Natural Products

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Functional Analysis of Synthetic Substructures of Polytheonamide B: A Transmembrane Channel-Forming Peptide**

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Polytheonamide B (**1**, chemical structure in Scheme 1) is an extremely cytotoxic marine natural product.[1,2] Compound **1** has a molecular weight of 5030 Da, and is the largest nonribosomal peptide identified to date.[3] The linear chain of 48 amino acid residues, including 13 different nonproteinogenic residues, is capped with 5,5-dimethyl-2-oxohexanoate (Ncap) at the N terminus. Intriguingly, the sequence has alternating D- and L-amino acid residues, except for the eight glycine units.[2] These unique structural features enable **1** to fold into a distinctive three-dimensional structure not found in ribosomal proteins. Extensive NMR and modeling experiments by Hamada et al. revealed that **1** adopts a tubular $\beta^{6.3}$-helical structure in 1:1 chloroform/methanol solution with a length of approximately 45 Å and a hydrophilic pore with a diameter of 4 Å (Figure 1).[4] It was proposed that this conformation is stabilized by 38 hydrogen bonds within the main amide chain, and is reinforced further by three hydrogen bonds between the four *N*-methylasparagines along the helix axis (residues 21, 27, 33, and 39).

The $\beta^{6.3}$ helix is believed to function as a membrane-spanning ion channel and is thus the biologically active three-dimensional structure of the peptide.[5] Experiments with planar bilayers demonstrated that a monomeric molecule of **1** unidirectionally inserts into the membrane and forms a channel selective for monovalent cations, with the highest selectivity for H^+.[6] Since typical ribosomally derived ion-channel proteins have molecular weights exceeding 100 000 Da and are composed of multiple domains,[7] **1** could serve as a minimum-structure platform for investigating requirements for ion-channel functions and designing novel transmembrane channels. The structure–function relationships of various analogues of **1** should shed light on the specific constituent amino acids and sequences that encode its unique channel function.[8] Herein we report the chemical synthesis of nine substructures of polytheonamide B, and identify the membrane-disrupting and ion-channel-forming sequences, as well as the cytotoxicity-enhancing sequence.

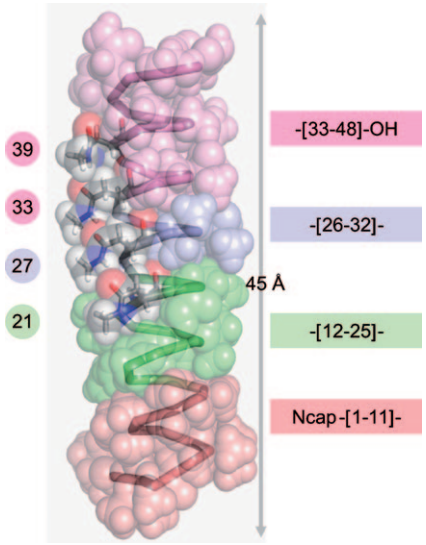

Figure 1. The $\beta^{6.3}$-helix structure of polytheonamide B (**1**) determined by NMR analysis in $CDCl_3/CD_3OD$ (1:1) (PDB 1D code: 2RQO). The color code corresponds to that in Scheme 1. Four *N*-methylasparagines, shown in the standard atom colors, participate in a hydrogen-bond network between the side chains along the helix axis.

We recently achieved the total synthesis of polytheonamide B (Scheme 1, **1**) by coupling four fragments using segment-coupling reactions.[9–11] Specifically, compounds **2**, **12**, **14**, and **16** were prepared by solid-phase peptide synthesis.[12] Carboxylic acids **2**, **12**, and **14** were converted to the corresponding thioesters **11**, **13**, and **15**. Then, three Ag^+-promoted condensations[13] of the four fragments at residues 11, 25, and 32, followed by acid-mediated global removal of the six protective groups (*t*Bu and Tr), gave rise to **1**. The protective-group and coupling strategies developed for the total synthesis were applied to produce eight new substructures of **1** (Table 1, **3**–**10**).[14] All of the synthesized compounds **1**–**10** were purified using HPLC conditions that were judiciously selected based on the polarities and the aggregation tendencies of the fragments.

