins of cyanobacteria and red algae and is reported to play a functional role in photosynthesis.[39] Although reported previously as products of chemical transfor-

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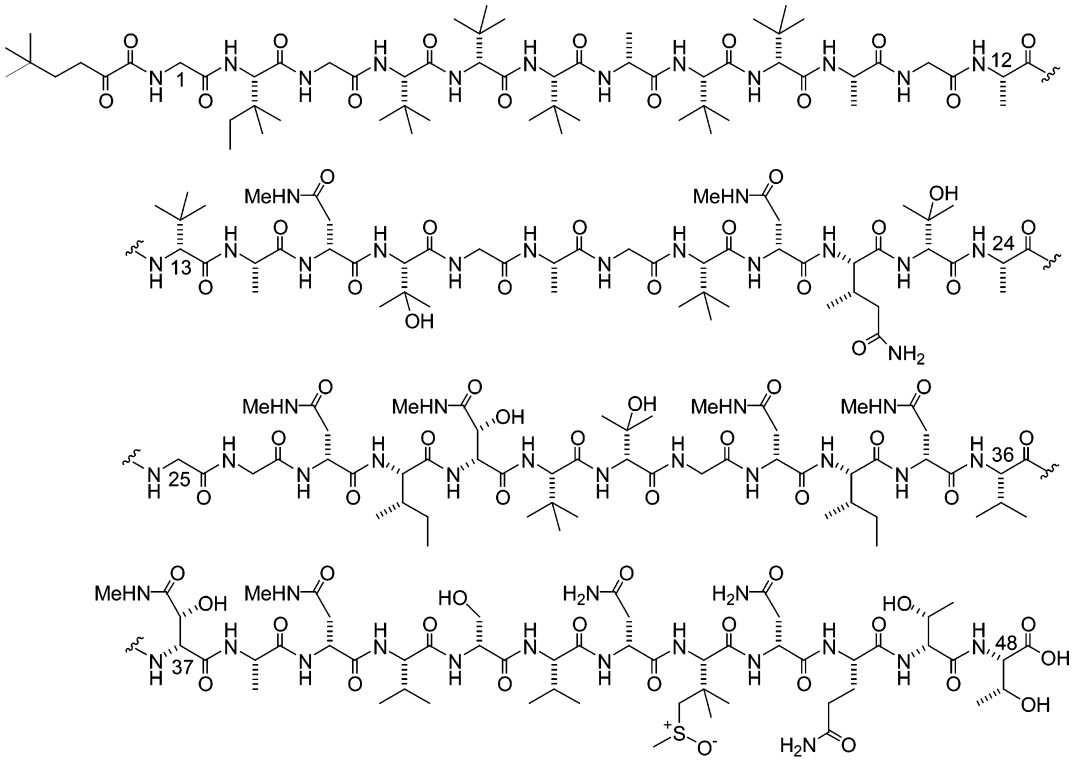
Chart 1. Structure of Polytheonamides A and B



Polytheonamides A and B

mations, γ-*N*-methylasparagine and γ-*N*-methyl-β-hydroxyasparagine have not been described as components of natural nonribosomal peptides.[40]

Polytheonamides are by far the largest nonribosomal peptides and their D/L alternating stereochemistry throughout the chain is only preceded by gramicidin A, a linear 15-residue peptide produced by *Bacillus brevis*.[41] This peptide is known to form monovalent-cation selective ion channels in lipid bilayers[42] and inhibit bacterial RNA polymerase and sporulation.[43] The modes of action of polytheonamides are currently under investigation. The origin of several nonribosomal peptides in lithistid sponges have been traced to symbiotic bacteria.[44] Perhaps, the polytheonamides are also derived from this source, judging from their highly unusual structural features.

Experimental Section

General Procedures. FAB and ESI mass spectra were acquired on a JEOL JMX-SX102/SX102 tandem mass spectrometer. Amino acid analyses were carried out with a Hitachi 835 amino acid analyzer. For NMR spectroscopy peptides are dissolved at concentrations of 1 mM and 4 mM in DMSO-d_6. NMR spectra were recorded on a Bruker Avance 600 or an Avance 800 spectrometer, and data processing was performed with XWINNMR software (Bruker) running on Silicon Graphics O_2 workstations. The DQF−COSY spectra were measured in phase-sensitive mode. The HOHAHA spectra were recorded with a mixing time of 66 ms. The NOESY spectra, with a mixing time of 400 ms, were measured at 300, 313, 323, and 333 K. HMBC and HMQC spectra were recorded essentially as described in the literature.

