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Chemical Construction and Structural Permutation of Potent Cytotoxin Polytheonamide B: Discovery of Artificial Peptides with Distinct Functions

HIROAKI ITOH AND MASAYUKI INOUE*

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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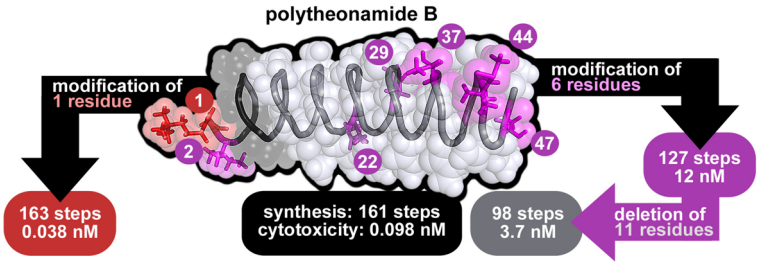

Polytheonamide B (1), isolated from the marine sponge *Theonella swinhoei*, is a posttranslationally modified ribosomal peptide (MW 5030 Da) that displays extraordinary cytotoxicity. Among its 48 amino acid residues, this peptide includes a variety D- and L-amino acids that do not occur in proteins, and the chiralities of these amino acids alternate in sequence. These structural features induce the formation of a stable $\beta^{6.3}$-helix, giving rise to a tubular structure of over 4 nm in length. In the biological setting, this fold is believed to transport cations across the lipid bilayer through a pore, thereby acting as an ion channel.

In this Account, we discuss the construction and structural permutations of this potent cytotoxin. First we describe the 161-step chemical construction of this unusual peptide 1. By developing a synthetic route to 1, we established the chemical basis for subsequent SAR studies to pinpoint the proteinogenic and nonproteinogenic building blocks within the molecule that confer its toxicity and channel function. Using fully synthetic 1, we generated seven analogues with point mutations, and studies of their activity revealed the importance of the N-terminal moiety. Next, we simplified the structure of 1 by substituting six amino acid residues of 1 to design a more synthetically accessible analogue 9. This dansylated polytheonamide mimic 9 was synthesized in 127 total steps, and we evaluated its function to show that it can emulate the toxic and ion channel activities of 1 despite its multiple structural modifications.

Finally, we applied a highly automated synthetic route to 48-mer 9 to generate 13 substructures of 27−39-mers. The 37-mer 12 exhibited nanomolar level toxicity through a potentially distinct mode of action from 1 and 9. The SAR studies of polytheonamide B and the 21 artificial analogues have deepened our understanding of the precise structural requirements for the biological functions of 1. They have also led to the discovery of artificial molecules with various toxicities and channel functions. These achievements demonstrate the benefits of total synthesis and the importance of efficient construction of complex molecules. The knowledge accumulated through these studies will be useful for the rational design of new tailor-made channel peptides and cytotoxic molecules with desired functions.

Introduction

Natural products continue to be a valuable source of tools for chemical biology and lead compounds for drug discovery.[1,2] They are secondary metabolites biosynthesized by living organisms and can be subdivided according to their chemical compositions as peptides, polysaccharides, isoprenoids, and alkaloids. Not only have these substances been optimized for a specific function during years of evolution, they also represent a promising basis for the development of substances with novel bioactivities.[3−5]

Peptidic natural products are classified as ribosomal and nonribosomal peptides according to their biosynthetic pathways. Ribosomal peptides are assembled from the 20 canonical proteinogenic amino acids by ribosomal machinery,

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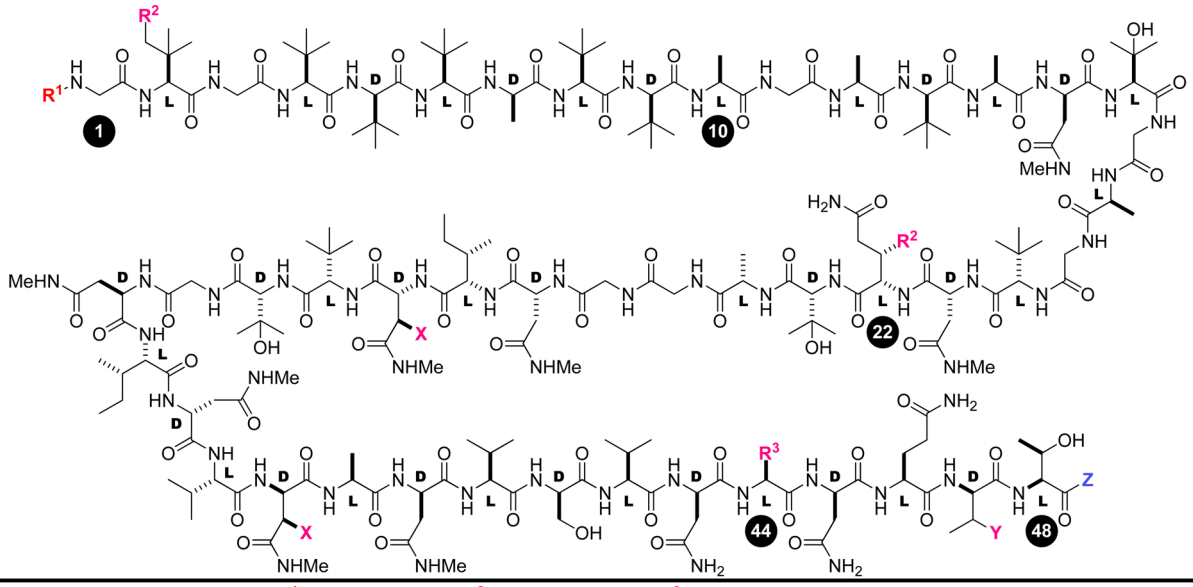


