Tetrahedron Vol. 47, No. 12/13, pp. 2169–2180, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00
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MARINE NATURAL PRODUCTS. XXV. BIOLOGICALLY ACTIVE TRIDECAPEPTIDE LACTONES FROM THE OKINAWAN MARINE SPONGE THEONELLA SWINHOEI (THEONELLIDAE)(1). STRUCTURE OF THEONELLAPEPTOLIDE ID

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(*Received in Japan* 10 *November* 1990)

Abstract --- Theonellapeptolide Id (1), a tridecapeptide lactone, was isolated from the Okinawan marine sponge Theonella swinhoei and the structure 1 was determined. A new HPLC analysis method for analyzing the amino acid composition of 1 containing N-methyl amino acids was devised.

During the course of our study on bioactive marine natural products, we have been investigating the chemical constituents of marine organisms inhabiting the coral reefs in Okinawa Prefecture.[1,2)]

From the marine sponge Theonella swinhoei (Theonellidae), we have isolated a potent cytotoxic dimeric macrolide swinholide A[3)] and five new tridecapeptide lactones named theonellapeptolides Ia, Ib, Ic, Id, and Ie. In this paper, we report the isolation of theonellapeptolides Ia, Ib, Ic, Id, and Ie and describe the full evidence consistent with the structure 1 of the major tridecapeptide lactone theonellapeptolide Id.[4,5)]

The acetone extract of the fresh marine sponge Theonella swinhoei, which was collected in July in the coral reef of Zamami-jima, Okinawa Prefecture, was partitioned into an ethyl acetate-water mixture and the ethyl acetate soluble portion was subjected to silica gel column chromatography to separate a macrolide fraction containing swinholide A and two peptide fractions (Chart 1). The more polar peptide fraction (theonellapeptolide I) was further purified by high-performance liquid chromatography (HPLC) to furnish theonellapeptolides Ia, Ib, Ic, Id (1), and Ie (0.04, 0.08, 0.05, 2.20, and 0.29% from the ethyl acetate soluble portion). Theonellapeptolides Ia, Ib, Ic, Id, and Ie were found to inhibit development of the fertilized eggs of the sea urchin Hemicentrotus pulcherrimus[6)] at 2, 2, 2, 50, and 10 μg/ml concentrations, respectively.

The major peptide theonellapeptolide Id (1), which was obtained as

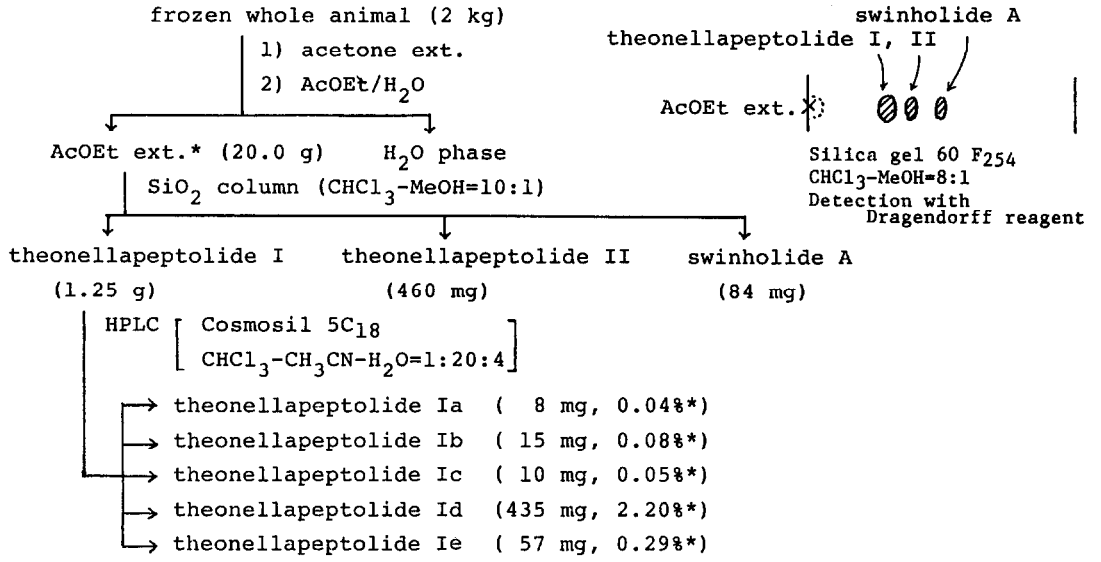
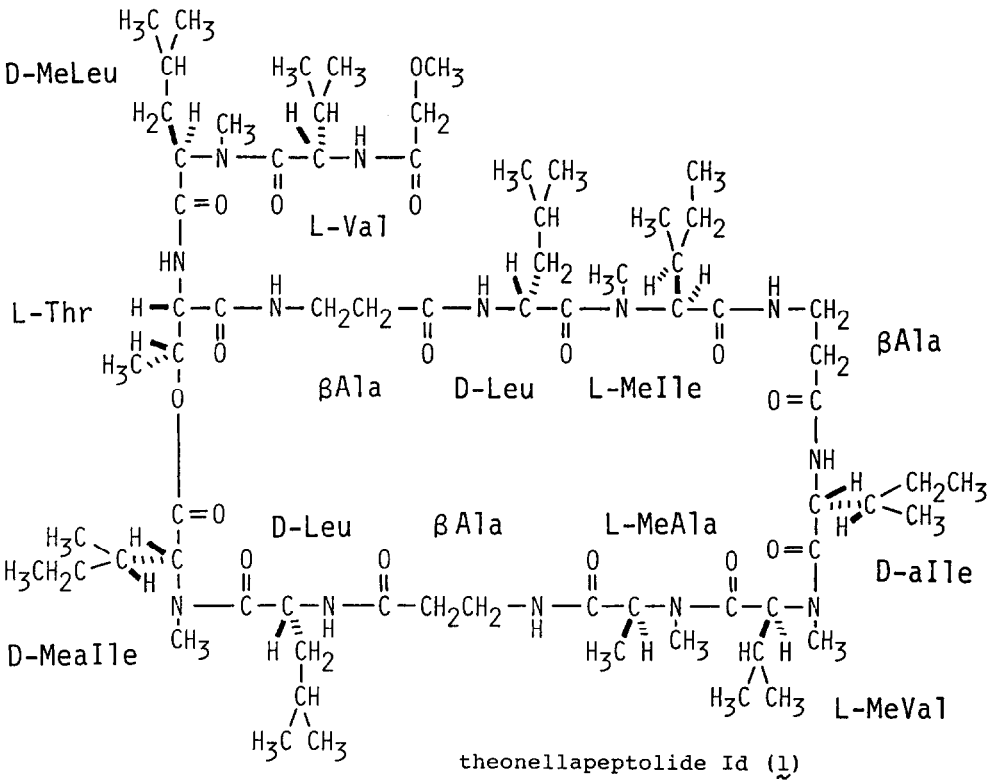