Biological Materials. The sponge samples were collected several times by hand using Scuba at depths of 10−20 m off Hachijo-jima Island (33° 06′ N; 139° 47′ E) and identified as *Theonella swinhoei* by Dr. R. W. M. van Soest of the University of Amsterdam.

Extraction and Isolation. The frozen sponge specimens (22 kg) were homogenized and extracted with EtOH (7 × 18 L). The combined EtOH extracts were concentrated in vacuo and partitioned between H_2O (3 L) and diethyl ether (15 L). The ether extract was evaporated to afford a brown gum (120 g) which was partitioned between MeOH/H_2O (9:1) and *n*-hexane. The aq MeOH layer (19.3 g) was subjected to ODS flash chromatography with MeOH/H_2O and MeOH/$CHCl_3$ systems. The fraction eluted with $CHCl_3$/MeOH (1:1) was gel-filtered on Sephadex LH-20 with $CHCl_3$/MeOH (3:2). Fractions (204 mg) eluted at the column void volume were collected and further purified by ODS HPLC on Cosmosil 5C_{18}AR with 45% *n*-propanol containing 0.05%TFA to furnish polytheonamides A, B, and C (3.8, 5.2, and 1.0 × 10^{-4}% based on wet weight, respectively).

Polytheonamide A: colorless amorphous solid; $C_{219}H_{376}N_{60}O_{72}S$; FABMS *m*/*z* 5033 $(M+H)^+$, 5055 $(M+Na)^+$; ^{1}H NMR (DMSO-d_6), see Table 3.

Polytheonamide B: colorless amorphous solid; $C_{219}H_{376}N_{60}O_{72}S$; FABMS *m*/*z* 5033 $(M+H)^+$, 5055 $(M+Na)^+$; ^{1}H NMR (DMSO-d_6), see Table 1. Elemental analysis for sulfur indicated the presence of 0.59% S (calcd for 0.64%).

Amino Acid Analysis of Polytheonamides. A 100 μg portion of the peptide was dissolved in 1.0 mL of 6N HCl in an evacuated sealed

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Table 2. Amino Acid Sequence of Peptide Fragments

fraction no.	FAB–MS (pos., *m*/*z*)	sequence
10	361	Val36 − OHAsm37-COOEt
19	233	Ser41 − Val42-COOEt
23	332	Ala10 − *t*-Leu13-COOH
	561	Ile28 − Gly32-COOH
25	702	Asm33 − Ala38-COOH
28	274	Ala12 − Ala14-COOH
	602	Ile34 − Ala38-COOEt
29	730	Gly19 − Gly25-COOEt
	659	Asm33 − OHAsm37-COOEt
30	430	Ala12 − Asm15-COOEt
31	602	Ala12 − Gly17-COOEt
	690	Asm27 − Gly32-COOH
32	589	Ile28 − Gly32-COOEt
33	731	Asm33 − Ala38-COOEt
	589	Ile28 − Gly32-COOEt
	518	Asm27 − *t*-Leu30-COOH
36	717	Asm27 − Gly32-COOEt
	774	Gly26 − Gly32-COOEt
38	602	Ala12 − Gly17-COOEt
39	674	Ala12 − Ala18-COOEt
	661	Asm27 − OHVal31-COOEt
40	717	Asm27 − Gly32-COOEt
42	830	Asm33 − Asm39-COOH
45	645	Ala18 − OHVal23-COOH
46	774	Ala18 − Gly25-COOH
	958	Asm33 − Val40-COOEt
47	401	*t*-Leu8 − Gly11-COOEt
52	344	*t*-Leu8 − Ala10-COOEt
59	1028	Ala7 − Gly17-COOEt
60	429	*t*-Leu4 − Ala7-COOH
61	585	*t*-Leu6 − Gly11-COOEt
65	656	*t*-Leu8 − Ala14-COOEt
69	671	Gly1 − Ala7-COOH
72	457	*t*-Leu4 − Ala7-COOEt
74	542	*t*-Leu4 − *t*-Leu9-COOH
80	698	Gly1 − Ala7-OEt
89	726	*t*-Leu4 − Ala10-COOH
90	726	*t*-Leu4 − Ala10-COOH
92	953	*t*-Leu4 − Ala12-COOH
93	1024	Gly1 − Gly11-COOH
94	967	*β*MeIle2 − Gly11-COOH
95	811	*t*-Leu4 − Gly11-COOEt
96	967	Gly1 − Ala10-COOH
97	910	*β*MeIle2 − Ala10-COOH
98	882	*t*-Leu4 − Ala12-COOEt
100	754	*t*-Leu4 − Ala10-COOEt
	1052	Gly1 − Gly11-COOEt
101	1052	Gly1 − Gly11-COOEt
102	995	Gly1 − Ala10-COOEt
103	938	*β*MeIle2 − Ala10-COOEt
	995	Gly1 − Ala10-COOEt
104	995	Gly1 − Ala10-COOEt
105	1066	*β*MeIle2 − Ala12-COOEt