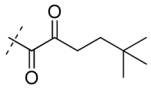
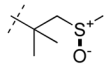
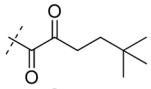
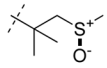
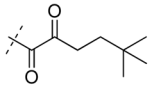
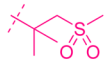
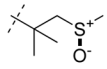
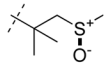
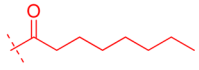
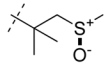
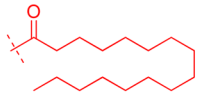
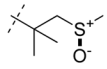
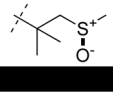
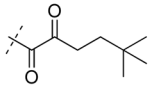
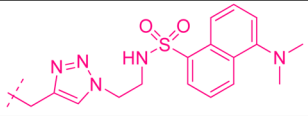
compounds	R^1	R^2	R^3	X	Y	Z
polytheonamide B						
Ncap-[1-48]-OH (**1**)		Me		OH	(*R*)-OH	OH
point mutated analogues						
Ncap-[1-48]-OMe (**2**)		Me		OH	(*R*)-OH	OMe
Ncap-[1-48(O)]-OH (**3**)		Me		OH	(*R*)-OH	OH
H-[1-48]-OH (**4**)	H	Me		OH	(*R*)-OH	OH
CH_3CO-[1-48]-OH (**5**)		Me		OH	(*R*)-OH	OH
$CH_3(CH_2)_6CO$-[1-48]-OH (**6**)		Me		OH	(*R*)-OH	OH
$CH_3(CH_2)_{14}CO$-[1-48]-OH (**7**)		Me		OH	(*R*)-OH	OH
$Me_3N^+(CH_2)_3CO$-[1-48]-OH (**8**)		Me		OH	(*R*)-OH	OH
dansylated polytheonamide mimic						
Ncap-[1-48]-OH (**9**)		H		H	(*S*)-OH	OH
truncated dansylated polytheonamide mimic						
H-[10-48]-OH (**10**), H-[11-48]-OH (**11**), H-[12-48]-OH (**12**), H-[13-48]-OH (**13**), H-[14-48]-OH (**14**), H-[15-48]-OH (**15**), H-[16-48]-OH (**16**), H-[17-48]-OH (**17**), H-[18-48]-OH (**18**), H-[19-48]-OH (**19**), H-[20-48]-OH (**20**), H-[21-48]-OH (**21**), H-[22-48]-OH (**22**)						

FIGURE 1. Structures of natural polytheonamide B (**1**), point-mutated polytheonamides (**2**–**8**), dansylated polytheonamide mimic (**9**), and truncated dansylated polytheonamide mimics (**10**–**22**). The sequences are abbreviated as R-[X-Y]-Z (R = functional group attached to N-terminal amine, X = N-terminal residue number, Y = C-terminal residue number, Z = functional group attached to C-terminal carboxylic acid). The residue numbers of **1** are written as plain text (R-[X-Y]-Z) and those of **9** are underlined (R-[X-Y]-Z).

while nonribosomal peptides are biosynthesized by a nonribosomal peptide synthetase, a large multifunctional protein complex, and contain many other building blocks in addition to the 20 amino acids.[6] Consequently, the structural diversity of

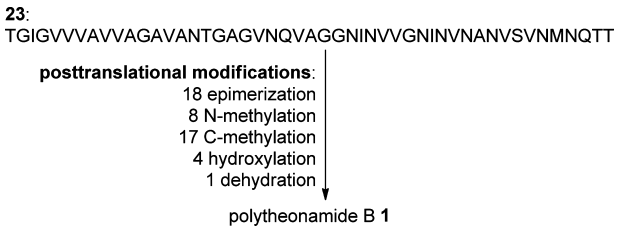


FIGURE 2. Posttranslational modifications of the ribosomally biosynthesized 49-mer peptide **23** to form polytheonamide B.

the ribosomal peptide natural products is limited in comparison to their nonribosomal counterparts. However, recent studies have shown that the ribosomal peptides can be posttranslationally modified extensively, and these modifications lead to products with many features resembling the nonribosomal peptides.[7] In fact, many of the modifications commonly thought of as nonribosomal are also found in ribosomally synthesized peptides.

Polytheonamide B (**1**, Figure 1), isolated from the Japanese marine sponge *Theonella swinhoei*, is an exceptionally cytotoxic linear peptide (IC_{50} = 0.098 nM, P388 mouse leukemia cells).[8,9] The structural elucidation of this peptide natural product by Fusetani and Matsunaga revealed that it has a molecular weight of 5030 Da, consists of 48 amino acid residues, and is capped at the N-terminus with 5,5-dimethyl-2-oxohexanoate (Ncap). The most striking structural features of **1** are that 13 out of the 19 different component amino acids of **1** are nonproteinogenic and its entire 48-mer peptide sequence is composed of alternating D- and L-amino acids, only interrupted by eight glycine residues.

By isolation of the biosynthetic genes from the sponge metagenome, Piel and co-workers proved that polytheonamide B is a posttranslationally modified ribosomal peptide (Figure 2).[10] The ribosomally synthesized 49-mer sequence **23** undergoes 18 epimerizations, 8 N-methylations, 17 C-methylations, and 4 hydroxylations by the action of only six enzymes. Consequently, **1** possesses various N-methylated, β-C-methylated, and β-C-hydroxylated derivatives of the corresponding proteinogenic amino acids as well as many D-configured amino acids and the highly methylated Ncap moiety.

These unusual amino acid components of **1** have pronounced effects on the conformation of its peptide backbone. Extensive solution NMR studies of **1** indicated that it folds into a β-helix of 6.3 residues per turn (Figure 3).[11] As a consequence, **1** forms a tubular structure with a length of approximately 4.5 nm and a hydrophilic pore with a diameter of 0.4 nm. This three-dimensional structure is considered to be stabilized by hydrogen-bonding interactions not only of the D,L-alternating main chain, but also of the side

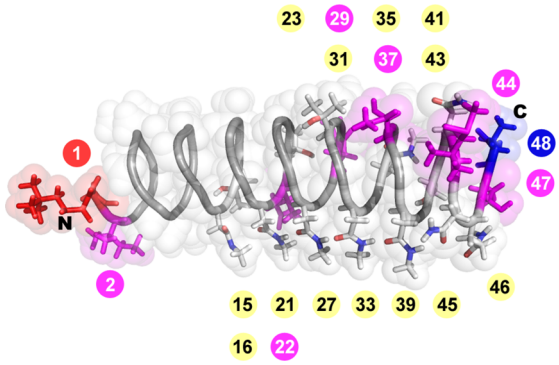


FIGURE 3. The $\beta^{6.3}$-helix structure of polytheonamide B (PDB1D code 2RQO). The residues are color-coded as follows: yellow, residues with side chains containing hydrogen bond donors or acceptors; red, Ncap and residue 1 at N-terminus; blue, residue 48 at C-terminus; purple, six residues substituted in dansylated polytheonamide mimic **9**.

chains of the residues oriented to point outside the helix. The hydroxy and amide groups of the side chains highlighted in yellow in Figure 3 are proposed to be involved in the hydrogen bond network that reinforces the $\beta^{6.3}$-helix of **1**.[11,12]

Since ion channel formation through a $\beta^{6.3}$-helical conformation is known for a head-to-head dimer of 15-mer D,L-alternating peptide gramicidin,[13−15] the 3-fold larger helix of monomeric **1** is believed to function as a transmembrane channel by itself. In fact, single channel recordings demonstrated that **1** formed a monovalent cation channel in a selectivity order of H^+, Na^+, K^+, Rb^+, and Cs^+.[16,17] Additionally, compound **1** was approximately 40 times more cytotoxic against P388 cells than gramicidin D (IC_{50} = 4.3 nM), and this exceedingly high toxicity correlated to the ability of **1** to efficiently form a highly stable ion channel.