The ten synthesized peptides were subjected to cytotoxicity assays against p388 mouse leukemia cells using the XTT method (Table 1).[15] The parent natural product **1** exhibited the most potent cytotoxicity, with an IC_{50} value of 0.098 nM. Surprisingly, the nine substructures with diverse molecular weights and structures all showed detectable cytotoxicities, and low micromolar-level activities ($IC_{50} = 0.80$–2.0 μM) were observed for five of them (**3**, **5**, **8**, **9**, and **10**). The three

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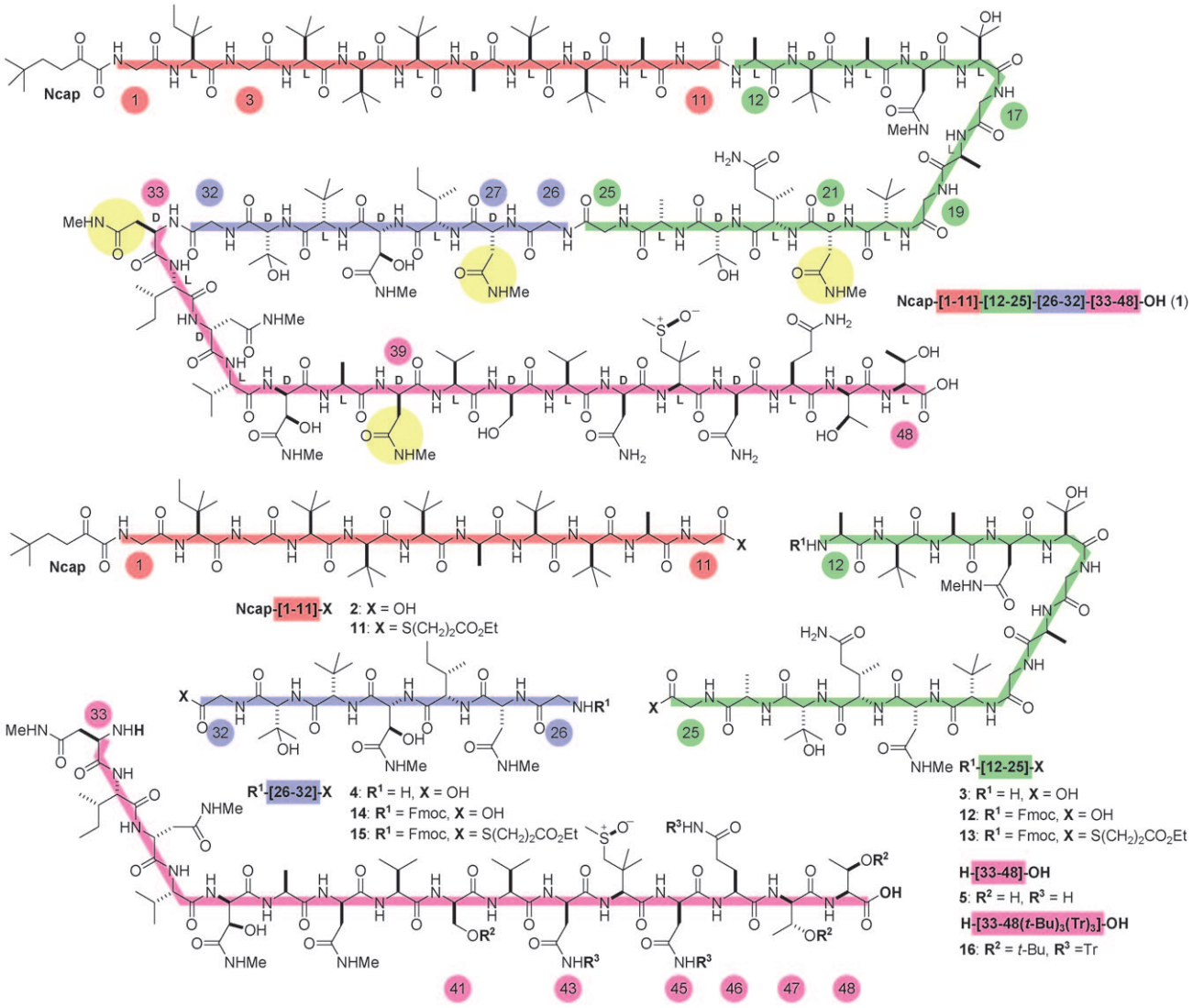

