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Total synthesis of the large non-ribosomal peptide polytheonamide B

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Polytheonamide B is by far the largest non-ribosomal peptide known at present, and displays extraordinary cytotoxicity (EC_{50} = 68 pg ml^{-1}, mouse leukaemia P388 cells). Its 48 amino-acid residues include a variety of non-proteinogenic D- and L-amino acids, and the absolute stereochemistry of these amino acids alternate in sequence. These structural features induce the formation of a stable β-strand-type structure, giving rise to an overall tubular structure over 30 Å in length. In a biological setting, this fold is believed to transport cations across the lipid bilayer through a pore, thereby acting as an ion channel. Here, we report the first chemical construction of polytheonamide B. Our synthesis relies on the combination of four key stages: syntheses of non-proteinogenic amino acids, a solid-phase assembly of four fragments of polytheonamide B, silver-mediated connection of the fragments and, finally, global deprotection. The synthetic material now available will allow studies of the relationships between its conformational properties, channel functions and cytotoxicity.

Polytheonamides A and B (epi-**1** and **1**, respectively; Fig. 1a) are exceptionally cytotoxic linear peptides (EC_{50} = 68 pg ml^{-1}, mouse leukaemia P388 cells)[1]. These compounds were isolated from the marine sponge *Theonella swinhoei*, and are believed to be produced by symbiotic microorganisms that have not yet been identified. The structural elucidation of this peptide by Fusetani, Matsunaga and colleagues revealed that it has a molecular weight of over 5,000 Da, consists of 48 amino-acid residues, and is capped at the N-terminus with 5,5-dimethyl-2-oxohexanoate. Remarkably, 13 of the 19 different component amino acids of **1** are non-proteinogenic (Fig. 1b), strongly indicating that these natural products are biosynthesized by non-ribosomal peptide synthases[2]. Accordingly, polytheonamides represent the largest non-ribosomal peptides known at present.

The unique amino-acid components of polytheonamides include eight γ-*N*-methyl asparagine derivatives (**17** and **19**, Fig. 1b) and fourteen β-methylated derivatives of proteinogenic amino acids such as β-methyl valine (**10** and **11**), β-methyl threonine (**14** and **15**), β-methyl isoleucine (**16**), β-methyl glutamine (**18**) and β,β-dimethyl methionine oxide (**20**). Sulfoxide-bearing amino acid **20** is unprecedented in both natural and synthetic products. Interestingly, polytheonamide A (epi-**1**) and B (**1**) are epimers at the sulfoxide moieties of this particular amino acid, although their absolute stereochemistries have not been determined (Fig. 1a).

The most striking structural feature of **1** is that its entire 48-residue peptide sequence is composed of alternating D- and L-amino acids, only interrupted by eight Gly residues (Fig. 1a). Extensive NMR studies of **1** in methanol/chloroform solution indicated that the peptide sequence with alternating chirality folded into a β-helix of 6.5 residues per turn, stabilized by inter-residue hydrogen bonding[3]. As a consequence, overall, it forms a tubular structure spanning over 30 Å, with all the side chains oriented to point outside the helix[4–7]. Polytheonamide B has been shown to conduct monovalent cations across a lipid bilayer and to have a voltage-dependent gating property similar to that of an ion channel protein[8]. Such specific ion channel characteristics have not been observed for any other known secondary metabolites, and it has therefore been proposed that the particular conformation of **1** is responsible for them. Because the molecular weights of channel proteins are typically over 100,000 Da, polytheonamide can be regarded as a minimalist transmembrane channel. At present, little is known about how the proteinogenic and non-proteinogenic building blocks serve to exert the specific channel function and the potent cytotoxicity of **1**. Understanding the structural requirements for its biological function would necessitate detailed structure–activity relationship (SAR) studies.

The unique function and structure of **1** motivated us to undertake its total chemical construction. The total synthesis of large polypeptides, including proteins, continues to pose significant challenges in chemistry[9]. 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 or biosynthesis. Polytheonamide B represents an ideal target molecule for expanding our knowledge regarding the reactivity and structural and functional properties of large polypeptides.

We report the first total synthesis of the largest non-ribosomal peptide, polytheonamide B (**1**), as well as the structural elucidation of the *R*-stereochemistry of its sulfoxide. This attempt to gain precise atom-by-atom control of the structure of **1** will provide the first chemical basis for systematically correlating its molecular structure and biological function[10–13].