The unusual structure, potent cytotoxicity, and specific channel characteristics motivated us to launch a program toward understanding and controlling the unique function of **1** using synthetic organic chemistry. We first developed the total synthesis route to 48-mer peptide polytheonamide B (Figure 1).[18−21] This achievement to gain precise synthetic control of the structure of **1** provided the first chemical basis for systematically correlating its molecular structure with biological functions. Specifically, structure−activity relationship (SAR) studies of the 21 fully synthetic variants, **2**−**22**, were performed to pinpoint the proteinogenic and nonproteinogenic building blocks within the molecule that were essential to the channel function and the toxicity.[22−24] These accomplishments are described in detail in this Account.

Total Synthesis of Polytheonamide B[18−21]

In general, the total synthesis of large polypeptides, including proteins, continues to pose significant challenges in

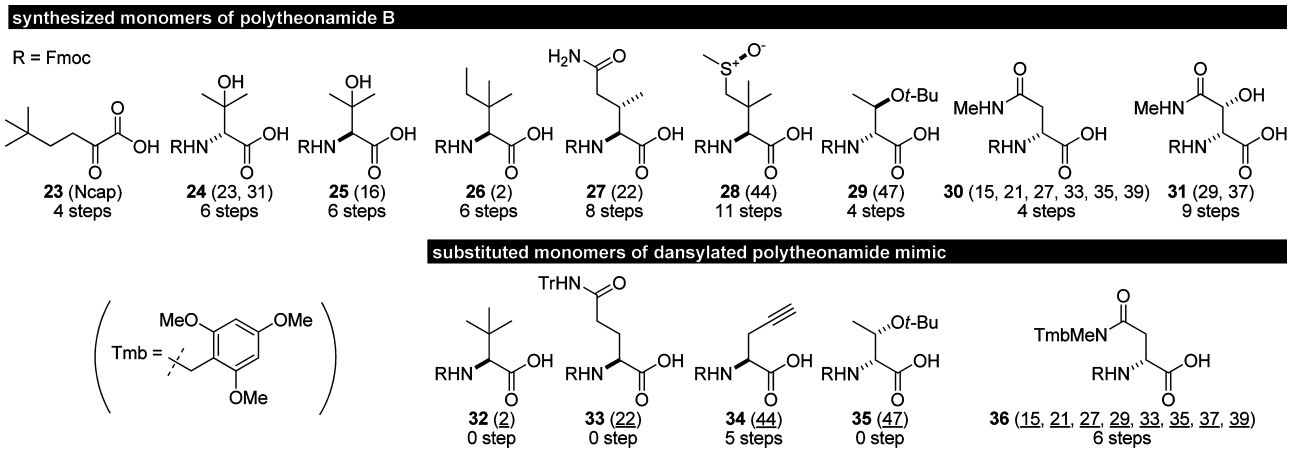


FIGURE 4. Synthetically prepared component monomers of polytheonamide B and dansylated polytheonamide mimic. The residue numbers are indicated in parentheses. *t*-Bu, *t*-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; Tmb, 2,4,6-trimethoxybenzyl; Tr, triphenylmethyl.

chemistry. This is particularly the case if the peptide molecules of interest are composed of unusual amino acids and are not easily amenable to automated solid-phase synthesis. Polytheonamide B represents an ideal target molecule for expanding our knowledge regarding the reactivity and structural and functional properties of large polypeptides.

Our total synthesis of **1** employed a flexible and general strategy, consisting of four independent stages: (1) synthesis of the nonproteinogenic amino acid monomers;[25] (2) construction of peptide segments by stepwise assembly using solid-phase peptide synthesis (SPPS) methods;[26] (3) convergent couplings of the peptide segments; and (4) global deprotection of the protected polytheonamide.

In the first stage, the nine building blocks, **23**–**31** (Figure 4), were synthesized from the corresponding commercially available materials in 58 total steps. In the second stage, Fmoc-based solid-phase peptide synthesis was adopted to construct the four peptide fragments. Preliminary studies showed that the assembly of amino acids on solid phase supports only proceeded up to 16 residues. Beyond this length, elongation of the peptide was impeded by the sterically hindered β-tetrasubstituted amino acids, which have low reactivity, and by asparagine and glutamine derivatives, the side chain amides of which have a strong tendency to form interstrand aggregates in the peptide–resin matrix.[27] Therefore, four segments containing 7–16 residues were designed (Figure 5): residues 1–11 (**37**), 12–25 (**38**), 26–32 (**39**), and 33–48 (**40**). The first three peptides included glycines as the C-terminal amino acid to eliminate the risk of epimerization during the third stage of the process. To maximize the yields of the elongation processes, the primary amide groups at residues 43, 45, and 46 and the hydroxyl groups at residues 41, 47, and 48 were protected with Tr and *t*-Bu groups, respectively.

Segments **37**, **38**, and **39** were synthesized from Fmoc-Gly-Wang resin **42** using HBTU/HOBt activation (Scheme 1). After cleavage from the Wang resin, the three segments were converted to the corresponding thioesters **45**, **44**, and **43**. Alternatively, the synthesis of segment **40** started from H-Thr(*t*-Bu)-2-chloro trityl resin **41**.[28] A more reactive reagent system (HATU/HOAt[29]) was applied for the stepwise elongation of **41**, because HBTU/HOBt conditions resulted in a significantly lower yield of the peptide. The highly acid-labile 2-chloro trityl resin linker allowed orthogonal cleavage from the resin under mildly acidic conditions without deprotection of the side chains of the elongated peptide, leading to **40**.

The third stage involved the assembly of the four structurally complex segments with numerous potentially reactive functionalities (Scheme 1). To realize the three challenging couplings, the Ag^+-mediated reaction between thioesters and amines[30] was employed because of its excellent chemoselectivity and high reactivity. Specifically, a reagent combination of $AgNO_3$ and HOOBt was applied for coupling between **43** and **40** to yield **46**. Piperidine treatment of the product gave rise to the half structure of the protected polytheonamide **47**. The obtained amine **47** was next coupled with **44** under similar reaction conditions, and the resulting compound was subsequently deprotected to produce **49**. Finally, the protected polytheonamide **50** was synthesized by Ag^+-mediated coupling of **49** with **45**.

The fourth stage of the total synthesis was the removal of the three *t*-Bu and three Tr groups. Exposure of **50** to TFA/H_2O under carefully controlled temperature and time delivered fully synthetic polytheonamide B without affecting acid-sensitive functionalities such as the β-tertiary hydroxyl groups of the side chains.