Chart 1. Isolation Procedure for Theonellapeptolides.
The % yields(*) were calculated from the AcOEt ext.



theonellapeptolide Id (1)

colorless needles of mp 168-169°C, is characterized by its lipophilic solubility. The fast atom bombardment mass spectrum (FAB-MS) of 1 gave a quasimolecular ion $(M+H)^+$ peak at m/z 1404, while the infra-red (IR) spectrum of 1 showed absorption bands due to amide groups (3330, 1680, 1540 cm^{-1}) and a lactone moiety (1740 cm^{-1}). The proton nuclear magnetic resonance (^1H NMR) spectrum of 1 showed the signals of eight amide protons, five N-methylprotons, and one methoxyacetyl group [δ 3.43 (3H,s), 3.97 (1H, d, J= 15.3 Hz), 3.90 (1H, d, J=15.3 Hz)]. The carbon-13 nuclear magnetic resonance (^{13}C NMR) spectrum of 1 showed complicated signals, some of which were assignable to amide and lactone carbonyls, together with signals due to a methoxyacetyl group [δ_c 60.4 (q), 73.2 (t)]. These findings led to a presumption that theonellapeptolide Id (1) is an oligopeptide containing five N-methyl amino acids and one methoxyacetyl moiety. Furthermore, 1 was negative in the ninhydrin test and was unaffected by diazomethane treatment. So that, the structure of 1 was presumed to be a peptide lactone (peptolide) in which the C-terminal amino acid participates in the lactone ring and the N-terminal amino acid is blocked with a methoxyacetyl group.

Complete acidic hydrolysis of theonellapeptolide Id (1) followed by amino acid analysis revealed only the composition of eight ordinary amino acids [threonine (Thr)(1), β-alanine (βAla)(3), valine (Val)(1), leucine (Leu)(2), alloisoleucine (aIle)(1)] except for the N-methyl amino acid constituents. In order to determine the total amino acid composition including the N-methyl amino acids and the absolute configurations of those α-amino acids, we have devised a new amino acid analysis method using HPLC. Thus, α-amino acid and N-methyl α-amino acid have a strong ultra-violet (UV) absorption maximum at ca. 185 nm due to $\pi \rightarrow \pi^*$ transition of the carboxyl group.[7] On the other hand, the circular dichroism (CD) spectrum of L-α-amino acid and N-methyl L-α-amino acid shows a positive CD maximum, whereas that of D-α-amino acid and N-methyl D-α-amino acid shows a negative CD maximum.[8] The acid hydrolysate of theonellapeptolide Id (1) obtained above was subjected to reversed phase HPLC using a Cosmosil $5C_{18}$ column eluting with water and detecting with UV absorption at 205 nm, which gave a chromatogram as depicted in Fig 1. Each amino acid eluted from the HPLC column was identified with the authentic sample. The retention time and the peak area of each peak indicate the amino acid composition. In addition, each peak was collected to measure the CD spectrum in 0.5 N aqueous HCl solution[8] for determination of the absolute configuration of the eluted amino acid. Consequently, the amino acid composition of theonellapeptolide Id (1) was determined as βAla (3), L-Thr (1), N-methyl-L-alanine (L-MeAla) (1), L-Val (1), N-methyl-L-valine (L-MeVal)(1), D-aIle (1), D-Leu (2), N-methyl-D-alloisoleucine (D-MeaIle)(1), N-methyl-L-isoleucine (L-MeIle)(1),