Results and discussion

Our total synthesis of polytheonamide made use of a flexible and general strategy consisting of four independent stages: (1) synthesis of the non-proteinogenic amino-acid monomers[14]; (2) construction of peptide segments by stepwise assembly using solid-phase synthesis methods[15]; (3) convergent couplings of the peptide segments; and (4) global deprotection of the protected polytheonamide. For the first stage, it was necessary to prepare the eight amino-acid monomers **13**–**20** from commercially available materials (Fig. 1b). In the second stage, the presence of multiple acid-labile amino-acid

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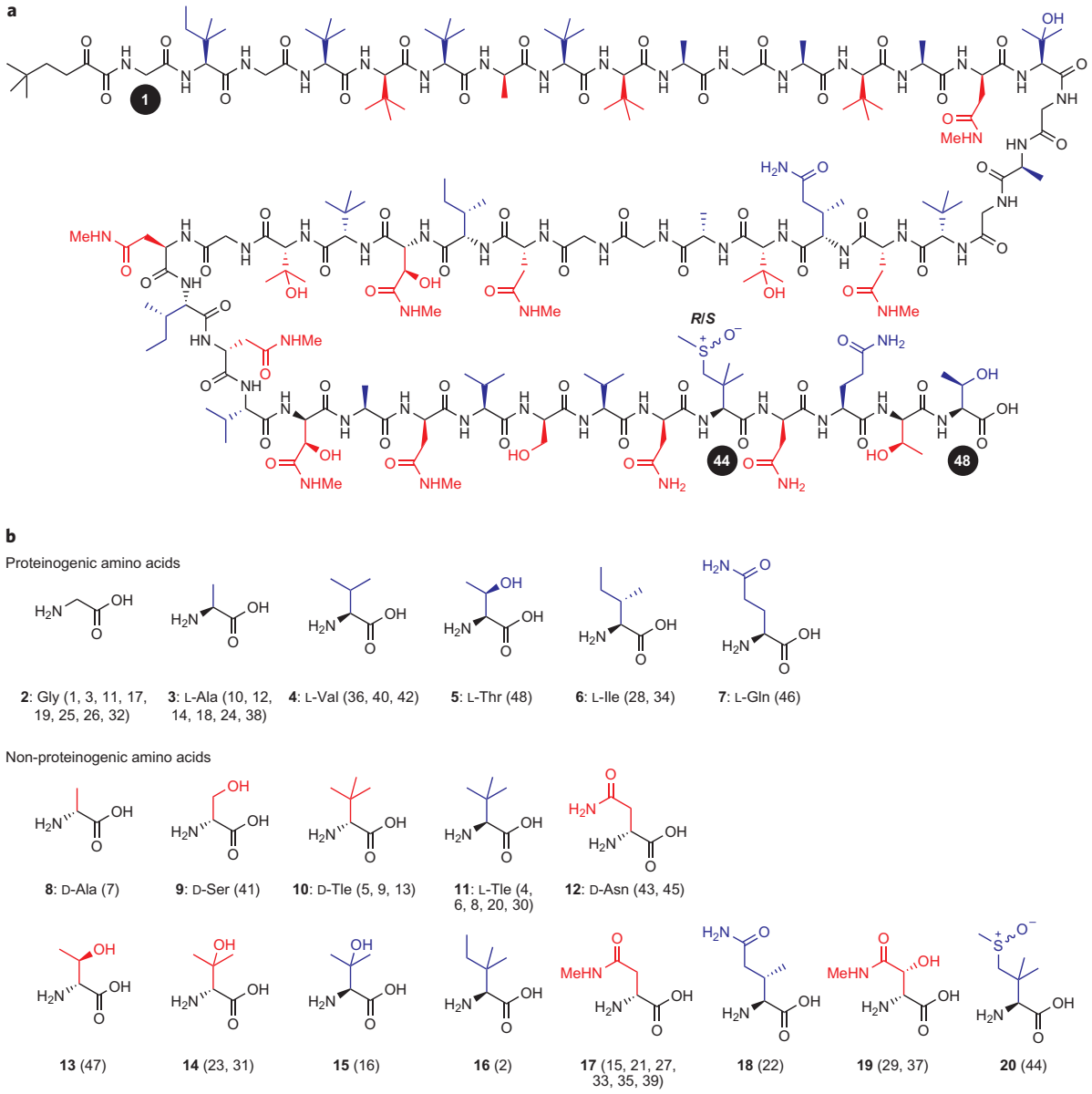