Scheme 1. Structure of polytheonamide B (**1**) and four peptide fragments for syntheses of **1** and its substructures. The main chain is color coded according to the four fragments. The four *N*-methylasparagines involved in the hydrogen-bond network between the side chains are highlighted in yellow.

Table 1: Ten synthetic peptides and their cytotoxicity.

Synthetic peptides		IC_{50} [nM][a]
1:	Ncap-[1-11]-[12-25]-[26-32]-[33-48]-OH	0.098
2:	Ncap-[1-11]-OH	14 000
3:	H-[12-25]-OH	1500
4:	H-[26-32]-OH	8000
5:	H-[33-48]-OH	2000
6:	Ncap-[1-11]-[12-25]-OH	2 000 000
7:	H-[12-25]-[26-32]-OH	4100
8:	H-[26-32]-[33-48]-OH	800
9:	Ncap-[1-11]-[12-25]-[26-32]-OH	1600
10:	H-[12-25]-[26-32]-[33-48]-OH	860

[a] IC_{50} values were deduced from the growth inhibition assay against p388 cells using the XTT method.

C-terminus-containing peptides **5**, **8**, and **10**, which share the same structure between residues 33 and 48, had comparable toxicities, despite the significant differences in their lengths. On the other hand, variable IC_{50} values, from mM to μM levels, were observed for the three N-terminus-containing peptides **2**, **6**, and **9**, which possess the same structure between residues 1 and 11. Most notably, a drastic increase in potency was observed when amide bonds were formed to generate full-length **1** (e.g. **2** + **10**, **5** + **9**, or **6** + **8**): Compound **1** is at least 10000 times more potent than all of the nine component structures. It is noteworthy that the effect of peptide extension on the biological activity is strongly dependent on the structural context. For instance, while the hydrophobic N-terminus fragment **2** decreased the cytotoxicity upon conjugation with **3** by 140-fold (**3** vs. **6**), the same compound **2** increased the potency upon attachment to **10** by 11000-fold (**10** vs. **1**). Our finding that there is no apparent correlation between cytotoxicity and the presence of particular substruc-

tures suggested that the modes of action for these ten peptides are not the same.

Although experiments with planar bilayers showed that parent compound **1** forms a monovalent-cation channel, the mode of activity of **1** toward liposomes, which are models of cell membranes,[16] has not been explored. We first evaluated the ion-transport activity and membrane-disrupting activity of **1** using liposomes encapsulating carboxyfluorescein (CF).[17] To evaluate the ion-transport activity, the liposomes were prepared with a transmembrane pH gradient with a pH of 6.5 inside the liposome and a pH of 5.5 outside; furthermore, the liposomes contained 20 μM CF as a pH indicator.[18] As expected, **1** (125 nM) effectively induced neutralization of the transmembrane pH gradient by cation exchange between internal Na^+ ions and external H^+ ions across the membrane, resulting in a rapid change in the fluorescence intensity of the encapsulated CF by protonation (Figure 2a). To evaluate the membrane-disrupting activity of **1**, liposomes were next prepared with a high concentration of encapsulated CF (20 mM), so that the fluorescence of CF was suppressed by self-quenching.[19] In this experiment, when the peptide disrupted the membrane structure sufficiently to allow passage of the CF molecule (5 Å), the encapsulated CF was released into the external buffer and diluted, and the intensity of fluorescence emission of CF increased. Compound **1** was found to have almost no membrane-disrupting activity at a concentration of 1 μM (Figure 2b). Therefore, this result excluded the possibility that the change in fluorescence observed in the ion-transport-activity assay originated from membrane disruption and following protonation of CF. These data thus indicate that full-length **1** also forms an ion channel in liposomes.