tube and heated at 110 °C for 20 h. After evaporation, the residue was dissolved in 0.4 mL of 0.01N HCl and subjected to amino acid analysis. Retention times in the amino acid analysis of the hydrolysate of polytheonamide B (min): OHAsp (7.4), Asp (19.4), Thr (26.8), Ser (28.7), Glu (32.2), Gly (57.1), Ala (62.9), Val (81.0), *t*-Leu (88.9), Ile (96.6), *β*MeIle (100.5), NH_3 (142.8), $MeNH_2$ (153.1).

Chiral GCMS Analysis. Chiral GC-MS was carried out using a Chirasil L-Val capillary column (0.25 mm × 25 m) with a JEOL DX303 mass spectrometer operating in the positive EI mode (scan range between *m*/*z* 50 and 600 with repetition times of 2 s). The acid hydrolysate of a peptide (500 *μ*g) was treated with 2N HCl in *i*-PrOH at 90 °C for 1h in a sealed tube. After drying in vacuo, to the residue were added CH_2Cl_2 and trifluoroacetic anhydride (0.3 mL, each), and the mixture heated at 100 °C for 1 h. The reaction mixture was dried in vacuo, and the residue was dissolved in CH_2Cl_2 and analyzed by GCMS. Retention times (min): D-Ala (8.0), L-Ala (9.3), D-*t*-Leu (10.0), L-*t*-Leu (10.6), L-Val (11.4), L-Thr (12.3), L-Ile (14.0), D-*a*Thr (14.9), D-Asp (20.4), L-Asp (20.8), D-OHVal (23.7), L-OHVal (24.6), and L-Glu (25.9).

Marfey Analysis. To 2.5 *μ*mol of the hydrolysate of peptide was added 100 *μ*L of 1 M $NaHCO_3$ followed by 2.8 *μ*mol of FDAA in 50 *μ*L of acetone. The mixture was heated at 80 °C for 3 min. After cooling to room temperature and the addition of 50 *μ*L of 2N HCl, the reaction mixture was analyzed by HPLC on Cosmosil $5C_{18}$ MS (0.5 × 25 cm): a linear gradient from 0.05% TFA to MeCN/H_2O/TFA (50:50:0.05) in 70 min at a flow rate of 1.0 mL/min: detection was with UV absorption at 340 nm. Each peak was identified by comparing the retention time with those of standard derivatives. The amino acid standards showed the following retention times (min): D-OHAsp (12.2), D-Ser (16.5), L-Thr (17.2), L-Asp (18.1), D-Asp, D-*a*Thr (19.1), L-Glu (20.6), L-Ala (22.4), D-Ala (26.2), L-Val (30.2), L-*t*-Leu (34.9), L-Ile (35.3), L-*β*MeIle (39.5), and D-*t*-Leu (40.7).

Partial Acid Hydrolysis. A solution of 10 mg of polytheonamide B in 2 mL each of EtOH and 4N HCl was heated at 70 °C for 2 h in a sealed tube. After cooling to room temperature, the solution was evaporated to dryness under reduced pressure. The product was dissolved in 0.5 mL of DMSO and subjected to ODS HPLC (column size, 0.5 × 25 cm; flow rate 1.5 mL/min; first eluted with 0.05% TFA for 20 min, and using a linear gradient from 0% to 40% MeCN in 0.05% TFA in 30 min followed by a linear gradient from 40% to 80% MeCN in 0.05% TFA in 20 min). One-hundred twenty fractions were obtained and analyzed by FABMS (pos.) and FABMS/MS.