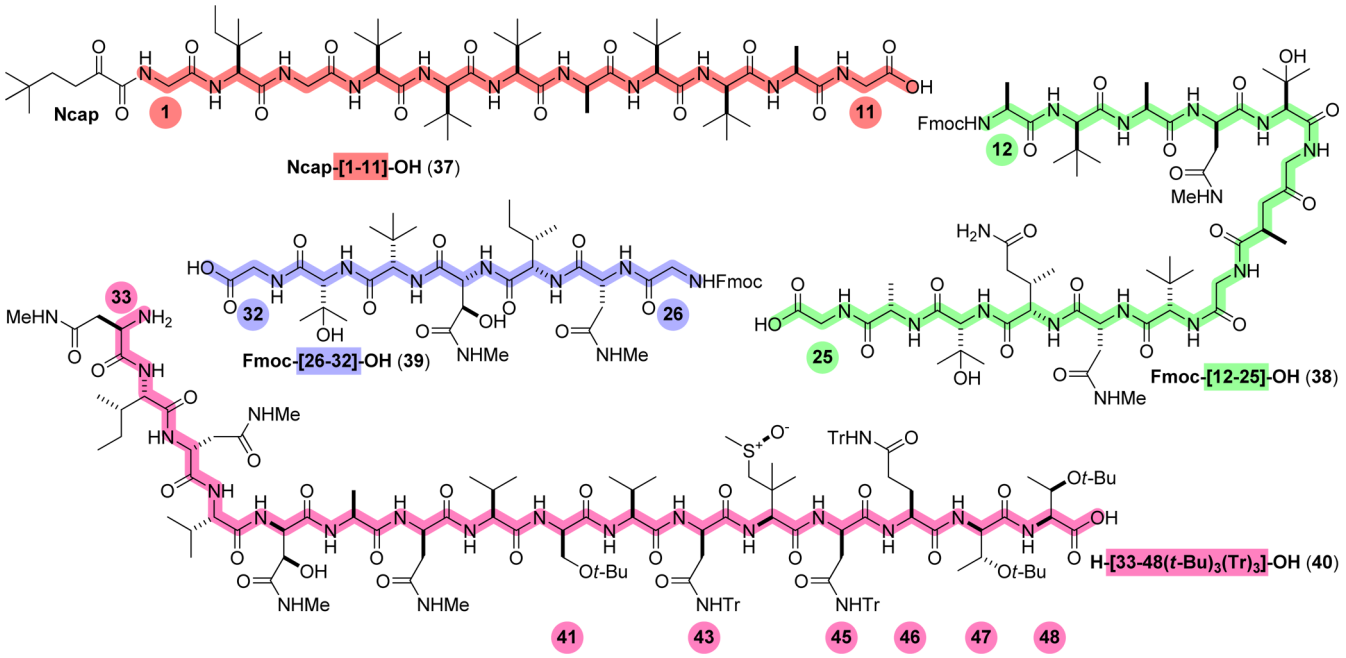
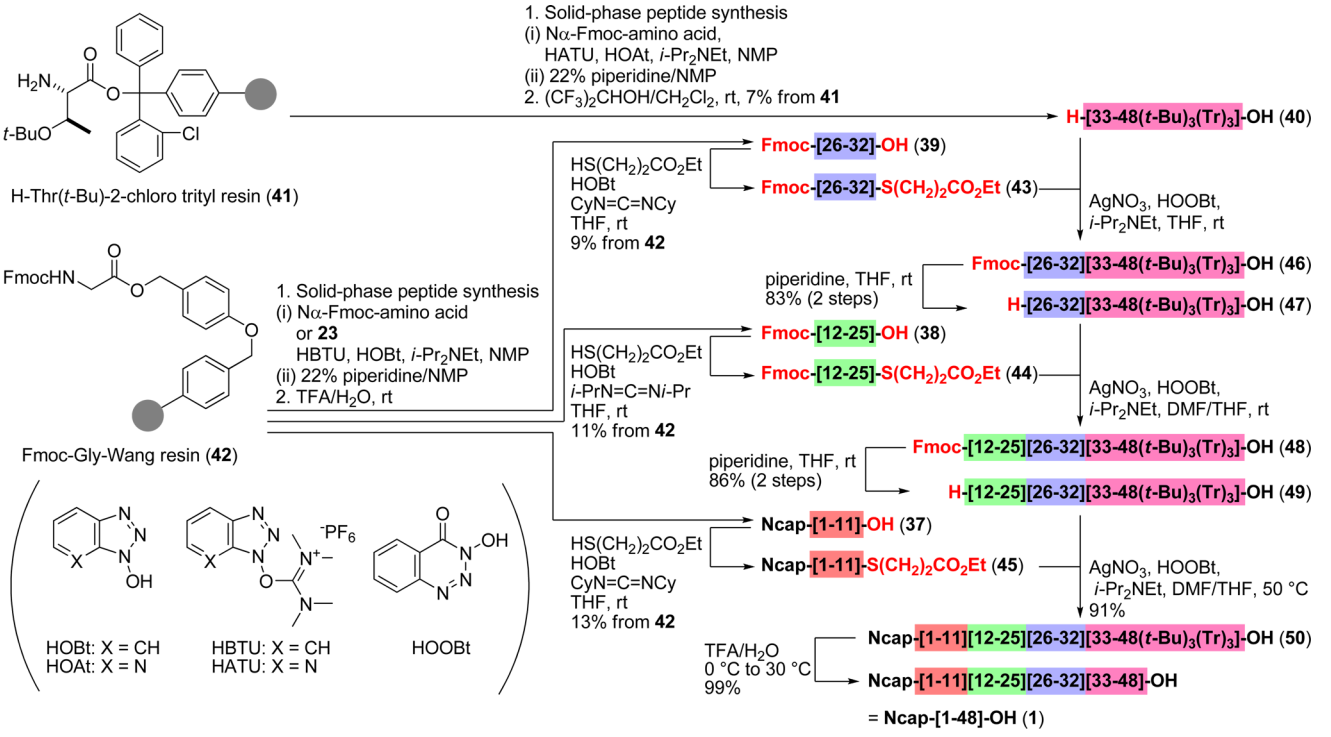


FIGURE 5. Structures of four peptide fragments of polytheonamide B.

SCHEME 1. Total Synthesis of Polytheonamide B[a]



[a]Colors of the four fragments correspond to those of Figure 5. Cy, cyclohexyl; HATU, *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydro-4-oxo-1,2,3-benzotriazine; TFA, trifluoroacetic acid.

Hence, the first total synthesis of polytheonamide B was achieved in 161 total steps by combination of the four key stages: synthesis of nonproteinogenic amino acids (58 steps), SPPS of the four complex peptides (94 steps), solution-phase functionalization and condensation of the fragments (8 steps) and, finally, global deprotection (1 step). The knowledge

obtained through the total synthesis, including the preparative methodologies and the physicochemical properties of the amino acids, the peptide segments, and the target molecules, permitted us to synthesize and functionally analyze the large peptide sequences described in the next sections.

