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Polytheonamides, Unprecedented Highly Cytotoxic Polypeptides from the Marine Sponge *Theonella swinhoei* 2. Structure Elucidation[1]

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Abstract: The structures of polytheonamides A-C were assigned to be linear 48-residue peptides with *N*-terminus blocked by a carbamoyl group mainly by interpretation of spectral data.

Polytheonamides comprise a novel class of cytotoxic polypeptides isolated from the marine sponge *Theonella swinhoei*.[1] Component amino acids of polytheonamide B were identified by amino acid analysis and 2D NMR analysis (COSY, HOHAHA, HMQC, and HMBC) of the total acid hydrolysate. The presence of Ala, Asp, Thr, *a*Thr, Ser, Glu, Val, Gly, Ile, *t*-Leu, β-methylGlu (β-MeGlu), β-methylIle (β-MeIle), β-hydroxyVal (OHVal), threo-β-hydroxyAsp (OHAsp), and γ-hydroxy-*t*-Leu (OH-*t*-Leu) was inferred from these experiments. In this paper we report the structure elucidation of polytheonamides on the basis of NMR and FAB mass spectral data.

With information of the component amino acids in hand, we sequenced polytheonamide B by extensive NMR experiments. Although signals in the ^{1}H NMR spectra measured in both $CDCl_3$-CD_3OH (1:1) and CF_3CD_2OH were well dispersed, the NOESY spectra in these solvents were hardly interpretable or did not give any sequential crosspeaks. Therefore, NMR data were collected in DMSO-d_6. In the HMQC spectrum, ^{13}C chemical shifts of α or β carbons were almost identical among the same type of amino acid residues, suggesting that polytheonamide B adopted random coil conformation in this solvent.[2] Importantly, NH-NH crosspeaks between most of adjacent residues were observed due to this conformation.

By tracing HOHAHA crosspeaks, signals for 35 residues — OHAsx (2), Asx (8), Thr or *a*Thr (1 each), Ser (1), Glx (1), β-MeGlx (1), Gly (8), Ala (7), Val (3), and Ile (2) were assigned.[3] Signals for thirteen residues — *t*-Leu (8), OHVal (3), β-MeIle (1), and OH-*t*-Leu (1) were assigned on the basis of intraresidual NOESY crosspeaks between α-H and γ-H. Eight additional crosspeaks between *N*-methyl and amide protons as well as between geminal protons of four primary amides provided confirming structural evidence (Table 1).

Sequence analysis of the 48 amino acid residues aforementioned was first carried out by tracing the NOESY crosspeaks between amide protons (Figure 1). Assignment of these crosspeaks were corroborated by NH/α-H and NH/β-H correlations. In the case of nearly identical chemical shifts of NH protons of adjacent residues, crosspeaks between NH/α-H and/or NH/β-H were used for sequencing. Interpretation of overlapping signals was made by measuring spectra at several temperatures (300 K, 313 K, and 333K) and at different concentrations (50 mg/0.5 mL and 18 mg/0.5 mL), since the chemical shift of NH protons is known to be temperature- and concentration-dependent.[3]

In the HMBC spectrum, both the amide and methylene protons of the *N*-terminal Gly residue were correlated with a carbon at δ 162.0, suggesting that the *N*-terminus was blocked by a carbamoyl group, which

was supported by a negative ninhydrin reaction.[4] On the other hand, hydrazinolysis[5] afforded Thr or *a*Thr as detected by the amino acid analysis, suggesting that either of these amino acids was at the *C*-terminus which was not blocked.[6]

Some of the amide groups in the sidechain of OHAsx, Asx, Glx, and β-MeGlx were *N*-methylated, which were distinguished from unsubstitued amides by NOESY data: the amides in the sidechain of Glx, MeGlx, Asx-43, and Asx-45 were unsubstituted, while those of the rest of Asx residues and two OHAsx residues were *N*-methylated.[7]