Determination of the Chirality of the *N*-Terminal Residue. To a fragment peptide (ca. 500 nM) were added 0.3 mL of 0.1M $NaHCO_3$−Na_2CO_3 buffer (pH 8.9) and 10 *μ*M dansyl chloride-acetone solution (0.3 mL). After stirring for 6 min at 70 °C, the solvent was removed under reduced pressure. The product was dissolved in a small amount of DMSO and separated by ODS HPLC (Cosmosil $5C_{18}$ MS; 0.5 × 25 cm column; linear gradient from MeCN/H_2O/TFA (20:80:0.1) to MeCN/H_2O/TFA (80:20:0.1) in 50 min; 1.5 mL/min; UV detection at 340 nm). The dansylated peptide was analyzed by FAB MS and subjected to total acid hydrolysis (6N HCl, 110 °C, 24 h) to liberate a dansylated amino acid, which was analyzed by chiral HPLC [Sumichiral OA-3200, 0.5 × 25 cm; elution with a solution of 0.01% $AcONH_4$ in MeOH (1 mL/min); detection was with UV absorption at 254 nm]. Each peak was identified by comparing the retention time with authentic samples.

Edman Degradation. A peptide (ca. 100 *μ*g) was dissolved in 10 *μ*L of EtOH/triethylamine/H_2O/phenyl isothiocyanate (7:1:1:1), and allowed to stand for 30 min at 50 °C under a nitrogen atmosphere. The reaction mixture was centrifuged after addition of water (2.5 *μ*L); the suspension was washed three times with *n*-heptane/EtOAc (15:1) and 4 times with *n*-heptane/EtOAc (1:1). The organic phases, containing excess reagent, were discarded and the aqueous phase was evaporated to dryness. The phenylthiocarbamylpeptide thus obtained was treated with neat TFA (10 *μ*L) under a nitrogen atmosphere at 50 °C for 7 min. The TFA was removed in vacuo, and the residue was kept at 50 °C for 2 min in MeOH (100 *μ*L). After concentration, the residue was mixed with 4 *μ*L of H_2O and 40 *μ*L of benzene/MeCN (2:1) and centrifuged. The lower layer containing the truncated peptide was evaporated to dryness and subjected to dansylation, as described above.

Reduction of Polytheonamide B with $NaBH_4$. To a 1.5 mL solution of polytheonamide B (20 mg) in EtOH was added $NaBH_4$ (5 mg), and the solution was stirred at room temperature for 1 h. After dilution with 5% AcOH in H_2O, the solution was subjected to reversed-phase HPLC with *n*-PrOH/H_2O/TFA (40:60:0.05) to afford dihydropolytheonamide B1 (4.6 mg) and dihydropolytheonamide B2 (2.5 mg). For the NMR spectra of these peptides, see Supporting Information.

***des*-Thr48-dihydropolytheonamide B2.** During purification of dihydropolytheonamide B2 by HPLC mentioned above, ca. 30% of the peptide had been converted to *des*-Thr48-dihydropolytheonamide

Scheme 1. Reactions for stereochemical assignment of $Me_2Met(O)$

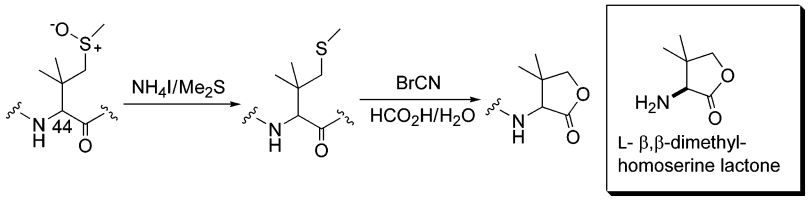

Table 3. ^{1}H NMR Chemical Shift of Polytheonamide A in DMSO-d_6 at 313K[a]