Next, the four shortest fragments, **2**–**5**, were subjected to these two liposome experiments at the same concentrations used for **1**. Whereas **2**–**4** induced no fluorescence change in either assay,[20] the C-terminus fragment **5** exhibited 6 times weaker H^+/Na^+ exchange activity and 5 times stronger membrane-disrupting activity than parent **1** (Figure 2a,b). These experiments allowed us to clarify that the mode of action of **5** toward the model cell membrane is distinct from that of **1**.

As we were particularly interested in the effect of peptide length on H^+/Na^+ exchange and membrane-disruption activities, the two C-terminus-containing peptides, **8** and **10**, which have cytotoxicities comparable to **5**, were subjected to the two liposome experiments (Figure 2c,d). Compound **8** showed a similar activity profile to **5**, and appeared to act as a membrane-disrupting molecule. Intriguingly, compound **10** induced higher ion exchange than either **5** or **8**, and negligible CF leakage, indicating that **10** acts solely as an ion transporter. We therefore concluded that introduction of the middle-region sequence [12–25] to the C-terminal sequence [26–48] switches the function of the C-terminus-containing peptides from a membrane disruptor to an ion transporter.

To further demonstrate that **10** forms an ion channel, and to compare its channel function to that of **1**, single-channel current recordings were performed using an artificial planar lipid bilayer.[21,22] Since **1** was reported to form a highly active H^+ channel,[6] transmembrane proton currents were evaluated in acidic aqueous

