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Selective Modification of the N-Terminal Structure of Polytheonamide B Significantly Changes its Cytotoxicity and Activity as an Ion Channel

Naoki Shinohara, Hiroaki Itoh, Shigeru Matsuoka, and Masayuki Inoue*[a]

Polytheonamide B (**1**; Scheme 1a) is an extremely cytotoxic natural product (IC_{50} = 0.098 nM, mouse P388 leukemia cells), and it is the largest non-ribosomal peptide described to date (MW = 5030 Da).[1,2] The linear-chain polypeptide **1** is formed of an N-terminal 5,5-dimethyl-2-oxohexanoate group (Ncap) and 48 amino acid residues of alternating D- and L-chirality, and the secondary structure was recently reported to be a remarkable $\beta^{6.3}$-helix with a length of approximately 45 Å (Figure 1).[3]

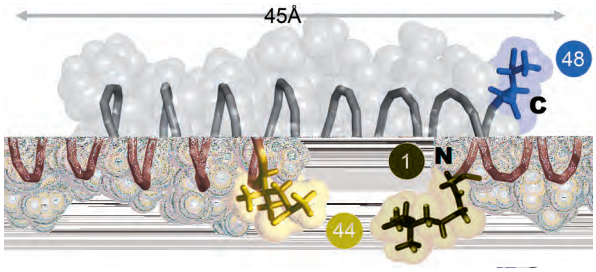

Figure 1. The $\beta^{6.3}$-helix structure of polytheonamide B (**1**) determined using NMR (PDB: 2RQO[3]). Labels N and C denote the N and C termini, respectively. Stick models of the Ncap and residues 1, 44, and 48 are shown. Colors correspond to those in Scheme 1.

This helical tube structure is believed to function as a transmembrane ion channel in biological settings, as the hydrophilic pore, which is 4 Å in diameter, creates a path for ion flow.[4] Planar bilayer experiments demonstrated that monomeric **1** forms a monovalent-cation-selective channel.[5] Therefore, cytotoxic peptide **1** not only poses a synthetic challenge, but also provide a unique structural platform for designing cytotoxic molecules and synthetic transmembrane channels.[6]

We recently reported the total synthesis of polytheonamide B,[7] as well as the synthesis of nine substructures of 7 to 37 amino acid residues. We found that all of the substructures are at least 10000-times less cytotoxic than the parent compound.[8,9] These studies suggest that the overall membrane spanning property of **1** is important for its biological activity. To further characterize the structure–function relationship of **1**, we planned to site-specifically modify monomer units of synthetic polytheonamide B and evaluate the biological activity of the newly generated analogues. We hypothesized that this type of molecular editing[10] would enable us to decipher the structural elements that are responsible for the unique biological functions of **1**. In the first set of experiments, the 44th, N- and C-terminal residues were selected as the modification points. Here, we report the synthesis of seven derivatives of polytheonamide B and discuss the significant effect of N-terminal modification on their cytotoxicity and ability to acts as ion channels in vitro.

Achieving selective and precise alteration of a complex structure such as that of polytheonamide B necessitates the use of highly chemoselective reactions, since the numerous native functional groups of **1** can cause undesired reactions. For instance, attempted methyl ester formation (**1**→**2**) at the C terminus resulted in generation of multiple methylated products when **1** was exposed to trimethylsilyldiazomethane ($TMSCHN_2$). Thus, methyl ester **2** was alternatively produced by treating **1** with 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylcarbodiimide (DIPCDI) in *N*,*N*-dimethylformamide (DMF) and methanol (Scheme 1b). The sulfoxide moiety of the 44th amino acid residue of **1** was chemoselectively oxidized to the sulfone group of **3** in 69% yield.[11,12] Then, a series of the N-terminal-modified analogues **5**–**8** was prepared from amine **4**, which was produced by taking advantage of the 1,2-dicarbonyl structure of Ncap in peptide **1**. Specifically, reaction between **1** and benzene-1,2-diamine under Dixon conditions[13] led to the formation of **4** and **9** without cleavage of the other numerous amide bonds. Amine **4** in turn was transformed into acetamide **5**, octanamide **6**, and palmitamide **7**, as well as trimethyl ammonium derivative **8** by applying the corresponding anhydride or mixed anhydrides as shown in Scheme 1b.[14]

The cytotoxicity of synthetic derivatives **2**–**8** was assessed using a colorimetric assay and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) in mouse P388 leukemia cells (Table 1).[15] While the cytotoxicity of methyl ester analogue **2** was comparable to the parent **1**, sulfone **3** was fivefold less toxic than **1**. Remarkably, the results indicate that the degree of toxicity is dependent upon the oxidation state of a single sulfur atom, suggesting that the highly polar

Table 1. Cytotoxicity of polytheonamide B (**1**) and analogues **2**–**8**.