residue	NH	$C_\alpha H$	$C_\beta H$	others
Gly1	8.54	3.80		
βMeIle2	7.81	4.28		$C_\gamma H_3$0.85; $C_\gamma H_3$0.85; $C_\gamma H_2$1.23; $C_\delta H_3$0.82
Gly3	8.29	3.60, 3.77		
t-Leu4	7.53	4.57		$C_\gamma H_3$0.85
t-Leu5	8.05	4.16		$C_\gamma H_3$0.89
t-Leu6	7.32	4.22		$C_\gamma H_3$0.82
Ala7	7.95	4.44	1.17	
t-Leu8	7.64	4.42		$C_\gamma H_3$0.84
t-Leu9	7.83	4.16		$C_\gamma H_3$0.87
Ala10	8.12	4.28	1.18	
Gly11	8.09	3.63, 3.76		
Ala12	7.90	4.43	1.18	
t-Leu13	7.73	4.24		$C_\gamma H_3$0.84
Ala14	8.20	4.24	1.14	
Asm15	8.14	4.63	2.36, 2.50	
OHVal16	7.70	4.18	1.04, 1.08	
Gly17	8.08	3.72		
Ala18	8.00	4.21	1.18	
Gly19	8.23	3.61, 3.88		
t-Leu20	7.65	4.10		$C_\gamma H_3$0.83
Asm21	8.32	4.59	2.35, 2.50	
βMeGln22	7.72	4.28	2.29	$C_\gamma H_3$0.77; $C_\gamma H_2$1.79, 2.09; NH_2 6.71, 7.22
OHVal23	8.02	4.24		$C_\gamma H_3$1.05; $C_\gamma H_3$1.12
Ala24	7.87	4.31	1.18	
Gly25	8.21	3.71		
Gly26	8.04	3.72		
Asm27	8.03	4.65	2.36, 2.45	
Ile28	7.67	4.26	1.65	$C_\gamma H_3$0.96; $C_\gamma H_2$1.29; $C_\delta H_3$0.69
OHAsm29	7.96	4.63	4.35	
t-Leu30	7.24	4.44		$C_\gamma H_3$0.83
OHVal31	8.02	4.27		$C_\gamma H_3$1.12; $C_\gamma H_3$1.12
Gly32	8.10	3.63, 3.81		
Asm33	8.06	4.62	2.37, 2.45	
Ile34	7.61	4.10	1.65	$C_\gamma H_3$0.98; $C_\gamma H_2$1.32; $C_\delta H_3$0.70
Asm35	8.17	4.65	2.35, 2.50	
Val36	7.64	4.06	1.87	$C_\gamma H_3$0.72; $C_\gamma H_3$0.72
OHAsm37	7.98	4.62	4.39	
Ala38	7.68	4.21	1.18	
Asm39	8.01	4.62	2.38, 2.51	
Val40	7.62	4.21	1.97	$C_\gamma H_3$0.76; $C_\gamma H_3$0.76
Ser41	8.05	4.40	3.51, 3.56	
Val42	7.80	4.21	1.93	$C_\gamma H_3$0.78; $C_\gamma H_3$0.78
Asn43	8.24	4.63	2.41, 2.52	NH_2 6.85, 7.25
Me_2Met(O)44	7.91	4.24		$C_\gamma H_3$1.02; $C_\gamma H_3$1.08; $C_\gamma H_2$2.75, 2.85; $C_\epsilon H_3$2.60
Asn45	8.35	4.54	2.42, 2.54	NH_2 6.85, 7.31
Gln46	7.86	4.29	2.06	$C_\gamma H_2$1.74, 1.88; NH_2 6.65, 7.11
*a*Thr 47	7.99	4.31	3.86	$C_\gamma H_3$1.03
Thr48	7.68	4.15	4.08	$C_\gamma H_3$1.02

[a] *N*-methyl amides in the side chain: NH/CH_3, 7.62/2.55, 7.63/2.55, 7.65/2.54, 7.66/2.52, 7.71/2.54, 7.72/2.54, 7.75/2.56, 7.79/2.55.

B2 [FABMS (negative mode, triethanolamine) *m*/*z* 4935 $(M-H)^-$] which eluted faster than dihydropolytheonamide B2.