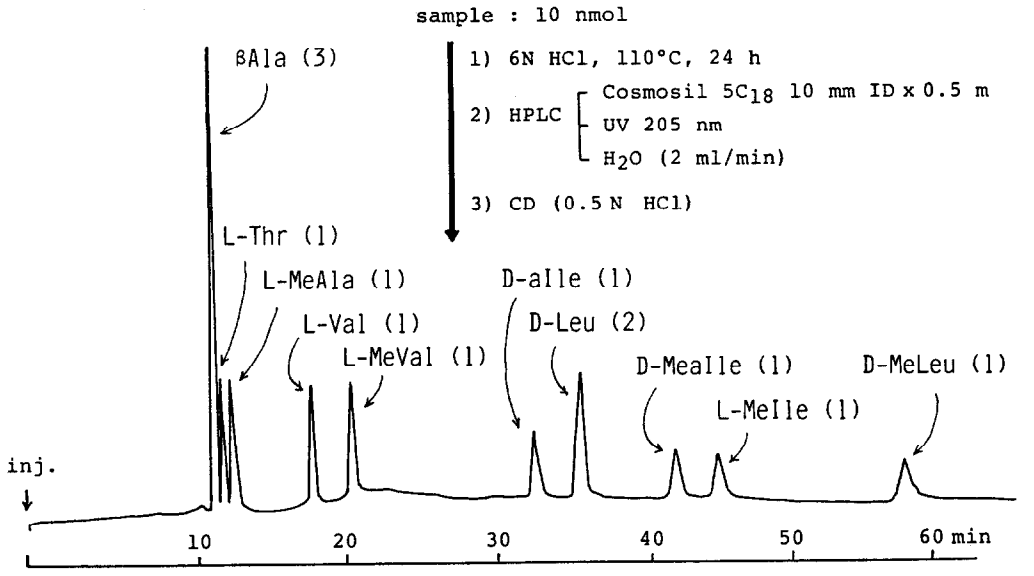


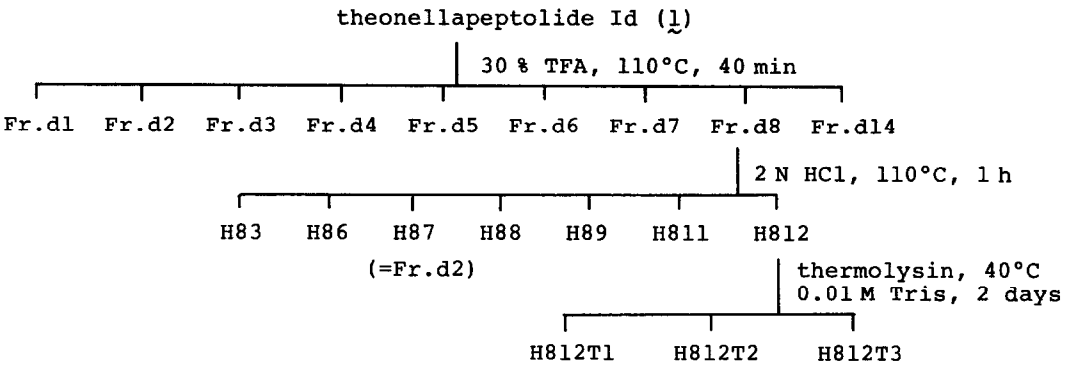
Fig. 1 HPLC of Complete Acidic Hydrolysate of Theonellapeptolide Id (1)

and N-methyl-D-leucine (D-MeLeu)(1).

In order to determine the amino acid sequence of theonellapeptolide Id (1), 1 was subjected to partial acidic hydrolysis with 30% aqueous trifluoro acetic acid (TFA) at 110°C[9] to afford various fragments which were separated by HPLC (ZORBAX ODS). Among these partial hydrolysates, Fr.d1 (2), Fr.d2 (12), Fr.d3 (3), Fr.d4 (4), Fr.d5 (5), Fr.d6 (6), Fr.d7 (7), Fr.d8 (8), and Fr.d14 (9) were analyzed. The chemical structures of these partial hydrolysates were determined on the basis of FAB-MS data, ^{1}H NMR analysis, N-terminal analysis of their dansyl derivatives, and above-described amino acid analysis using HPLC, respectively.

For example, the N-terminal amino acid of Fr.d1 (2) [the amino acid composition: βAla (1), aIle (1)] was determined βAla by dansyl derivation analysis of 2. The structure of Fr.d3 was elucidated as 3 by analyzing the FAB-MS spectrum which showed fragment ion peaks at m/z 132 $[M-(\beta Ala+aIle)+H]^+$ and m/z 245 $[M-\beta Ala+H]^+$ together with the quasimolecular ion peak at m/z 316 $(M+H)^+$ and also by the N-terminal analysis. The structures of Fr.d4 (4) and Fr.d5 (5) were determined in the similar manner as for Fr.d3 (3), respectively. Thus, the presence of a partial sequence A [Leu → MeIle → βAla → aIle → MeVal] in 1 was substantiated.

The FAB-MS of Fr.d8 (8) gave the $(M+H)^+$ ion peak at m/z 998, while the ^{1}H NMR spectrum of 8 showed a lower-shifted signal (δ5.34, 1H, m) assignable to a proton geminal to a hydroxyl group in Thr and those of a methoxyacetyl group (δ 3.43, 3H, s; δ 3.97, 2H, s). From these findings, it was presumed



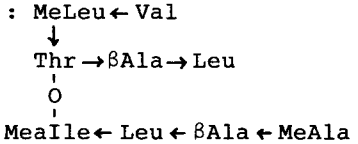
Fr.d1 (2): βAla → aIle

Fr.d3 (3): βAla → aIle → MeVal

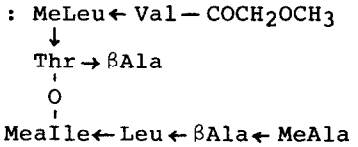
Fr.d4 (4): MeIle → βAla → aIle

Fr.d5 (5): Leu → MeIle → βAla → aIle

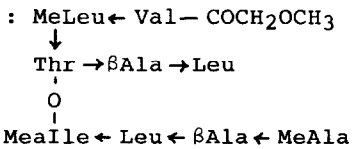
Fr.d6 (6):



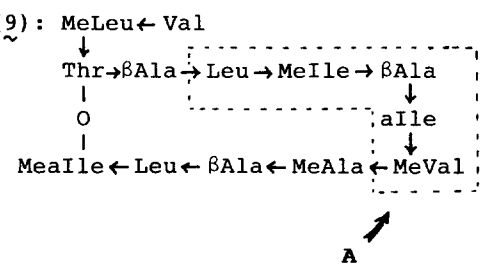
Fr.d7 (7):



Fr.d8 (8):

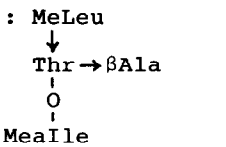


Fr.d14 (9):



H83 (10): Val — $COCH_2OCH_3$

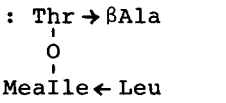
H86 (11):



H87 (12) (= Fr.d2):

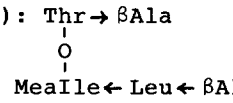
Leu ← βAla ← MeAla

H88 (13):

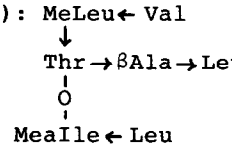


H89 (14): MeLeu ← Val

H811 (15):



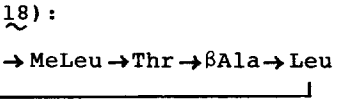
H812 (16):



H812T1 (17):

Thr → βAla → Leu

H812T2 (18):



H812T3 (19): MeaIle ← Leu

Chart 2

that Fr.d8 (8) is a peptide comprising two peptide chains and the hydroxyl group of threonine in one peptide chain is esterified with a carboxyl group of the C-terminal of another one. Comparison of the amino acid composition of Fr.d6 (6), Fr.d7 (7), and Fr.d8 (8) led us to presume that the N-terminal of one of the peptide chains in 8, which is blocked by a methoxyacetyl group, is Val and the C-terminal of the same peptide chain is Leu.