Chemical Point Mutations of Polytheonamide B[22]

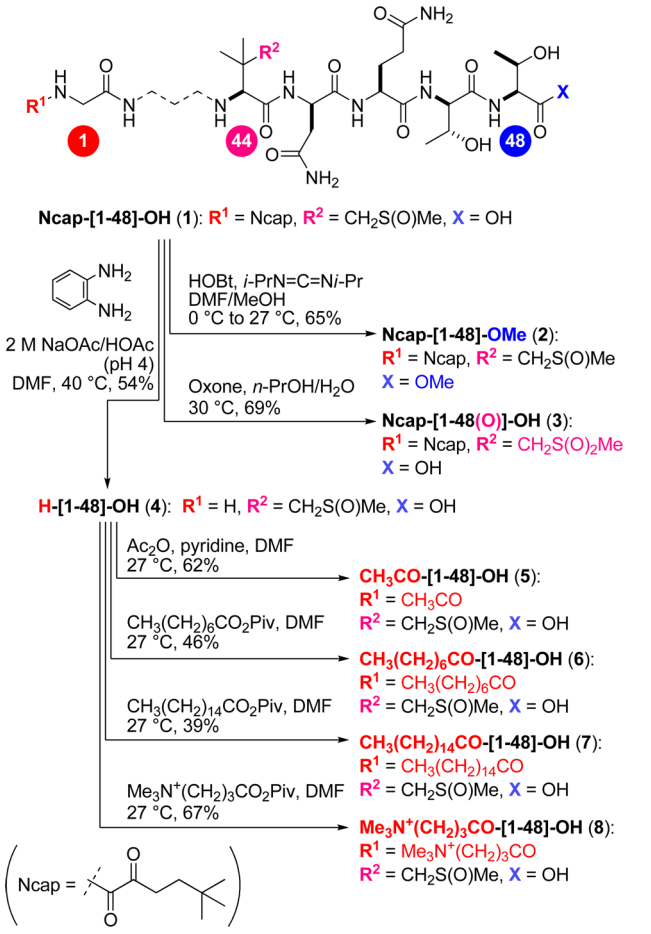
To decipher the building blocks that are responsible for the unique biological function of polytheonamide B, we planned to site-specifically modify the monomer units of synthetic **1** and to evaluate the biological activity of those synthetically generated point-mutated analogues. Residues 1, 44, and 48 were selected as the modification points, since these terminal structures would be placed at the entrance of the pore in the polytheonamide channel, and their structural perturbations could influence the ion channel function (see Figure 3).

Achieving selective and precise alteration of a complex structure such as that of **1** necessitates the use of highly chemoselective reactions, since its numerous native functional groups can cause undesired reactions (Scheme 2). After various unsuccessful attempts, **1** was chemoselectively converted to methyl ester **2** and sulfone **3**.[31] The four N-terminal modified analogues **5**–**8** were produced by acylation of amine **4**, which was prepared from **1** by employing a chemoselective reaction between the 1,2-dicarbonyl structure of Ncap and benzene-1,2-diamine.[32]

The cytotoxicity of the seven derivatives was assessed (Table 1). While the cytotoxicity of methyl ester **2** was comparable to **1**, sulfone **3** was 5-fold less toxic than **1**. Remarkably, the degree of toxicity was dependent upon the oxidation state of a single sulfur atom, suggesting that the highly polar nature of the sulfoxide group of residue 44 played a favorable role in toxicity.[31,33]

Substitution of the Ncap of **1** had an even more profound effect on cytotoxicity. The degree of toxicity was ordered according to the hydrophobicity of incorporated functionalities. The less hydrophobic **4** and **5**, and the comparably hydrophobic **6**, were 240-fold, 480-fold, and 5-fold less toxic than **1**, respectively, whereas the more hydrophobic **7** was even more toxic than potent **1**. The apparent correlation between hydrophobicity and cytotoxicity among compounds **1**, **4**, **5**, **6**, and **7** indicated that the size of the hydrophobic N-terminal moieties was critical for cytotoxicity. The most drastic decrease in cytotoxicity (2500-fold) was exhibited when the Ncap of **1** was switched to the hydrophilic trimethyl ammonium structure of **8**. These results raise the possibility that hydrophobic substitution at the N-terminus is required for targeting cell membranes as well as for orienting **1** for effective membrane insertion, where it exerts its cytotoxic activity.

SCHEME 2. Chemical Point Mutation of Polytheonamide B[a]



[a]Residues 2–43 are omitted in the structure of **1**. Piv = pivaloyl.

TABLE 1. Cytotoxicity of **1** and Analogues **2**–**8** against P388 Mouse Leukemia Cells

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

To compare the channel functions of representative more toxic **7** and less toxic **8** to that of **1**, transmembrane H^+ currents were recorded in acidic aqueous solution using an artificial planar lipid bilayer (Figure 6).[34,35] Whereas the conductance obtained from current–voltage curves for **1** and **7** were comparable, **8** exhibited negligible H^+ conductance.

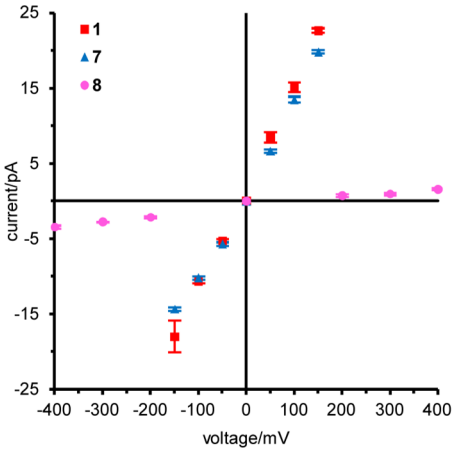


FIGURE 6. Current−voltage (*I*−*V*) curves for H^+ channels formed by **1**, **7**, and **8**. The single-channel currents were recorded in DPhPC planar lipid bilayer in 0.1 M HCl. DPhPC = diphytanoyl phosphatidylcholine.

These data indicated that the increased hydrophobicity at the N-terminal region of **7** was inconsequential as far as impacting the H^+ channel formation of **1** and that the mere presence of the ammonium group in **8** almost completely eliminated the H^+ 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, resulting in blockage of ion conductance.[36−38] The decreased ion channel activity of **8** may also correlate with its significantly lower cytotoxicity than that of **1**.

Therefore, the chemical point mutations highlighted the effects of residues 1, 44, and 48 on the cytotoxicity and the channel activities. Importantly, we demonstrated for the first time that increased hydrophobicity of the N-terminal structures of residue 1 strongly correlated to increased cytotoxicity. Furthermore, the drastic deactivating effect of the trimethyl ammonium moiety of **8** suggested that the ion current can be controlled merely by changing the terminal functional group of the Ncap moiety. These discoveries should facilitate efforts to rationally design tailor-made transmembrane ion channels.