Synthetic peptides	IC_{50}[a] [nM]
Ncap-[1-48]-OH (**1**)	0.098
Ncap-[1-48]-OMe (**2**)	0.12
Ncap-[1-48(O)]-OH (**3**)	0.46
H-[1-48]-OH (**4**)	24
CH_3CO-[1-48]-OH (**5**)	47
$CH_3(CH_2)_6CO$-[1-48]-OH (**6**)	0.50
$CH_3(CH_2)_{14}CO$-[1-48]-OH (**7**)	0.038
$Me_3N^+(CH_2)_3CO$-[1-48]-OH (**8**)	250

[a] IC_{50} values were determined from the results of growth inhibition assays (XTT method) performed on mouse P388 leukemia cells.

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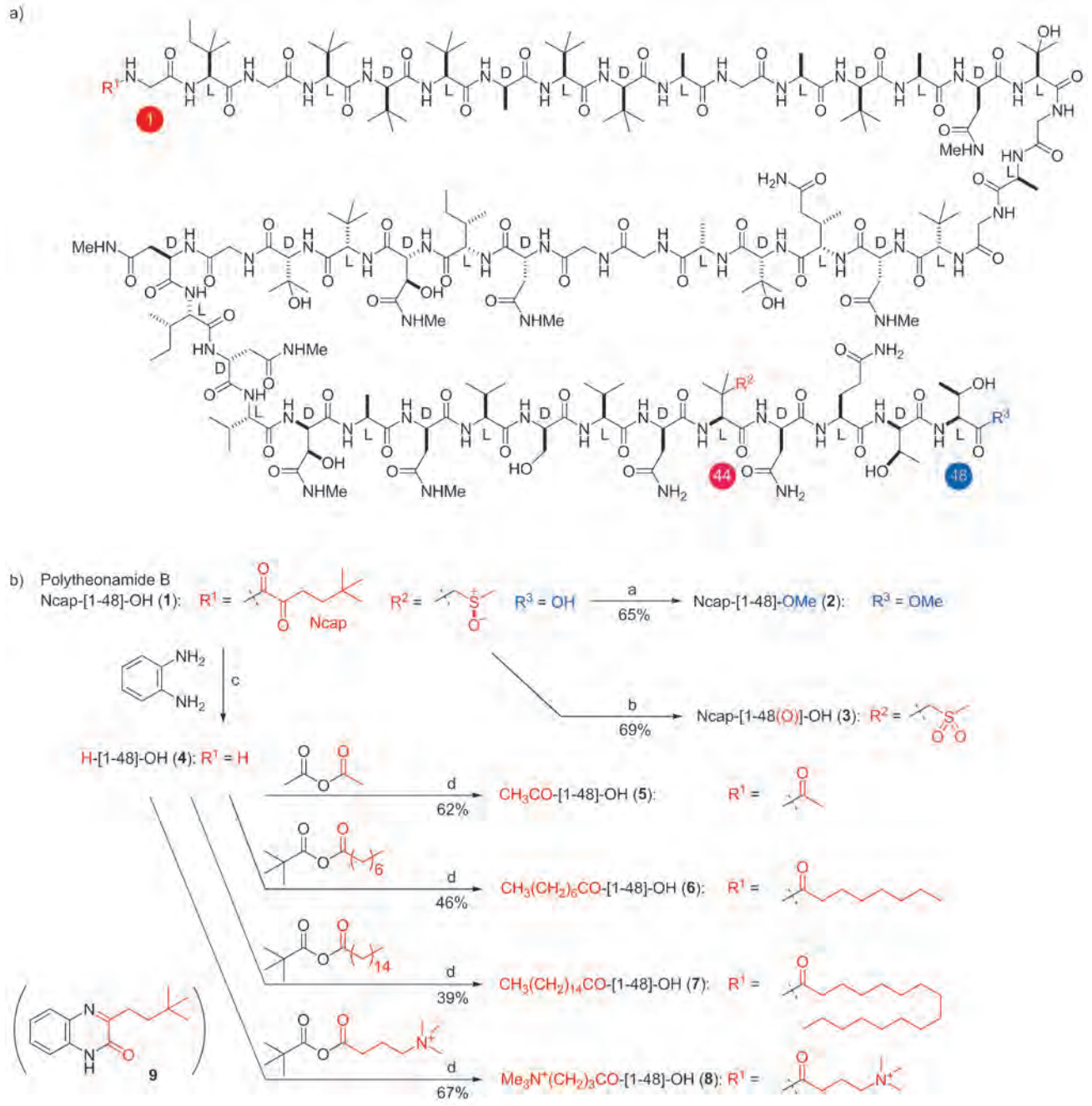