Figure 1 | Structure of polytheonamide. D-Chirality and L-chirality of the amino acids are indicated by red and blue, respectively. **a**, Polytheonamide A (epi-**1**) and B (**1**) are the epimers of the sulfoxide of the 44th amino acid. **b**, Component amino acids of polytheonamide. Polytheonamide contains six proteinogenic amino acids and thirteen non-proteinogenic amino acids. The residue numbers are indicated in parentheses. Compounds **13**–**20** required synthetic preparation.

residues, such as β-hydroxy amino acids, required the use of Fmoc (9-fluorenylmethoxycarbonyl) chemistry[16] for solid-phase peptide synthesis (SPPS) rather than Boc (*t*-butoxycarbonyl) chemistry[17], which would require harsh acidic conditions for global deprotection. Design of the peptide segments by dissection of the entire molecule was crucial for the success of the synthesis. Preliminary studies showed that solid-phase synthesis was only efficient up to 16 residues. Beyond this length, smooth elongation of the peptides 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[18]. Therefore, four segments of 7 to 16 residues were designed (Fig. 2): residues 1–11 (**21**: Ncap-[1–11]-OH), 12–25 (**22**: Fmoc-[12–25]-OH), 26–32 (**23**: Fmoc-[26–32]-OH) and 33–48 (**24**: H-[33–48 $(t\text{-Bu})_3(\text{Tr})_3$]-OH). The first three peptides included glycine as the C-terminal amino acid to eliminate the risk of epimerization during the third stage of the process, segment condensation. To maximize the yields of the elongation processes, the primary amides at residues 43, 45 and 46, and the alcohols at residues 41, 47 and 48, were planned to be protected with triphenylmethyl (Tr) and *t*-butyl (*t*-Bu) groups, respectively. In the third stage, Ag^+-mediated amide formation between thioesters and amines[19,20] was selected for assembly of the gigantic peptides because of the high chemoselectivity and reactivity of this reaction. After derivatization of the three segments to thioesters and subsequent condensation from the C-terminal segment, the three Tr and three *t*-Bu groups of the protected polytheonamide were simultaneously removed under acidic conditions in the fourth stage.

In the first stage of total synthesis, the eight non-proteinogenic amino acids **13**, **14**, **15**, **16**, **17**, **18**, **19** and **20** were synthesized as protected forms, together with 5,5-dimethyl-2-oxohexanoic acid (Ncap-OH) conjugated at the N-terminus (see Supplementary Information for details). To determine the unknown sulfoxide