Design, Synthesis, and Functional Analysis of Dansylated Polytheonamide Mimic[23]

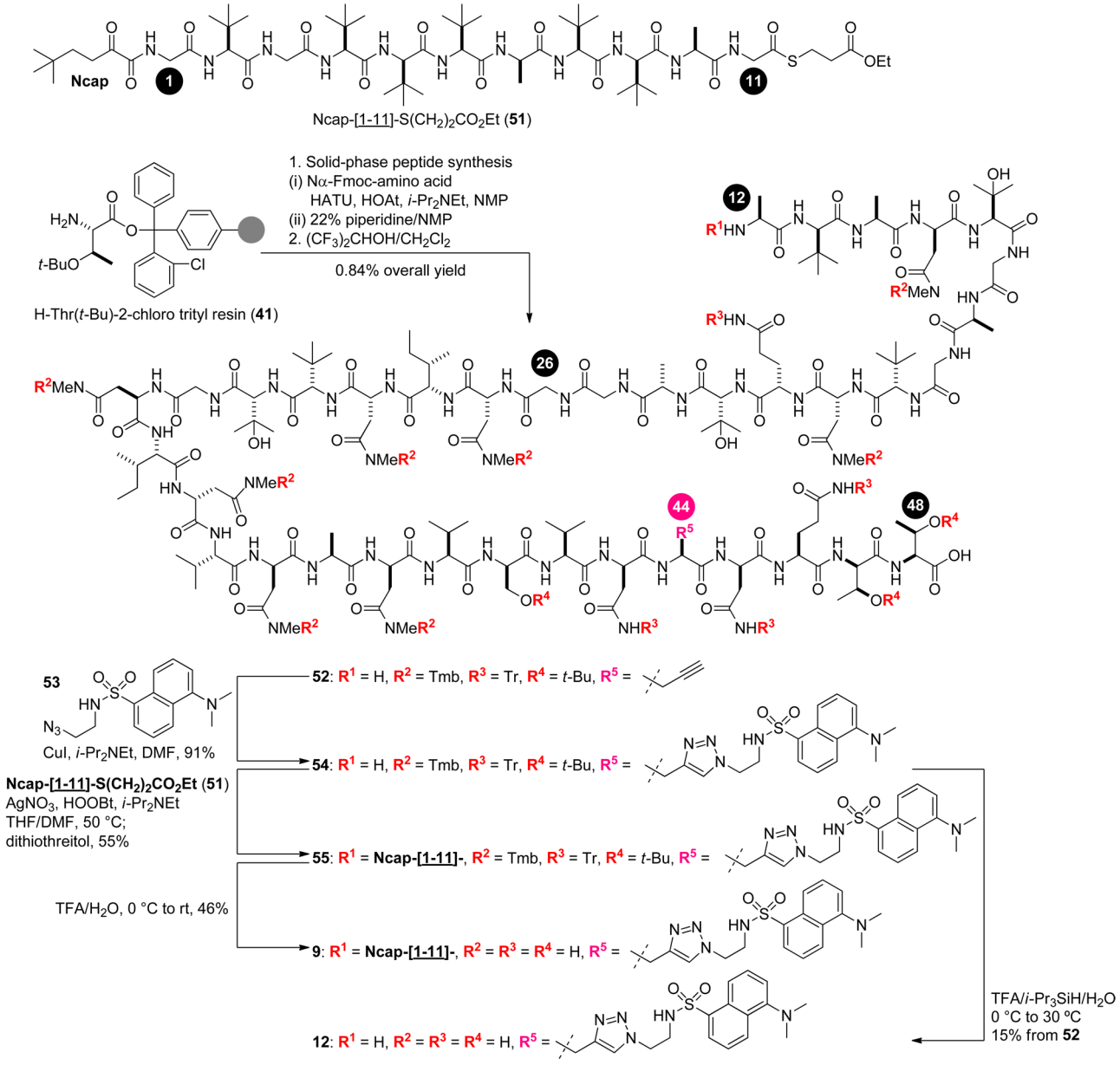
The functional analyses of the point-mutated polytheonamides provided us with a useful set of SAR information. For performing further systematic biological studies, it is particularly important to supply polytheonamide B structures in a more efficient fashion by reducing the required synthetic steps for preparation of the amino acids. Thus, the next aim of our research was the design of new and simplified analogues that would retain the functions of the original natural product and would be prepared by shorter routes.

Multiple mutations of the residues in natural peptide sequences often lead to loss of their original biological activity, because the structures and order of their constituent amino acids encode their functions. Depending on peptide length and residue composition, linear D,L-peptides can in fact adopt diverse single-stranded or double-stranded β-helices of various pore sizes.[39−42] Therefore, judicious incorporation of the function-determining features of **1** is crucial for simplifying the entire structure of **1**. Because the $\beta^{6.3}$-helix formation of **1** is considered to be stabilized by hydrogen-bonding interactions of both the main chain and the side chains (see Figure 3), we hypothesized that retaining these interresidue interactions would be essential for mimicking the structure and function of **1**.

These considerations guided us in the design of the synthetically more accessible polytheonamide mimic **9**, in which six amino acid residues of **1** were replaced (**9**, Figure 1). While maintaining alternating D- and L-α-chiral centers of the main chain of **1** and the hydroxy and amide groups of the side chains, the γ-methyl group of residue 2 and β-substituents of residues 22, 29, and 37 were removed, and residue 47 was changed from D-*allo*-threonine to D-threonine. These minimal modifications significantly reduced the number of steps for preparation of the amino acid monomers (Figure 4). Additionally, residue 44 (β,β-dimethyl L-methionine oxide), which is expected to be placed at the entrance of the channel pore, was replaced by L-propargyl glycine. This particular alteration would enable the incorporation of various probes and controlling elements that potentially influence the ion channel function at the late stage of the synthesis through click chemistry.[43] We first selected the dansylated polytheonamide mimic **9** (Ncap-[1-48]-OH, the residue numbers are underlined for this series of peptides) as the target molecule, because the fluorescent dansyl group could be utilized as a probe for investigation of the physicochemical behavior of **9** under biological settings.

Among the six substituted amino acid residues, only **34** and **36** required synthetic preparation (Figure 4). Thus, the total number of steps for the monomer syntheses was decreased to 27 steps for **9** from 58 steps for **1**. The secondary amide of D-*N*-methyl aspargine derivative **36** was prepared as its Tmb protected form, although Tmb was not used in the total synthesis of **1**. It was envisaged that this group would be simultaneously removed with the two other protective groups (Tr and *t*-Bu) upon acid activation at the last step of the total synthesis of **9**. More importantly, capping the secondary amide with Tmb was expected to inhibit formation of nonreactive aggregates in the peptide resin, and thus to increase efficiency of the SPPS.

SCHEME 3. Total Synthesis of Dansylated Polytheonamide Mimic **9** and Its Truncated Derivative **12**



In contrast to the fact that the assembly of amino acids only proceeded up to 16 residues in the total synthesis of **1** (Scheme 1), the longer sequence of **9** was found to be elongated by the SPPS, demonstrating the significance of the presence of the Tmb group on the efficient assembly. These results permitted us to adopt a highly automated synthetic strategy for assembly of **9** (Scheme 3). Namely, 37-mer peptide **54** (residues 12−48) and 11-mer peptide **51** (residues 1−11) were planned to be separately prepared by the SPPS method and then to be coupled at the reactive 11th glycine to generate the full length mimic.