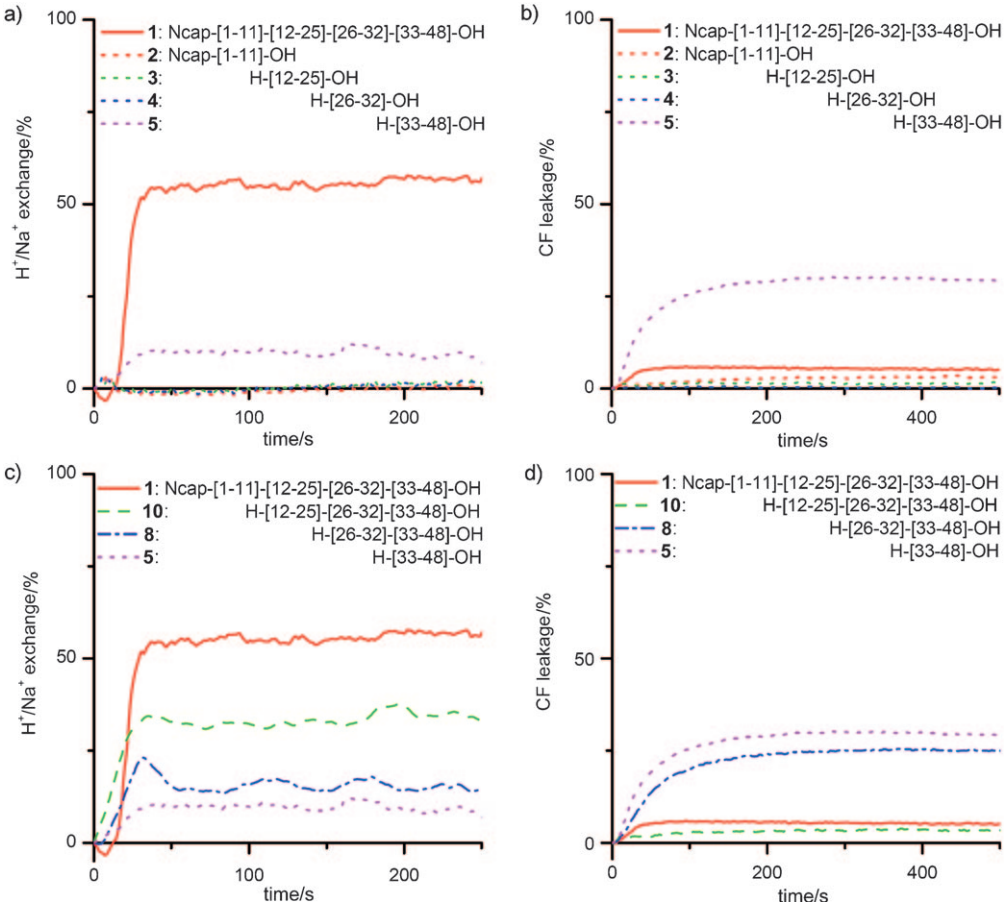

Figure 2. Ion-transport activity and membrane-disrupting activity of peptides assayed using carboxyfluorescein (CF)-encapsulating liposomes. a) Time course of H^+/Na^+ exchange across lipid bilayers composed of unilamellar vesicles (LUVs, containing 20 μM CF and with a pH gradient) caused by **1**–**5** (125 nM). b) Time course of CF leakage from LUVs (20 mM CF) caused by **1**–**5** (1 μM). c) H^+/Na^+ exchange across lipid bilayers of LUVs (20 μM CF and with a pH gradient) caused by **1**, **10**, **8**, and **5** (125 nM). d) Time course of CF leakage from LUVs (20 mM CF) caused by **1**, **10**, **8** and **5** (1 μM).

solution (0.1M HCl) to achieve high signal-to-noise ratios. Single-channel activity appeared immediately after addition of peptides **1** and **10** to the planar lipid bilayer from the external aqueous solution. The channel current for **1** was observed to fluctuate rapidly between the open and closed state on the millisecond timescale (Figure 3a).[23] Compound **10** also showed a current trace with distinct open and closed states, similar to those of **1**. As shown in Figure 3b, current–voltage (*I–V*) curves for **1** and **10** were comparable with slight differences. Full-length **1** generates a nearly linear curve with an almost constant value of conductance of approximately 80 pS in the voltage range ±150 mV. On the other hand, **10** shows weak voltage dependency, where a smaller conductance of roughly 40 pS was observed under low voltage (±100, ±50 mV), and a larger conductance of about 80 pS was observed under high voltage (±150 mV). Overall, compound **10** exhibited H^+-channel activity analogous to that of **1** except for its weak voltage dependency. Most importantly, it was found that the hydrophobic N-terminal region Ncap-[1–11], which is responsible for the significant difference in the cytotoxicity of **1** and **10**, is not an essential component for channel formation.