Scheme 1. a) Structure of polytheonamide B (**1**). b) The synthesis of polytheonamide analogues **2**–**8**. *Reagents and conditions*: a) HOBt, DIPCDI, DMF/MeOH (1:1), 0→27 °C; b) oxone, *n*PrOH/H_2O (3:1), 30 °C; c) 2 M NaOAc/HOAc (pH 4), DMF, 40 °C; d) pyridine (for **5** only), DMF, 27 °C. Abbreviations: *N*,*N*′-diisopropylcarbodiimide (DIPCDI), *N*,*N*-dimethylformamide (DMF), 1-hydroxybenzotriazole (HOBt), 5,5-dimethyl-2-oxohexanoic acid (NcapOH), $2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$ (oxone).

nature of the sulfoxide group of the 44th residue plays a favorable role in the toxicity.[12,16]

Structural modification of the N terminus had an even more profound effect on cytotoxicity. The less hydrophobic compounds amine **4** and acetylamide **5**, and the comparably hydrophobic octanamide **6**, were 240-fold, 480-fold, and fivefold less toxic than the parent 5,5-dimethyl-2-oxohexanamide **1**, respectively, whereas the more hydrophobic palmitamide **7** was even more cytotoxic than **1**. The apparent correlation between hydrophobicity and cytotoxicity among compounds **1**, **4**, **5**, **6** and **7** indicates that the size of the hydrophobic N-terminal moieties is critical for their cytotoxicity. The most drastic decrease in cytotoxicity (2500-fold) was exhibited when the Ncap group of **1** was switched to a trimethyl ammonium structure (compound **8**). The ammonium cation appears to have a strongly negative impact on the intrinsic potency of **1**. These results suggest that hydrophobic substitution at the N terminus is required for targeting cell membrane as well as orienting **1** for effective membrane insertion, where it exerts its cytotoxic activity.

To compare the function of the ion channel formed by polytheonamide B derivatives **2**–**8** with that of **1**, transmembrane proton (single-channel) currents were recorded by patch clamp assay in acidic aqueous solution using an artificial planar lipid bilayer.[17–19] The results showed that channels with distinct open and closed states formed immediately in the planar lipid bilayer after addition of **1** and derivatives **2**–**7** (Figure 2a). In contrast, virtually no channel current was observed when trimethyl ammonium derivative **8** was added to the planar lipid bilayer (Figure 2b).

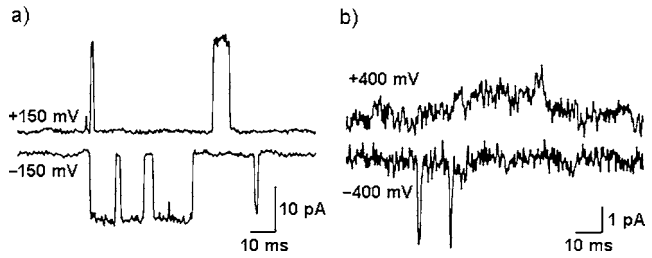

Figure 2. Single-channel current recordings for analogues **7** and **8** made with the Port-a-Patch patch clamp system from Nanion Technologies. Traces show proton current flow through a channel formed by a) **7** or b) **8** in a planar diphytanoyl phosphatidylcholine (DPhPC) bilayer in 0.1 M HCl.

Accordingly, the current–voltage curves for all polypeptide analogues except **8** were comparable, with a constant conductance in the range of ±150 mV (Figure 3a,b). Analogue **8** blocked the proton current in a higher voltage range (± 400 mV; Figure 3c). Our data indicate that the structural differences in analogues **2**–**7** are inconsequential as far as impacting the in vitro activity of the proton channel formed by **1**. In contrast, the mere presence of the ammonium group in **8** almost completely eliminates the proton channel activity. It is possible that the positively charged group at the N terminus of **8** mimics a monovalent cation and thus strongly binds to the cation entrance of the tubular structure (see Figure 1), resulting in a "closed end" and so blockade of ion passage.[20] The negligible ion channel activity of **8** correlates with its significantly lower cytotoxicity of **8** as compared with compounds **1**–**7**, suggesting that the two activities are related. Overall, we have demonstrated that both the cytotoxicity and ion channel activity can be dramatically altered by varying the structure of the N terminus.