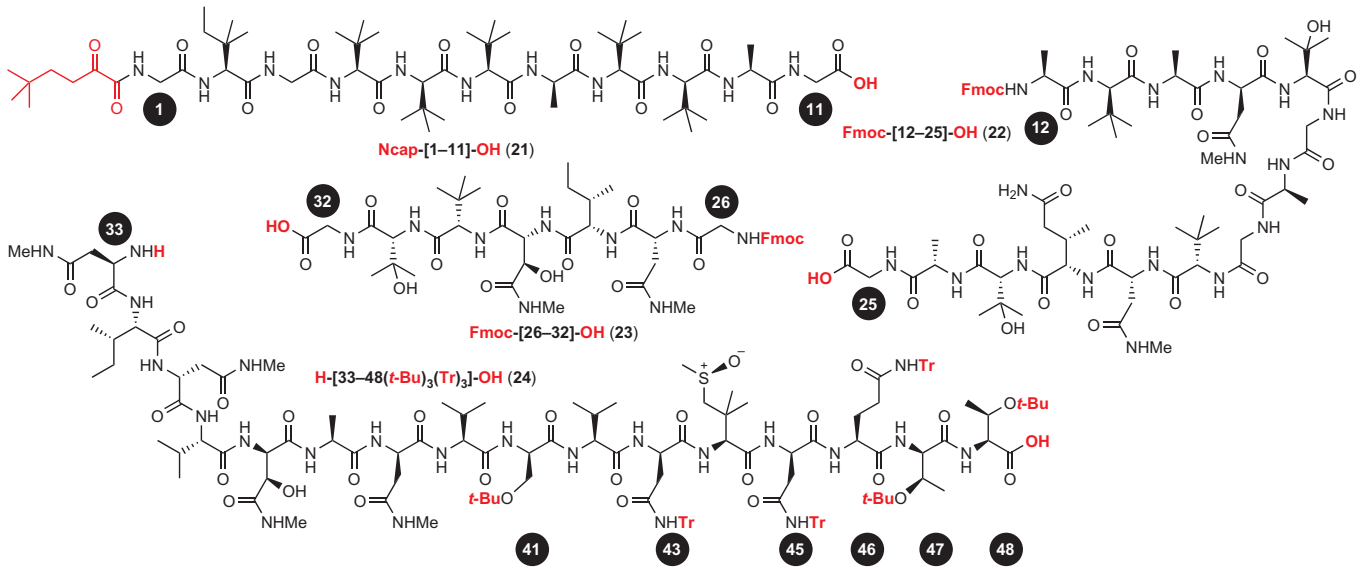

Figure 2 | Four peptide segments for the total synthesis of polytheonamide. Segments Ncap-[1–11]-OH (**21**), Fmoc-[12–25]-OH (**22**) and Fmoc-[26–32]-OH (**23**) have glycine as the C-terminal amino acid to eliminate the risk of epimerization during segment condensation. The primary amides at residues 43, 45 and 46, and the alcohols at residues 41, 47 and 48 in the C-terminus segment **24** were protected with Tr and *t*-Bu groups, respectively. Fmoc = 9-fluorenylmethoxycarbonyl; Ncap = $Me_3C(CH_2)_2(CO)CO$; *t*-Bu = *t*-butyl; Tr = triphenylmethyl.