Acidic hydrolysis of Fr.d8 (8) with 2 N aqueous HCl at 110°C yielded various fragments which were separated by HPLC (ZORBAX ODS). Among these fragments, seven fragments H83 (10), H86 (11), H87 (12), H88 (13), H89 (14), H811 (15), and H812 (16) were utilized for the structure elucidation of Fr.d8 (8).

The structure of H83 was elucidated as 10 from the ^{1}H NMR spectrum which showed signals of Val and a methoxyacetyl group (δ 3.43, 3H, s; δ 4.07, 2H, s). So that, it was concluded that the N-terminal of theonellapeptolide Id (1) is Val whose amino group is blocked with a methoxyacetyl group. Next, the ^{1}H NMR spectra of H86 (11) and H88 (13) showed lower-shifted proton signals (δ 5.58, 1H, m in 11 and δ 5.50, 1H, m in 13, respectively) which were assigned as the β-proton of threonine. Furthermore, the structure of H87 (12), which was identical with Fr.d2, was elucidated by examination of the solvent-induced shift[10] in the ^{1}H NMR spectra of 12. Thus, the α-proton signal of Leu in H87 measured in DCl-D_2O (pH 1) was observed at lower field (ca. 0.15 ppm) as compared to that measured in D_2O, and the α-proton signal of MeAla in H87 measured in NaOD-D_2O (pH 14) was observed at higher field (ca. 0.7 ppm) as compared to that measured in D_2O. Consequently, comparison of the amino acid composition of H86, H87, H88, H89 (14), and H811 led us to presume that the structures of H86, H88, and H811 were respectively expressed as 11, 13, and 15, in which the location of βAla was substantiated by comparison with the structure of H812T1 (17) described below.

In order to determine the structure of H812 (16) which possesses two N-terminals of Val and Leu, 16 was further subjected to enzymatic hydrolysis using thermolysin to furnish three fragments: H812T1 (17), H812T2 (18), and H812T3 (19). Taking into consideration of the structural relation of H86 (11) and H88 (13), the structure of H812T1 was determined as 17. The FAB-MS spectra of H812T2 (18) and H812T3 (19) showed the $(M+H)^+$ ion peaks at m/z 512 and m/z 241, respectively, but the N-terminal analysis of 18 and 19 gave a negative result. So that, it was considered that H812T2 (18) and H812T3 (19) possess cyclic structures, respectively and H812T2 (18) and H812T3 (19) were presumed to be secondary products formed during the process of enzymatic hydrolysis.

Based on the elucidated amino acid sequence of H83 (10), H86 (11), H87 (12), H88 (13), H89 (14), H811 (15), and H812T1 (17), the structures of H812

and Fr.d8 were determined to be expressed as 16 and 8, respectively.

Finally, combination of the partial sequence A (vide supra) and Fr.d8 (8) led us to determine the amino acid sequences of Fr.d6 (6), Fr.d7 (7), Fr.d14 (9), and theonellapeptolide Id (1). Fr.d14 (9) corresponds to a desmethoxyacetyl derivative of 1.

Furthermore, in order to make clear the absolute configuration of two Leu units contained in theonellapeptolide Id (1), Fr.d2 (12) and H812T1 (17) were hydrolyzed with 6 N HCl at 110°C and the hydrolysates were subjected to above-mentioned HPLC analysis, respectively. The CD spectra taken in 0.5 N HCl of both Leu, obtained from 12 and 17, showed a negative maximum at 210 nm which indicated both Leu to be a D-α-amino acid.

In addition, treatment of theonellapeptolide Id (1) with sodium methoxide in methanol furnished the acyclic methyl ester 20. The FAB-MS of 20 provided several fragment ion peaks which were consistent with the amino acid sequence of theonellapeptolide Id (1) as shown in Fig. 2.

Theonellapeptolide Id (1) was a limited number of examples of peptides comprising N-methyl amino acid and D-amino acid in high ratio.[13] It is interestingly mentioned here that theonellapeptolide Id (1) showed ion-transport activities for Na^+, K^+, and Ca^{++} ions.[11,12] Furthermore, Ohizumi and his group reported independently that 1 exhibited a significant inhibitory effect against Na^+,K^+-ATPase (ED_{50} 6 x 10^{-6} M).[14,15]