The synthesis of **9** is illustrated in Scheme 3. The N-terminal segment **51** was synthesized similarly to the corresponding portion of **1** (**45**, Scheme 1). As planned, the 37-mer peptide **52** was elongated by the action of the HATU/HOAt reagent system from **41** including the eight condensations of the Tmb-protected amino acid **36**. Next, cleavage from the 2-chloro trityl resin was achieved under mild acidic conditions without deprotecting the side chains to generate **52**, which is the longest D,L-alternating sequence prepared to date through the SPPS.[44] Thus obtained segment **52** underwent smooth Cu^{+}-promoted 1,3-dipolar addition with **53** to produce the dansylated compound **54**.

After syntheses of the two segments **51** and **54**, only two reactions, coupling and global deprotection, remained to be

established for the total synthesis of **9** (Scheme 3). When thioester **51** and amine **54** were treated with $AgNO_3$ and HOOBt, the protected 48-mer **55** was successfully obtained. Finally, by careful tuning of the acidic conditions, **55** was converted to dansylated polytheonamide mimic **9** through simultaneous removal of the eight Tmb, four Tr, and three *t*-Bu groups from **55**. Hence, the simplified mimic **9** was synthesized via a considerably more practical and efficient route in comparison to the 161-step synthesis of original **1**. The total 127 steps were comprised of 27 steps of monomer syntheses, 96 steps of automated solid-phase syntheses, and only 4 steps of technically demanding reactions of the large peptides, demonstrating the high operational superiority of the construction of **9**.

Completion of the synthesis of **9** enabled biological and biophysical studies to be performed. Compound **9** exhibited strong cytotoxicity with an IC_{50} value of 12 nM. Although the toxicity of **9** was approximately 100-times weaker than that of **1** (IC_{50} = 0.098 nM), it was remarkable that multiple structural modifications of the six residues of **1** did not result in complete loss of toxicity, indicating that our designed molecule maintained the activity-determining structural features of **1**.

To demonstrate that **9** forms ion channels for Na^+ ions and protons and to compare its channel behavior to that of **1**, single channel recording experiments were performed. A planar lipid bilayer was formed either in neutral solution (1 M NaCl, pH 7.4) or in acidic aqueous solution (0.1 M HCl, pH 1.0), and then Na^+ or H^+ current across the membrane was measured. Interestingly, while the shapes of the Na^+ current–voltage curves of **1** and **9** were comparable (Figure 7a), their H^+ current–voltage relationships were found to be significantly different (Figure 7b). Considering the similar activities of **1** and **9** for the Na^+ channels, the much smaller H^+ conductance of **9** can be attributed to the lower pH necessary for the H^+ channel experiments in comparison to the Na^+ channel. Since the dansyl group of residue 44 would be located close to the ion entrances of the channel, it is likely that the basic amine of the dansyl group undergoes protonation from the aqueous phase at pH 1. The protonated cationic amine could in turn lower the channel activities through binding to the ion entrance or by inducing a repulsive electrostatic effect toward incoming protons.[22,36–38] This suggests an exciting possibility of pH-driven control of the channel functions of **9**, though future detailed studies would be necessary to further prove this hypothesis.

In this work, we realized the design, total synthesis, and functional analysis of dansylated polytheonamide mimic **9**,

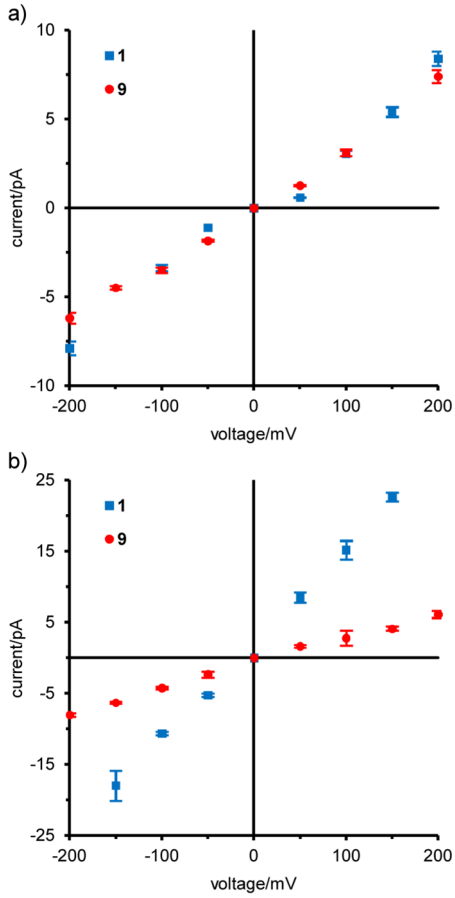


FIGURE 7. (a) Current–voltage (*I*–*V*) curves for Na^+ channels formed by **1** and **9**. The single-channel currents were recorded in DPhPC planar lipid bilayer in 1 M NaCl (pH 7.4). (b) Current–voltage (*I*–*V*) curves for H^+ channels formed by **1** and **9**. The single-channel currents were recorded in DPhPC planar lipid bilayer in 0.1 M HCl (pH 1.0).

an artificial ion channel. In designing **9**, the sequence of the original natural product **1** was simplified by replacing six residues. Accordingly, the synthetic route to **9** required significantly fewer synthetic steps in comparison to **1**. Despite the multiple substitutions, **9** emulated the cytotoxic and ion channel activities of **1**. Due to the practicality in the total synthesis and efficiency in introduction of diverse functional structures via click chemistry, our polytheonamide mimic will serve as a novel platform for optimizing and controlling cytotoxic and channel activities and for bestowing artificial functions that the original natural product does not possess.

Synthesis and Functional Analysis of Truncated Dansylated Polytheonamide Mimic[24]

In the total synthesis of the 48-residue sequence of **9**, assembly of the D,L-alternating 37 amino acid sequence from residues 12 to 48 was realized through a single automatic SPPS. The optimized SPPS technology enabled us to

TABLE 2. Cytotoxicity of **9** and Truncated Dansylated Polytheonamide Mimics **10**–**22** against P388 Mouse Leukemia Cells

compound	IC_{50} (nM)
9, Ncap-[1-48]-OH	12
10, H-[10-48]-OH	>420
11, H-[11-48]-OH	>420
12, H-[12-48]-OH	3.7
13, H-[13-48]-OH	81
14, H-[14-48]-OH	100
15, H-[15-48]-OH	>420
16, H-[16-48]-OH	140
17, H-[17-48]-OH	>450
18, H-[18-48]-OH	190
19, H-[19-48]-OH	>410
20, H-[20-48]-OH	390
21, H-[21-48]-OH	>250
22, H-[22-48]-OH	>420

initiate a program directed toward efficient automated preparation of 13 substructures, **10**–**22** (H-[X-48]-OH, X = 10–22) of **9** in the search for biologically active compounds with minimum molecular complexity.