In conclusion, polytheonamide B (**1**) was structurally modified at the 44th, N- or C-terminal amino acid residue to generate derivatives **2**–**8**, which were evaluated in cytotoxicity assays and single-channel recording experiments. We have demonstrated for the first time that the hydrophobicity of the N-terminal structures of **3**–**7** correlates strongly with the cytotoxicity exhibited by the compound, and that palmitamide **7** is more potent than the parent natural product (**1**) despite its similar behavior as a proton channel. Furthermore, the drastic deactivating effect of the trimethyl ammonium moiety of **8** on both the cytotoxicity and proton current was revealed. These data suggest that hydrophobic N-terminal moieties function as membrane-targeting anchors critical for cytotoxicity and that an ammonium cation in the same position acts as an intramolecular channel blocker. The results highlight the functional roles of the 44th, N-, and C-terminal residues on the cytotoxicity and channel activities of these polypeptides, and should facilitate efforts to rationally design tailor-made transmembrane ion channels based on the structure of polytheonamide B.

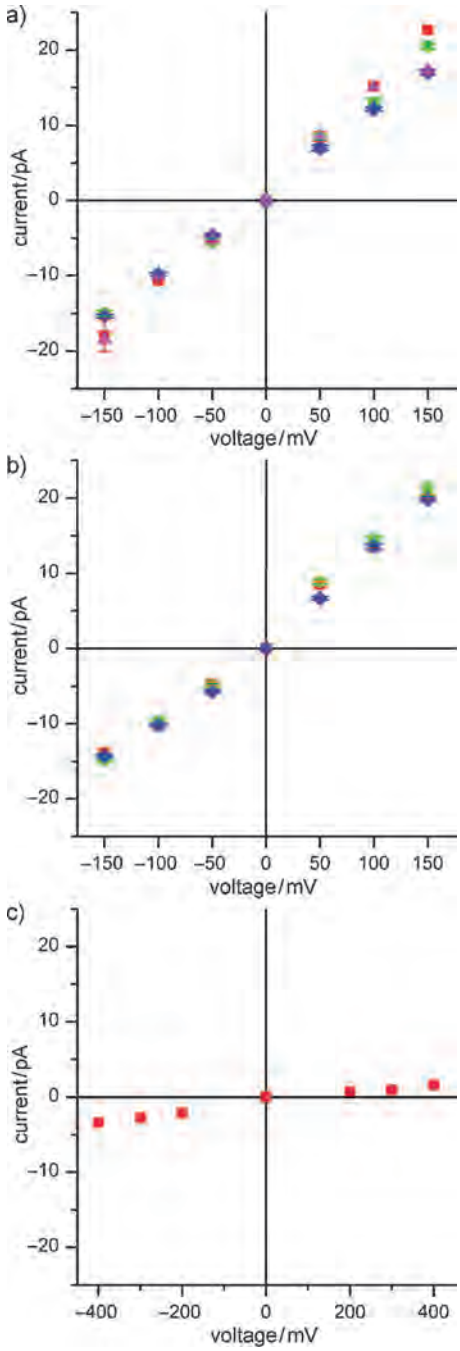

Figure 3. Current (I)–voltage (V) curves for proton channels formed by a) Ncap-[1–48]-OH (**1**: ■), Ncap-[1–48]-OMe (**2**: ●), Ncap-[1–48(O)]-OH (**3**: ◆), H-[1–48]-OH (**4**: ▲), b) MeCO-[1–48]-OH (**5**: ■), $Me(CH_2)_6CO$-[1–48]-OH (**6**: ●), $Me(CH_2)_{14}CO$-[1–48]-OH (**7**: ◆), and c) $Me_3N^+(CH_2)_3CO$-[1–48]-OH (**8**: ■).

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Keywords: cytotoxic agents · natural products · nonribosomal peptides · structure–activity relationships · synthetic ion channels

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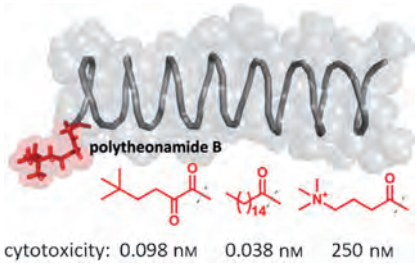
Chemical point mutation: Polytheonamide B is a naturally occurring polypeptide containing 48 amino acids. It both displays potent cytotoxicity and acts as a monovalent cation channel in vitro. Chemoselective methods to modify the 44th, N-, and C-terminal residues of the natural product have been developed, and evaluation of the resultant derivatives suggests that the intrinsic activities of the peptide can only be altered by switching its N-terminal substitution.



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