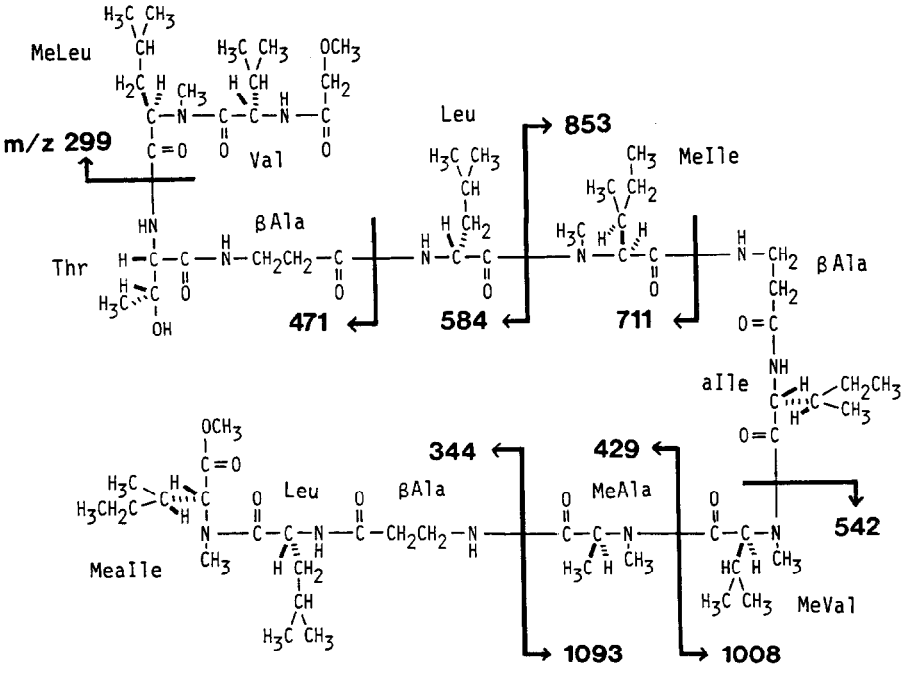


Fig. 2 Mass Spectral Fragmentation Patterns of 20

EXPERIMENTAL

Instrumentation IR spectra were obtained using a Hitachi 260-30 grating spectrometer. Optical rotations were measured with a JASCO DIP-181 digital polarimeter. ^{1}H NMR and ^{13}C NMR spectra were measured with JEOL JNM FX-500S (500 MHz and 125 MHz) spectrometers with Me_4Si as the internal standard. UV spectra were obtained using a Hitachi 330 spectrometer. CD spectra were taken on a JASCO J-500A spectrometer and a JASCO DP-501 data processor. EIMS and FAB-MS spectra were measured with a JEOL D-300 mass spectrometer and a JEOL JMS-HX100 mass spectrometer. Melting points were measured using a Yanagimoto Micro-meltingpoint Apparatus and recorded uncorrected. HPLC was carried out using Shimadzu LC-6A and Chromatopak C-R6A chromatographs.

Isolation of theonellapeptolides Ia, Ib, Ic, Id (1), and Ie Fresh whole bodies of the marine sponge Theonella swinhoei (2 kg, collected in July in Zamami-jima, Okinawa Prefecture) were cut and extracted with acetone three times (5 l each) at room temperature. Removal of the solvent under reduced pressure from the combined extracts provided the acetone extract which was partitioned into an ethyl acetate-water mixture. The ethyl acetate phase was separated and evaporated under reduced pressure to furnish the ethyl acetate soluble portion (20 g). The ethyl acetate soluble portion was subjected repeatedly to column chromatography (Kieselgel 60, n-hexane-AcOEt=1:2 and $CHCl_3$-MeOH=10:1) to furnish a macrolide fraction[3] (84 mg) and two peptide fractions (less-polar one 460 mg, more-polar one 1.25 g). The more polar peptide fraction (1.25 g) was further subjected to HPLC (Cosmosil $5C_{18}$ 10 mm x 25 cm, $CHCl_3$-CH_3CN-H_2O=1:20:4) to furnish theonellapeptolides Ia (8 mg), Ib (15 mg), Ic (10 mg), Id (435 mg), and Ie (57 mg). Theonellapeptolide Ia, colorless needles, mp 156-157°C (MeOH-H_2O), $[\alpha]_D^{20}$ -58° (MeOH, c=1.4). Theonellapeptolide Ib, colorless needles, mp 159°C (MeOH-H_2O), $[\alpha]_D^{20}$ -54° (MeOH, c=1.8). Theonellapeptolide Ic, colorless needles, mp 147°C (MeOH-H_2O), $[\alpha]_D^{20}$ -50° (MeOH, c=1.1). Theonellapeptolide Id (1), colorless needles, mp 168-169°C (MeOH-H_2O), $[\alpha]_D^{20}$ -68°C (MeOH). FAB-MS: 1404 $(M+H)^+$. IR ($CHCl_3$): 3330, 1740, 1680, 1540 cm^{-1}. ^{1}H NMR ($CDCl_3$-CD_3OD=10:1, δ): amide protons [8.70 (1H, d, J=9.5 Hz), 8.51 (1H, d, J=9.5 Hz), 8.43 (1H, d, J=9.2 Hz), 8.35 (1H, d, J=8.5 Hz), 7.42 (1H, d, J=8.9 Hz), 7.38 (1H, brd, J=5.2 Hz), 7.22 (1H, brd, J=7.6 Hz), 7.18 (1H, t-like)], N-methyl protons [3.30, 3.29, 3.24, 3.20, 2.75 (all 3H, s)], methoxyacetyl [3.43 (3H, s), 3.97 (1H, d, J=15.3 Hz), 3.90 (1H, d, J=15.3 Hz)]. ^{13}C NMR (CD_3OD, δ_C): 176.7, 176.5, 176.1, 175.7, 175.0, 174.6, 174.2, 173.5, 173.2, 173.1, 172.5, 172.4, 172.1, 171.0 (all s), 73.2 (t), 60.4 (q) (methoxyacetyl). Theonellapeptolide Ie, colorless needles, mp 153-155°C (MeOH-H_2O), $[\alpha]_D^{20}$ -62° (MeOH, c=0.2).

Complete acidic hydrolysis of theonellapeptolide Id (1) 10 nM of 1 was treated with 6 N aqueous HCl (250 µl) and after degasing the reaction mixture was heated in a sealed tube at 110°C for 24 h. After cooling the reaction mixture was evaporated *in vacuo* to give the residue which was analyzed by an auto-amino acid-analysis system to reveal the amino acid composition as Thr (1), βAla (3), Val (1), Leu (2), and aIle (1). 10 nM of other partial hydrolysates were also hydrolyzed in the same manner as described here.

Dansyl derivation analysis procedure 1) Each sample (*ca.* 5 nM) was treated with 0.2 M aqueous $NaHCO_3$ (10 µl) and the mixture was evaporated *in vacuo*. The residue was treated with water (10 µl) and a 10 mM dansyl chloride-acetone solution (10 µl) and after mixing the reaction mixture was kept at 37°C for 1 h. Removal of the solvent from the reaction mixture under reduced pressure gave a crude product. The crude product was subjected to complete acidic hydrolysis (6 N aqueous HCl, 110°C, 24 h, in a sealed tube) to furnish a dansyl derivative of the N-terminal amino acid. The dansyl derivative was analyzed by polyamide TLC plate [Polyamide Precoated for Thin-layer Chromatography, Aluminum Sheet (Merck), developed in

two dimensions one with benzene-acetic acid (1:1) and another with 1.5 % aqueous HCOOH]. 2) Each sample (ca. 10 nM) was subjected to complete acidic hydrolysis as described above. The residue obtained by evaporation of the reaction mixture under reduced pressure was treated with 0.2 M aqueous $NaHCO_3$ (10 µl) and a 10 mM dansyl chloride-acetone solution (10 µl). After mixing the reaction mixture was kept at 37°C for 1 h. Evaporation under reduced pressure of the reaction mixture gave dansyl derivatives of respective amino acids which were analyzed by polyamide TLC as described above.