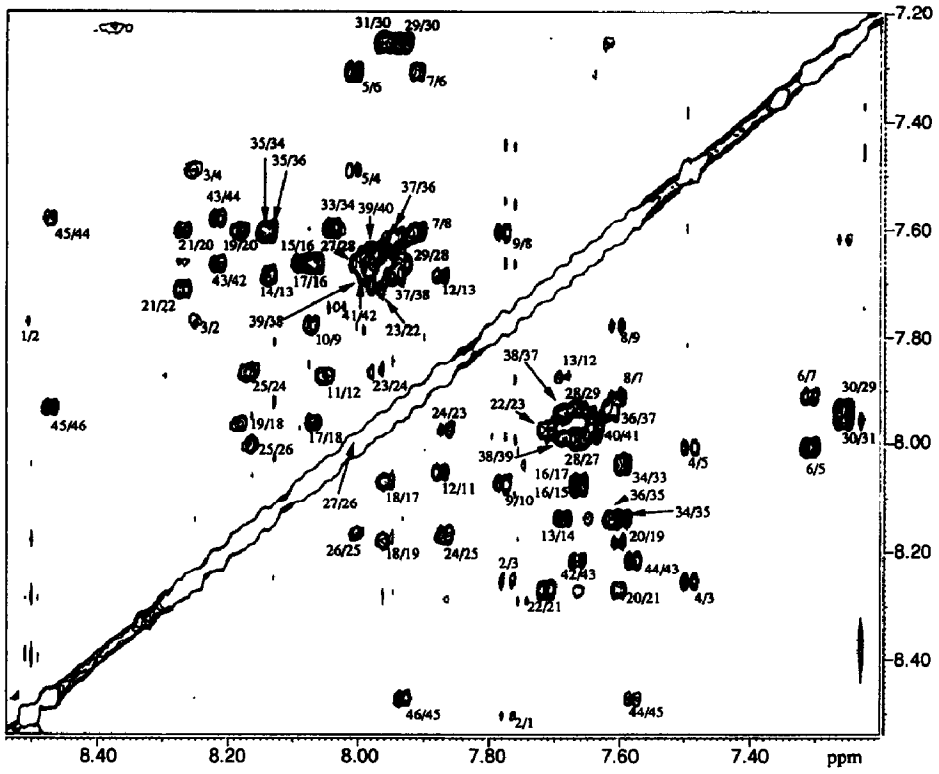


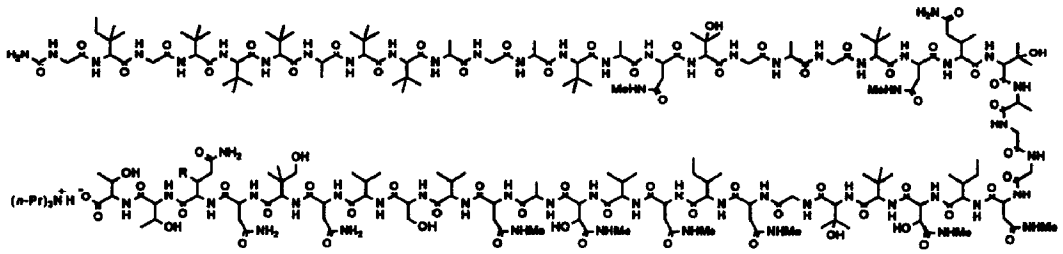
Figure 1: NH-NH region of the NOESY spectrum of polytheonamide B (2) in DMSO-d6 at 313K. Cross peaks showing NH(i)-NH(i+1) connectivities are labeled.

Table 1: Chemical Shifts of the Assigned Proton NMR Resonances of Polytheonamide B (2) in DMSO-d_6 at 313K[a, b]

residue	NH	$C^{\alpha}H$	$C^{\beta}H$	others
Gly-1	8.50	3.82, 3.82		
βMeIle-2	7.77	4.30		$C^{\gamma}H_3$ 0.83; $C^{\gamma}H_3$ 0.87; $C^{\gamma}H_2$ 1.28; $C^{\delta}H_3$ 0.76
Gly-3	8.26	3.62, 3.80		
t-Leu-4	7.49	4.57		$C^{\gamma}H_3$ 0.88
t-Leu-5	8.01	4.18		$C^{\gamma}H_3$ 0.92
t-Leu-6	7.31	4.23		$C^{\gamma}H_3$ 0.85
Ala-7	7.92	4.47	1.20	
t-Leu-8	7.61	4.41		$C^{\gamma}H_3$ 0.87
t-Leu-9	7.78	4.17		$C^{\gamma}H_3$ 0.92
Ala-10	8.08	4.29	1.21	
Gly-11	8.04	3.68, 3.78		
Ala-12	7.88	4.43	1.22	
t-Leu-13	7.68	4.24		$C^{\gamma}H_3$ 0.87
Ala-14	8.14	4.27	1.18	
Asm-15	8.09	4.65	2.40, 2.53	
OHVal-16	7.67	4.22	1.08, 1.12	
Gly-17	8.08	3.76, 3.76		
Ala-18	7.96	4.29	1.21	
Gly-19	8.18	3.64, 3.89		
t-Leu-20	7.60	4.13		$C^{\gamma}H_3$ 0.87
Asm-21	8.27	4.62	2.39, 2.51	
βMeGln-22	7.71	4.29	2.31	$C^{\gamma}H_3$ 0.81; $C^{\gamma}H_2$ 1.83, 2.13; NH_2 6.73, 7.25
OHVal-23	7.97	4.27		$C^{\gamma}H_3$ 1.09; $C^{\gamma}H_3$ 1.13
Ala-24	7.87	4.33	1.22	
Gly-25	8.17	3.74, 3.74		
Gly-26	8.00	3.74, 3.74		
Asm-27	8.00	4.67	2.41, 2.48	
Ile-28	7.67	4.27	1.70	$C^{\gamma}H_3$ 0.74; $C^{\gamma}H_2$ 1.01, 1.34; $C^{\delta}H_3$ 0.75
OHAsm-29	7.93	4.67	4.38	
t-Leu-30	7.26	4.44		$C^{\gamma}H_3$ 0.87
OHVal-31	7.97	4.29		$C^{\gamma}H_3$ 1.14; $C^{\gamma}H_3$ 1.14
Gly-32	8.08	3.68, 3.84		
Asm-33	8.04	4.64	2.40, 2.48	
Ile-34	7.59	4.13	1.71	$C^{\gamma}H_3$ 0.75; $C^{\gamma}H_2$ 1.03, 1.36; $C^{\delta}H_3$ 0.75
Asm-35	8.14	4.67	2.39, 2.52	
Val-36	7.62	4.10	1.91	$C^{\gamma}H_3$ 0.75; $C^{\gamma}H_3$ 0.75
OHAsm-37	7.95	4.65	4.42	
Ala-38	7.68	4.25	1.21	
Asm-39	7.98	4.63	2.42, 2.54	
Val-40	7.64	4.21	2.01	$C^{\gamma}H_3$ 0.81; $C^{\gamma}H_3$ 0.81
Ser-41	7.98	4.38	3.55, 3.60	
Val-42	7.67	4.22	1.97	$C^{\gamma}H_3$ 0.81; $C^{\gamma}H_3$ 0.81
Asn-43	8.22	4.65	2.42, 2.54	NH_2 6.88, 7.29
OH-*t*-Leu-44	7.58	4.59		$C^{\gamma}H_3$ 0.98; $C^{\gamma}H_3$ 1.05; $C^{\gamma}H_2$ 2.68, 2.95
Asn-45	8.47	4.59	2.47, 2.59	NH_2 6.90, 7.36
Gln-46	7.93	4.29	2.09, 2.09	$C^{\gamma}H_2$ 1.78, 1.91; NH_2 6.69, 7.16
Thr-47	7.96	4.31	3.89	$C^{\gamma}H_3$ 1.09
*a*Thr-48	7.63	4.18	4.07	$C^{\gamma}H_3$ 1.05
(*n*-Pr)$_3$N		2.97	1.63	$C^{\gamma}H_3$ 0.90