All the compounds, **10**–**22**, were prepared by a unified protocol, and the synthetic route to **12** (H-[12-48]-OH) is illustrated as a representative example in Scheme 3. After synthesis of **54** through SPPS and the dansyl introduction, simultaneous removal of the side chain protective groups from **54** delivered **12**. This methodology was applicable for efficient preparations of longer **10** and **11** and shorter **13**–**22**.

The collection of the 39–27-mer analogues **10**–**22** allowed us to determine the cytotoxicities of this series (Table 2). Six out of thirteen structures showed detectable toxicities, and submicromolar level activities were observed for five (**13**, **14**, **16**, **18**, and **20**). Surprisingly, compound **12** was found to be at least 20-fold more toxic (IC_{50} = 3.7 nM) than the other twelve analogues, and its toxicity was even three times higher than the longer **9**. It is noteworthy that a drastic increase in potency was observed when only one amino acid was detached from 38-mer **11** or attached to 36-mer **13**. These results strongly suggested the significance of the specific structure of 37-mer **12** for potent cytotoxicity.

A key question was whether **12** shared the same mechanism of action as natural product **1** and mimic **9**. Therefore, ion transport activity of **12** was compared with those of **1** and **9** using liposomes, which are models of cell membranes (Figure 8). The liposomes were prepared in NaCl-containing buffer with a pH gradient, and pyranine, a fluorescent pH indicator, was incorporated into the liposomes.[45] Addition of the peptide channels then permitted exchange between protons and Na^+ ions across the lipid bilayer to preserve the ionic milieu, leading to the fluorescence increase

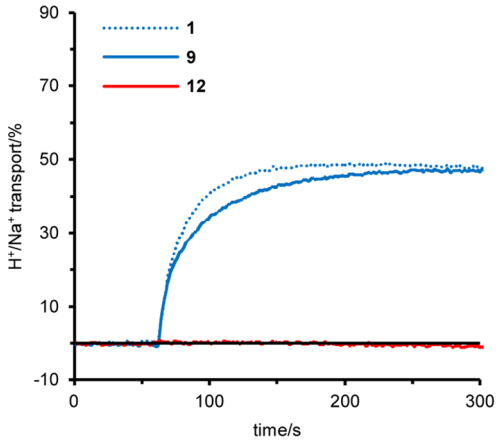


FIGURE 8. Time-course of H^+/Na^+ exchange across lipid bilayers of pH-gradient liposomes caused by **1**, **9**, and **12**. The ion transports were evaluated as the pH-dependent fluorescence from pyranine standardized against the maximum exchange by Triton X-100. In all the experiments, **1**, **9**, or **12** was added at 60 s. Pyranine = trisodium 8-hydroxypyrene-1,3,6-trisulfonate.

caused by deprotonation of pyranine. In contrast to **1** and **9**, which induced an increase of the fluorescence over time through H^+/Na^+ exchange, addition of peptide **12** resulted in no change, clarifying that **12** had no ion transport activity in this assay. The data strongly suggested that compound **12** exerted its cytotoxicity through a different mechanism of action from **1** and **9**, although **12** shares 2/3 of its sequence with ion-channel forming **9**.

Hence the discovery of potently toxic 37-mer peptide **12** with its distinct activity was achieved through development of the highly automated procedure for comprehensive syntheses of 13 substructures of **9** and their subsequent functional analyses. Future detailed biological studies will be performed to uncover the unknown mechanism of action of **12**.

Conclusion

The first total synthesis of polytheonamide B, a 48-mer natural peptide, was achieved. Our synthesis of 161 steps involved 58 steps of monomer syntheses, 94 steps of the automatic SPPS, and 9 steps of functionalizations, couplings, and deprotection of the large peptides. The obtained **1** was utilized for preparation of seven point-mutated analogues. Through their biological evaluation, the N-terminal moiety was found to be an extremely important structure for cytotoxicity and ability to function as an ion channel: the more hydrophobic analogue **7** had more potent activity than **1**, and the ammonium cation analogue **8** exhibited weak cytotoxic and negligible ion channel activities.

Furthermore, the simplified polytheonamide mimic **9** was designed and synthesized as a new platform structure for

further SAR studies. This molecule required significantly fewer synthetic steps (127 steps), consisting of minimized monomer synthesis and peptide manipulations (31 steps) and maximized automated SPPS (96 steps). The 48-mer mimic **9** emulated the potent cytotoxicity and the ion channel activity of the original **1**. The highly optimized SPPS methodology for **9** was then applied for unified preparation of its 13 substructures. Among these 27−39-mer peptides, 37-mer **12**, which was synthesized in 98 total steps, was discovered to possess nanomolar toxicity. Unexpectedly, **12** did not exhibit ion channel activity, suggesting that **12** exerted its potent cytotoxicity through a distinct mode of action from **1** and **9**.

The SAR studies of polytheonamide B and the 21 artificial analogues taken together not only have deepened our understanding of the precise structural requirements for the biological functions of polytheonamide but also have led to the discovery of artificial molecules with distinct toxicity and channel functions. These achievements demonstrate the benefits of total synthesis endeavors and the importance of efficient construction of complex molecules and offer a unique opportunity for further exploration in chemical biology studies and drug discovery efforts. Further studies on polytheonamide B will lead to rational generation of new tailor-made channel peptides and new cytotoxic molecules with desired functions.

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BIOGRAPHICAL INFORMATION

Hiroaki Itoh was born in Mie, Japan, in 1985. He received a B.Sc. degree in Pharmaceutical Sciences from The University of Tokyo in 2008. He is currently pursuing a Ph.D. under the supervision of Prof. M. Inoue. His work focuses on developing a novel polytheonamide-based artificial peptide ion channel and biologically active peptides.

Masayuki Inoue was born in Tokyo, Japan, in 1971. He received a B.Sc. degree in Chemistry from The University of Tokyo in 1993. In 1998, he obtained his Ph.D. from the same university, working under the supervision of Prof. K. Tachibana. After spending 2 years with Prof. S. J. Danishefsky at the Sloan-Kettering Institute for Cancer Research, he joined the Graduate School of Science at Tohoku University as an assistant professor in the research group of Prof. M. Hirama in 2000. At Tohoku University, he was promoted to associate professor in 2004. In 2007, he moved to the Graduate School of Pharmaceutical Sciences, The University of Tokyo, as a full professor. His research interests include the synthesis, design, and study of biologically important molecules, with particular emphasis on the total synthesis of structurally complex natural products.

FOOTNOTES

*E-mail: inoue@mol.f.u-tokyo.ac.jp.
The authors declare no competing financial interest.

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