Hydrolysis with DCl/D_2O. A 200 μg portion of the peptide was hydrolyzed with 6N DCl at 110 °C for 10 h. The acid hydrolysate was processed in the same way as mentioned for the chiral GCMS analysis. The retention times of all amino acid derivatives were identical with those mentioned above. GCMS of D-Asp: *m*/*z* 255 $[M^+ - 59\ (OC_3H_7)]$, 227 $[M^+ - 87\ (CO_2C_3H_7)]$, 213 $[M^+ - 101\ (CO_2C_3H_7 + CH_3 - 1)]$, 185 $[M^+ - 129\ (CO_2C_3H_7 + C_3H_7 - 1)]$, 140 $[M^+ - 174\ (2 \times CO_2C_3H_7)]$, 69 $[(CF_3)^+]$. GCMS of L-Asp: *m*/*z* 228 $[M^+ - 87\ (CO_2C_3H_7)]$, 214 $[M^+ - 101\ (CO_2C_3H_7 + CH_3 - 1)]$, 186 $[M^+ - 129\ (CO_2C_3H_7 + C_3H_7 - 1)]$, 141 $[M^+ - 174\ (2 \times CO_2C_3H_7)]$, 69 $[(CF_3)^+]$.

Reduction of Polytheonamides A and B with NH_4I/Me_2S. To a solution of polytheonamide B in TFA was added NH_4I (0.8 mg) and Me_2S (2 μL) and the mixture kept at 0 °C for 1h and 4 °C overnight. After addition of 10 mg of NH_4OAc, the mixture was diluted with H_2O and subjected to ODS column chromatography. Inorganic salts were eluted with *n*-PrOH/H_2O (2:8) and the peptides were eluted with *n*-PrOH/H_2O (3:1). The latter fraction was further purified by ODS HPLC with *n*-PrOH/H_2O/AcOH (4:6:0.2) to afford the sulfide [FABMS (positive, nitrobenzyl alcohol with CsI), *m*/*z* 5151 $(M + Cs)^+$, 5282 $(M + 2Cs-H)^+$]. Polytheonamide A (2 mg) was similarly processed to afford a peak indistinguishable from the one prepared from polytheonamide B.

BrCN treatment of the sulfide. To a solution of the above-mentioned sulfide in HCO_2H/H_2O (7:3) was added BrCN (7.8 mg), and the mixture kept at room temperature overnight in the dark. The reaction mixture was evaporated and subjected to ODS HPLC with *n*-PrOH/H_2O/AcOH (45:55:2) to afford a single peak [FABMS (positive, nitrobenzyl alcohol with CsI), *m*/*z* 4657 $(M+Cs)^+$].

Oxidation of Polytheonamides. To a solution of polytheonamide B (2 mg) in *n*-PrOH/H_2O (3:1, 0.5 mL) was added Oxone (5 mg in 100 μL of H_2O) and the mixrue left at room temperature for 2 h. The solution was diluted with H_2O and subjected to ODS HPLC with *n*-PrOH/H_2O/AcOH (45:55:2) to afford a single peak [FABMS (negative, nitrobenzyl alcohol with CsI), *m*/*z* 5047 $(M - H)^-$, 5308 $(M + CsI-H)^-$] which eluted between polytheonamides A and B. Polytheonamide A was processed in the same way to afford a product indistinguishable from the one prepared from polytheonamide B in HPLC, MS, and ^{1}H NMR data (Figure S53).

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Supporting Information Available: (1) spectral data and ^{13}C NMR chemical shift Table of polytheonamides B, (2) spectral data of polytheonamides A, (3) amino acid analysis, elemental analysis, spectral data and ^{1}H NMR chemical shift Table of acid hydrolysate of polytheonamide B, (4) spectral data of dihydropolytheonamide B1, (5) chiral GCMS data of trifluoroacetyl isopropyl ester derivatives and HPLC charts of FDAA derivatives of polytheonamide B, (6) FABMS/MS spectra of

fragment peptides of polytheonamide B by the partial acid hydrolysis [4N HCl/EtOH (1:1), 70 °C, 30 min.], (7) *N*-terminal analysis, Marfey analysis and chiral GCMS data for determining chiralities of amino acid residues in polytheonamide B, (8) Experimental Section describing synthesis of βMeGlu, (9) spectral data of *des*-Thr48-dihydropolytheonamide B2, (10) GCMS data of D- and L-Asp derivatives after DCl hydrolysis, (11) spectral data for determining the stereochemistry of Me_2Met(O)44 in polytheonamide B, (12) spectral data of the oxidized products of polytheonamides A and B by Oxone (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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