Amino acid analysis using HPLC for complete acidic hydrolysates
10 nM of the acidic hydrolysate obtained by complete acidic hydrolysis of theonellapeptolide Id (1) described above was subjected to HPLC analysis [Cosmosil $5C_{18}$ 10 mm x 50 cm; elution with water (2 ml/min); detection with UV absorption at 205 nm]. The amino acid composition of 1: βAla (peak area: 26646): t_R=12'44"; Thr (9606): t_R= 13'10"; MeAla (9754): t_R=13'51"; Val (9203): t_R=18'20"; MeVal (10853): t_R=21'09"; aIle (8319): t_R=32'22"; Leu (18772): t_R=35'25"; MeaIle (9346): t_R=41'00"; MeIle (8754): t_R=43'57"; MeLeu (8841): t_R=55'22". Each peak was identified with an authentic sample. The peak area was calculated with Chromatopak C-R6A (Shimadzu). Each amino acid eluted from the column was collected repeatedly and the CD spectrum was measured in 0.5 N HCl. The absolute configuration of each amino acid was determined by the sign of the CD maximum observed at 210 nm. Pos. max.: L-Thr, L-MeAla, L-Val, L-MeVal, L-MeIle. Neg. Max.: D-aIle, D-Leu, D-MeaIle, D-MeLeu.

Synthesis of N-methyl-L-valine (L-MeVal) L-Valine (50 mg) was treated with 2 N aqueous NaOH (2 ml) and acetic anhydride (2 ml) at 20°C for 1 h. The reaction mixture was neutralized with 6 N aqueous HCl and the solvent was evaporated off under reduced pressure. A solution of the residue in tetrahydrofuran (THF)-dimethylformamide (DMF)(10:1, 5 ml) was treated with CH_3I (2 ml) and NaH (0.3 g) and the reaction mixture was refluxed for 24 h. The reaction mixture was then poured into water and the whole was extracted with ether. The ether extract was washed with water and dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a crude product which was purified by silica gel column chromatography (n-hexane-AcOEt=10:1). The product obtained was treated with 6 N aqueous HCl (0.5 ml) at 110°C for 24 h in a sealed tube. A residue obtained by evaporation under reduced pressure of the reaction mixture was purified by silica gel column chromatography (AcOEt→CH_3CN→H_2O) and HPLC (Cosmosil $5C_{18}$, water) to furnish N-methyl-L-valine (12 mg)(confirmed by CD).

Syntheses of N-methyl-D-alloisoleucine (D-MeaIle), N-methyl-L-isoleucine (L-MeIle), and N-methyl-D-leucine (D-MeLeu) D-Alloisoleucine (73 mg) was dansylated as described above [0.2 M aqueous $NaHCO_3$ (200 ml), dansyl chloride (500 mg) in acetone (200 ml), 37°C, 1 h]. The reaction mixture was evaporated in vacuo and then extracted with methanol (5 ml). The residue obtained by evaporation under reduced pressure of the methanol solution was dissolved in THF (10 ml) and treated with CH_3I (10 ml) and NaH (500 mg) at 20°C for 1 h. The reaction mixture was poured into water and the whole was extracted with ethyl acetate. The ethyl acetate phase was separated and washed with water and evaporated under reduced pressure to give a crude product which was purified by silica gel column chromatography ($CHCl_3$-MeOH= 20:1). The product was hydrolyzed with conc. HCl (1 ml) at 110°C for 24 h in a sealed tube. The reaction mixture was evaporated under reduced pressure and the residue was subjected to silica gel column chromatography (AcOEt→CH_3CN→H_2O) to give N-methyl-D-alloisoleucine (17 mg). N-Methyl-L-isoleucine (13 mg) and N-methyl-D-leucine (16 mg) were also synthesized in the same manner as described here from L-isoleucine (50 mg) and D-leucine (50 mg), respectively.