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.

b see note 7.



Polytheonamide A R=H
Polytheonamide B R=H
Polytheonamide C R=Me

The 48-residue chain has a molecular weight of 4887, which was consistent with an FABMS ion at *m/z* 5033 (average of isotopic clusters) corresponding to an ion incorporating an $(n\text{-Pr})_3N$ molecule present as a counter ion: the NMR spectra indicated the presence of one equivalent of $(n\text{-Pr})_3N$.[8] Thus, the structure of polytheonamide B is as shown.

Similarly polytheonamide A was analyzed by spectroscopic method leading to the same amino acid sequence as polytheonamide B. Chemical shift discrepancy of NH protons between polytheonamides A and B was observed in residues 41-48, suggesting that the stereochemistry of amino acids in this region may differ between the two compounds. Polytheonamide A gave the same $(M+Pr_3N)^+$ ion in the FABMS.

Polytheonamide C gave the $(M+Pr_3N)^+$ ion 14 mass units larger than polytheonamide B. Interpretation of HOHAHA and NOESY data led to the amino acid sequence with Gln-46 residue in 2 being replaced by β-MeGln.

Polytheonamides contain several unusual α-amino acid residues and apparently is a member of an entirely new class of peptides. The biosynthesis of polytheonamides, whether synthesized ribosomally or enzymatically,[9] poses an interesting question.[10]

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References and Notes:

1. Bioactive Marine Metabolites. 56. Part 55: Fusetani, N.; Hamada, T.; Sugawara, T.; Matsunaga, S., a proceeding paper in this issue.
2. Wishart, D. S.; Sykes, B. D.: Richards, F. M. *J. Mol. Biol.* **1991**, *222*, 311.
3. Wuthrich, K. *NMR of Proteins & Nucleic Acids*, Wiley, New York, **1986**.
4. At present we were unable to locate the NH_2 signals of the carbamoyl group, propably due to peak broadening. The chemical shift of 162.0 ppm and the FABMS data strongly suggested the presence of $CONH_2$ at the *N*-terminus.
5. Akabori, S.; Ohno, K.; Narita, K. *Bull. Chem. Soc. Jpn.*, **1952**, *25*, 314.
6. Thr and *a*Thr gave an identical retention time in the amino acid analysis.
7. For convenience Asn and OHAsn residues with an *N*-methyl amide group in the side chain were designated as Asm and OHAsm, respectively. The sequence of the *C*-terminal Thr-*a*Thr was tentatively assigned on the basis of proton chemical shift values: the difference between the chemical shifts of α- and β- protons are larger in Thr.[1]
8. Attempts at the preparation of free carboxylic acid by partitioning between 1N HCl and $CHCl_3$ or by ion exchange resin were unsuccessful.
9. Bacterial antibiotic peptides containing lanthionine residues such as epidermin are ribosamally synthesized followed by post-translational modification (Schnell, N.; Entian, K.; Schneider, U.; Gotz, F.; Zahner, H.; Kellner, R.; Jung, G. *Nature* **1988**, *333*, 276), while some cyclic peptides such as gramicidins are synthesized by a multienzymic cycle (Kleinkauf, H.; Dohren, H. *Eur. J. Biochem.* **1990**, *192*, 1).
10. Details of structure elucidation will be published in a full paper.

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