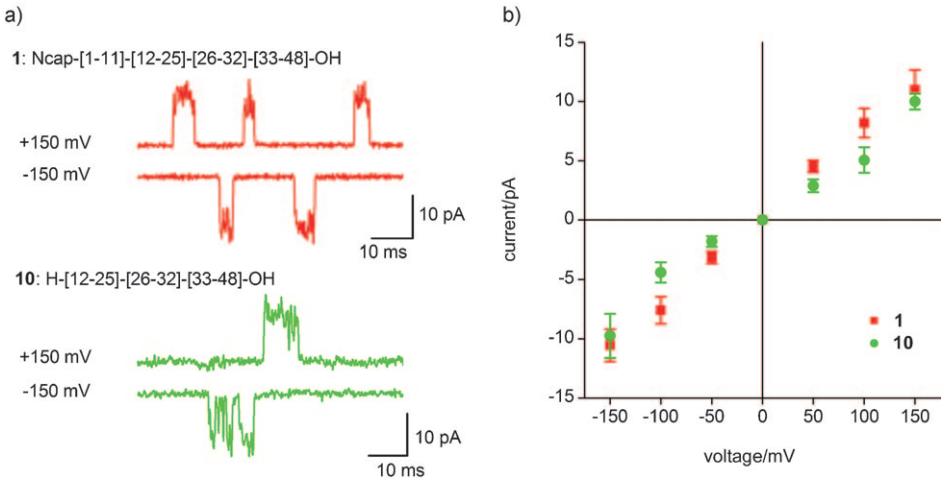

Figure 3. Single-channel current recording on the Port-a-Patch patch-clamp system for **1** and **10**. a) Traces showing proton current flow through a planar diphytanoyl phosphatidylcholine (DPhPC) bilayer containing a channel formed by **1** (top) or **10** (bottom) in 0.1M HCl. b) Current–voltage (*I–V*) curves were obtained for **1** and **10** from proton channels observed in (a).

The above functional analyses of the synthetic substructures uncovered the hierarchy of functions in the amino acid sequence of **1** (Scheme 1). The hydrophilic C-terminal region **5**, where the longest D-,L-alternating sequence is located, is responsible for the membrane-disrupting activity. The sequence of the C-terminal region is composed of hydrophilic residues with a polar side chain (e.g., amide, alcohol, sulfoxide) and hydrophobic residues with an alkyl side chain. Consequently, **5** is amphiphilic and acts as a surfactant to disrupt the lipid bilayer. The addition of the [26–32] sequence (e.g. **4**+**5**→**8**) changes the activity only slightly because of its short length and the similar amphiphilic nature of the residues. However, the sequence of [12–25] in **10** is capable of changing the function of the peptide from membrane disruption to ion channel formation. Assuming that these peptides adopt a $\beta^{6.3}$-helix structure (Figure 1), the axial length of **10** is approximately 35 Å and that of **8** is approximately 22 Å. Since the thickness of the hydrophobic region of a lipid bilayer is approximately 30 Å, **10** is long enough to span the bilayer, while **8** is too short. This would be the major reason for the difference in the modes of action of **10** and **8**. Additionally, the side-chain hydrogen-bond network would contribute to stabilizing the channel formation of **10**, because **10** possesses four *N*-methylasparagines (residues 21, 27, 33, and 39) that reinforce the $\beta^{6.3}$-helix conformation of **1**. Interestingly, although the N-terminal-region peptide **2** is weakly cytotoxic and is not required for ion-channel formation, conjugation of **2** to **10** significantly increased the cytotoxicity. It is highly likely that the hydrophobic N-terminal region of **1**, with seven fully β-branched alkyl side chains, would play a crucial role in efficiently concentrating **1** in the hydrophobic lipid environment, where **1** then forms a bioactive ion channel.

In summary, the functions of the chemically synthesized structures of polytheonamide B (**1**) were evaluated using four different experiments: assays of cytotoxicity, H^+/Na^+ exchange activity, membrane-disrupting activity, and recordings of the single-channel current. These experiments revealed a switch in the functional properties of peptides upon elongation from the C-terminal fragments. While the C-terminus substructures are characterized by membrane-disrupting activity up to the length of **8**, the N-terminally elongated peptides as in **10** and **1** are conferred with ion-channel activity and devoid of membrane-disrupting activity. Furthermore, the hydrophobic Ncap-[1–11] region of **1** was found responsible for rendering **1** 11000 times more cytotoxic than **10**, presumably by aiding the peptide to partition into the hydrophobic membrane environment. Therefore, the channel-forming property of **10** and the role of the N-terminus region of **1** in membrane partitioning represent the two most important findings, which provide new design principles for the synthesis of artificial ion-channel-forming molecules. The discoveries described here demonstrate the benefits of total synthesis and the power of constructing complex molecules in elucidating the structural basis for the biological activity of natural products.

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