Partial acidic hydrolysis of theonellapeptolide Id (1) 1 (100 mg) was

dissolved in 30 % aqueous TFA (5 ml) and after degassing the whole was heated at 110°C for 40 min in a sealed tube. The residue obtained by evaporation of the reaction mixture under reduced pressure was subjected to HPLC[ZORBAX ODS, eluted gradiently with 0.1 % aqueous TFA→2-PrOH-CH_3CN (7:3) containing 0.1 % TFA] to furnish partial hydrolysates: Fr.d1 (2) (3 mg), Fr.d2 (12) (3 mg), Fr.d3 (3) (2 mg), Fr.d4 (4) (2 mg), Fr.d5 (5) (1 mg), Fr.d6 (6) (5 mg), Fr.d7 (7) (4 mg), Fr.d8 (8) (13 mg), and Fr.d14 (9) (1 mg). Fr.d1 (2), FAB-MS: m/z 203 $(M+H)^+$, [amino acid composition: βAla (1), aIle (1)], N-terminal amino acid: βAla. ^1H NMR (D_2O, δ): βAla [3.26 (2H, t, J=6.5 Hz, β-H), 2.77 (2H, t, J=6.5 Hz, α-H)], aIle [4.46 (1H, d, J=4.6 Hz, α-H), 2.02 (1H, m, β-H), 1.36, 1.27 (both 1H, m, γ-H), 0.92 (3H, d, J=7.0 Hz, β-CH_3), 0.88 (3H, t, J=7.3 Hz, γ-CH_3)]. Fr.d3 (3), FAB-MS: m/z 316 $(M+H)^+$, [βAla (1), MeVal (1), aIle (1)], N-terminal: βAla. ^1H NMR (D_2O, δ): βAla [3.25 (2H, m, β-H), 2.76 (2H, m, α-H)], aIle [4.85 (1H, d, J=5.5 Hz, α-H), 2.28 (1H, m, β-H), 1.39, 1.29 (both 1H, m, γ-H), 0.92 (3H, t, J=7.3 Hz, γ-CH_3), 0.90 (3H, d, J=6.7 Hz, β-CH_3)], MeVal [4.45 (1H, d, J=9.8 Hz, α-H), 1.92 (1H, m, β-H), 1.02, 0.86 (both 3H, d, J=6.7 Hz, β-CH_3)]. Fr.d4 (4), FAB-MS: m/z 330 $(M+H)^+$, [βAla (1), aIle (1), MeIle (1)], N-terminal: MeIle. ^1H NMR (D_2O, δ): βAla [3.62 (2H, m, β-H), 3.50 (2H, m, α-H)], aIle [4.43 (1H, d, J=4.9 Hz, α-H), 2.00 (1H, m, β-H), 1.36, 1.26 (both 1H, m, γ-H)], MeIle [3.67 (1H, d, J=5.2 Hz, α-H), 2.66 (3H, s, N-CH_3), 1.95 (1H, m, β-H), 1.50, 1.21 (both 1H, m, γ-H)]. Fr.d5 (5), FAB-MS: m/z 443 $(M+H)^+$, [βAla (1), aIle (1), Leu (1), MeIle (1)], N-terminal: Leu. ^1H NMR (D_2O, δ): βAla [3.53, 3.44 (both 1H, m, β-H), 2.57 (2H, m, α-H)], aIle [4.40 (1H, d, J=4.9 Hz, α-H), 2.00 (1H, m, β-H)], Leu [4.47 (1H, dd, J=10.1, 3.6 Hz, α-H), 1.75 (2H, m, β and γ-H), 1.64 (1H, m, β-H), 1.01 (3H, d, J=6.1 Hz, γ-CH_3), 0.99 (3H, d, J=6.4 Hz, γ-CH_3)], MeIle [4.59 (1H, d, J=11.3 Hz, α-H), 2.09 (1H, m, β-H)]. Fr.d6 (6), FAB-MS: m/z 927 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla, Val. Fr.d7 (7), FAB-MS: m/z 885 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (1), MeaIle (1), MeLeu (1)], N-terminal: MeAla. Fr.d8 (8), FAB-MS: m/z 998 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla. ^1H NMR (D_2O, δ): methoxyacetyl [3.43 (3H, s), 3.97 (2H, s)], Thr [5.34 (1H, m, β-H)]. Fr.d14 (9), FAB-MS: m/z 1333 $(M+H)^+$, [βAla (3), Thr (1), MeAla (1), Val (1), MeVal (1), aIle (1), Leu (2), MeaIle (1), MeIle (1), MeLeu (1)], N-terminal: Val.

Partial acidic hydrolysis of Fr.d8 (8) giving H83 (10), H86 (11), H87 (12), H88 (13), H89 (14), H811 (15), and H812 (16) 8 (30 mg) was treated with 2 N aqueous HCl (2 ml) and after degasing the reaction mixture was heated at 110°C for 2 h in a sealed tube. The reaction mixture was evaporated under reduced pressure and the product was subjected to HPLC [ZORBAX ODS, eluted gradiently with 0.1 % aqueous TFA→2-PrOH-CH_3CN (7:3) containing 0.1 % TFA] to give H83 (10), H86 (11), H87 (12), H88 (13), H89 (14), H811 (15), and H812 (16). H83 (10), FAB-MS: m/z 190 $(M+H)^+$, [Val], N-terminal: none. ^1H NMR (D_2O, δ): methoxyacetyl [3.43 (3H, s), 4.07 (2H, s)], Val [4.33 (1H, d, J=5.8 Hz, α-H), 2.23 (1H, m, β-H), 0.97, 0.96 (both 3H, d, J=7.0 Hz, β-CH_3)]. H86 (11), FAB-MS: m/z 445 $(M+H)^+$, [βAla (1), Thr (1), MeaIle (1), MeLeu (1)], N-terminal: MeaIle, MeLeu. ^1H NMR (D_2O, δ): βAla [3.46, 3.42 (both 1H, m, β-H), 2.57 (2H, m, α-H)], Thr [5.58 (1H, m, β-H), 4.71 (1H, d, J=3.1 Hz, α-H), 1.36 (3H, d, J=6.7 Hz, β-CH_3)], MeaIle [4.07 (1H, d, J=3.7 Hz, α-H), 2.08 (1H, m, β-H), 1.49, 1.27 (both 1H, m, γ-H)], MeLeu [4.08 (1H, t-like, α-H), 1.82, 1.77, 1.65 (each 1H, m, β and γ-H)], N-methyl [2.76, 2.72 (both 3H, s)]. H87 (12) (= Fr.d2), FAB-MS: m/z 288 $(M+H)^+$, [βAla (1), MeAla (1), Leu (1)], N-terminal: MeAla. ^1H NMR (D_2O, δ): βAla [3.53 (2H, t-like, β-H), 2.53 (2H, t-like, α-H)], MeAla [3.86 (1H,

q, J=6.7 Hz, α-H), 2.68 (3H, s, N-CH_3), 1.50 (3H, d, J=6.7 Hz, α-CH_3)], Leu [4.25 (1H, t-like, α-H), 1.60 (3H, m, β and γ-H), 0.92 (3H, d, J=6.1 Hz, γ-CH_3), 0.88 (3H, d, J=5.5 Hz, γ-CH_3)]. H88 (13), FAB-MS: m/z 431 $(M+H)^+$, [βAla (1), Thr (1), Leu (1), MeaIle (1)], N-terminal: Thr, Leu. ^{1}H NMR (D_2O, δ): βAla [3.44 (2H, m, β-H), 2.53 (2H, m, α-H)], Thr [5.50 (1H, m, β-H), 4.63 (1H, m, α-H), 1.36 (3H, d, J=6.7 Hz, β-CH_3)], Leu [4.52 (1H, m, α-H), 1.73 (2H, m, β and γ-H), 1.60 (1H, m, β-H)], MeaIle [4.18 (1H, d-like, α-H), 2.10 (1H, m, β-H), 1.28, 1.15 (both 1H, m, γ-H), 3.05 (3H, s, N-CH_3)]. H89 (14), FAB-MS: m/z 245 $(M+H)^+$, [Val (1), MeLeu (1)], N-terminal: Val. ^{1}H NMR (D_2O, δ): Val [4.28 (1H, d, J=4.9 Hz, α-H), 2.15 (1H, m, β-H), 0.95, 0.84 (both 3H, d, J=7.0 Hz, β-CH_3)], MeLeu [4.91 (1H, dd, J=11.6, 4.6 Hz, α-H), 1.75, 1.60, 1.28 (each 1H, m, β and γ-H)]. H811 (15), FAB-MS: m/z 502 $(M+H)^+$, [βAla (2), Thr (1), Leu (1), MeaIle (1)], N-terminal: Thr, βAla. ^{1}H NMR (D_2O, δ): βAla [3.42, 3.33 (both 2H, m, β-H), 2.74, 2.36 (both 2H, m, α-H)], Thr [5.64 (1H, m, β-H), 4.71 (1H, d, J=3.7 Hz, α-H), 1.35 (3H, d, J=6.7 Hz, β-CH_3)], Leu [5.19 (1H, m, α-H), 1.88 (1H, m, β-H)], MeaIle [4.45 (1H, d, J=4.9 Hz, α-H), 3.15 (3H, s, N-CH_3), 2.05 (1H, m, β-H)], Leu [5.19 (1H, m, α-H), 1.88 (1H, m, β-H)]. H812 (16), FAB-MS: m/z 770 $(M+H)^+$, [βAla (1), Thr (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: Val, Leu. ^{1}H NMR (D_2O, δ): Thr [5.53 (1H, m, β-H)].

Enzymatic hydrolysis of H812 (16) A solution of 16 (2 mg) in 0.01 M tris buffer (pH 7.7)(1 ml) was treated with thermolysin (0.8 mg, activity: 7000 PU/mg, WAKO) and incubated at 40°C for 48 h. The reaction mixture was subjected to HPLC [ZORBAX ODS, eluted gradiently with 0.1 % aqueous TFA →2-PrOH-CH_3CN (7:3) containing 0.1 % TFA] to give H812T1 (17), H812T2 (18), and H812T3 (19). H812T1 (17), FAB-MS: m/z 304 $(M+H)^+$, [Thr (1), βAla (1), Leu (1)], N-terminal: Thr. ^{1}H NMR (D_2O, δ): βAla [3.54 (2H, t-like, β-H), 2.55 (2H, t-like, α-H)], Thr [4.11 (1H, m, β-H), 3.77 (1H, d, J=6.4 Hz, α-H), 1.27 (3H, d, J=6.4 Hz, β-CH_3)], Leu [4.28 (1H, t, J=7.0 Hz, α-H), 1.62 (3H, m, β and γ-H), 0.92, 0.89 (both 3H, d, J=5.8 Hz, γ-CH_3)]. H812T2 (18), FAB-MS: m/z 512 $(M+H)^+$, [βAla (1), Thr (1), Val (1), Leu (1), MeLeu (1)], N-terminal: none. ^{1}H NMR (D_2O, δ): βAla [3.50 (2H, m, β-H), 2.54 (2H, m, α-H)], Thr [4.31 (1H, m, β-H), 1.29 (3H, d, J=6.1 Hz, β-CH_3)], Val [4.08 (1H, m, α-H), 2.49 (1H, m, β-H)], Leu [4.48 (1H, m, α-H)], MeLeu [4.20 (1H, m, α-H), 3.03 (3H, s, N-CH_3)]. H812T3 (19), FAB-MS: m/z 241 $(M+H)^+$, [Leu (1), MeaIle (1)], N-terminal: none. ^{1}H NMR (D_2O, δ): Leu [4.11 (1H, dd, J=8.9, 4.6 Hz, α-H), 1.79 (1H, m, γ-H), 1.71 (2H, m, β-H)], MeaIle [3.97 (1H, d, J=4.3 Hz, α-H), 3.00 (3H, s, N-CH_3), 2.03 (1H, m, β-H), 1.55, 1.19 (both 1H, m, γ-H)].

NaOMe treatment of theonellapeptolide Id (1) A solution of 1 (10 mg) in methanol (2 ml) was treated with 28 % NaOMe in methanol (0.2 ml) at room temperature for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with ethyl acetate. The ethyl acetate phase was washed with brine and evaporated under reduced pressure to furnish a crude product (8 mg). The crude product was subjected to silica gel column chromatography ($CHCl_3$-MeOH=8:1) to give the methyl ester 20 (5 mg). 20, amorphous powder, FAB-MS: as shown in Fig. 2. IR ($CHCl_3$): 3420, 3330, 1733, 1665, 1540 cm^{-1}. ^{1}H NMR (CD_3OD, δ): methoxyacetyl [3.33 (3H, s), 3.86 (2H, s)], 3.58 (3H, s, $COOCH_3$), 3.12, 3.04, 3.01, 2.94, 2.87 (each 3H, s, N-methyl), Thr [4.21 (1H, m, β-H), 1.05 (3H, d, J=6.1 Hz, β-CH_3)].

Acidic hydrolysis of Fr.d2 (12) and H812T1 (17) 12 (1 mg) and 17 (1 mg) were hydrolyzed respectively under the same conditions as described above for the complete hydrolysis of theonellapeptolide Id (1). The resulting amino acid mixtures were subjected to HPLC (Cosmosil 5C_{18}, eluted with water). The CD spectra of resulting amino acids were taken in 0.5 N aqueous HCl. Fr.d2 (12) afforded D-Leu (neg. max.) and L-MeAla (pos. max.). H812T1 (17) yielded L-Thr (pos. max.) and D-Leu (neg. max.).

Acknowlegement The authors are grateful to Prof. N. Fusetani, the University of Tokyo for undertaking the bioassay. They are also indebted to the Ministry of Education, Science and Culture of Japan (Grant-in-Aid for Cancer Research No. 02151037) and Yamada Science Foundation for financial support.

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15) After we reported the structure elucidation of theonellapeptolide Id,[5] a report on the structure of theonellamine B having the same structure 1 was presented by H. Nakamura, et al [Tetrahedron Letters, **1986**, 27, 4319]. Afterwards, a sample of theonellamine B was provided by Dr. Y. Ohizumi, one of the authors of theonellamine B, and was identified with theonellapeptolide Id by comparing TLC, HPLC, and ^{1}H NMR. Dr. Y. Ohizumi agreed with us to use theonellapeptolide Id in place of theonellamine B hereafter.