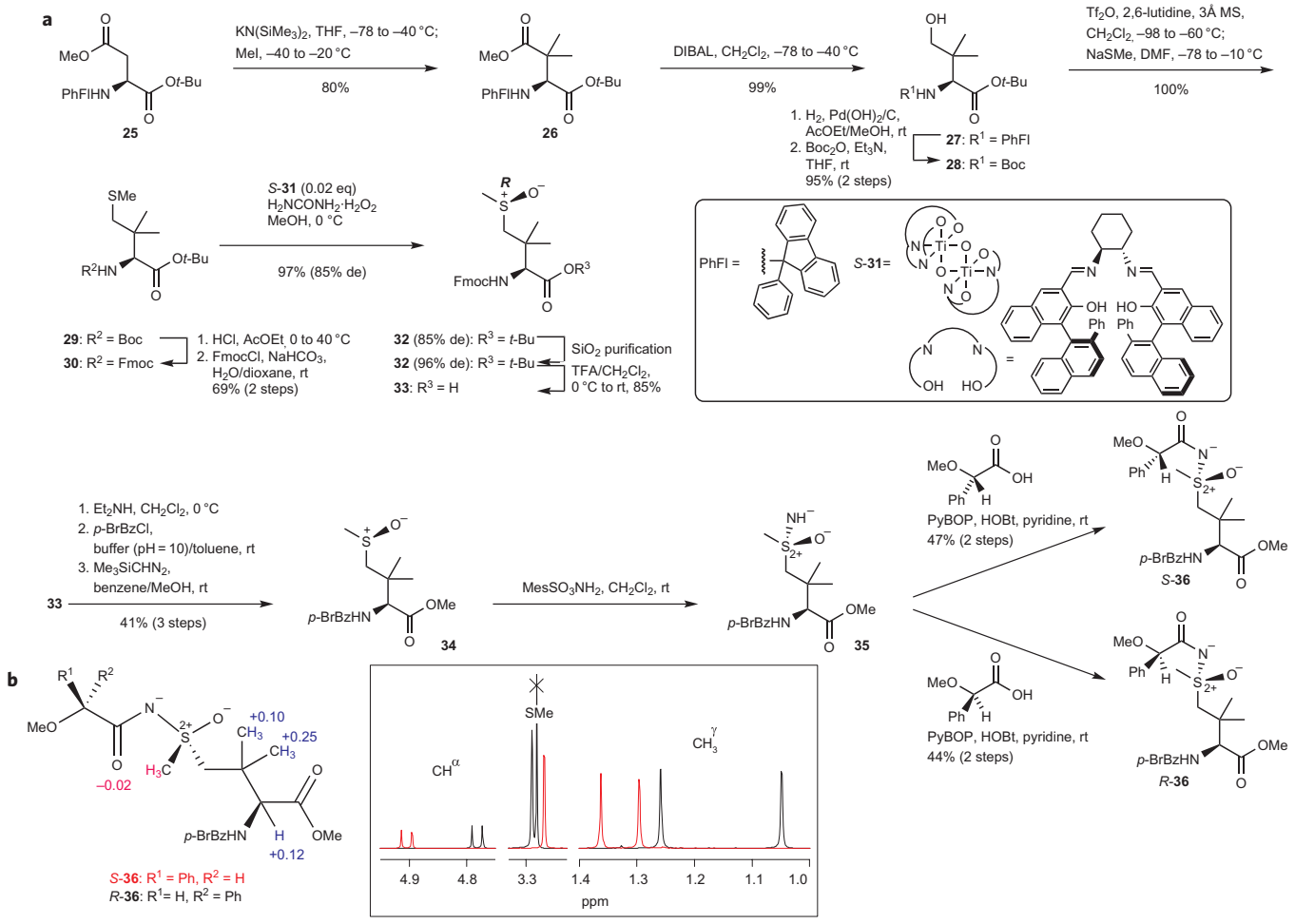

Figure 3 | Synthesis and structural determination of the Fmoc-protected amino acid of residue 44. a, Stereoselective synthesis of **33**, and derivatization of **33** to *S*-**36** and *R*-**36**. **b**, Determination of the absolute configuration of the sulfoxide. The coloured numbers are differences ($\Delta\delta$) in the ^{1}H chemical shifts between *S*-**36** and *R*-**36** ($\Delta\delta = \delta$ (*S*-**36**)–δ (*R*-**36**)). Boc = *t*-butoxycarbonyl; DIBAL = diisobutylaluminium hydride; Fmoc = 9-fluorenylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole; 2,6-lutidine = 2,6-dimethylpyridine; Mes = mesityl = 2,4,6-trimethylphenyl; MS = molecular sieves; PyBOP = benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate; Tf = trifluoromethanesulfonyl; TFA = trifluoroacetic acid.

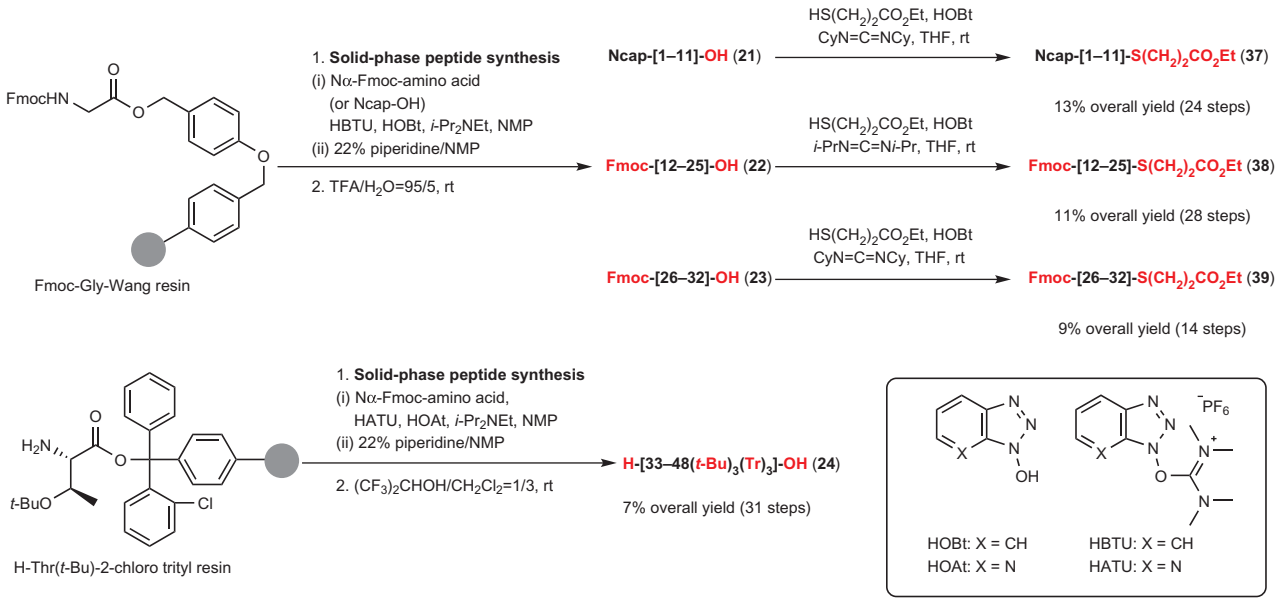

Figure 4 | Solid-phase synthesis of the four peptide segments. Segments Ncap-[1–11]-OH (**21**), Fmoc-[12–25]-OH (**22**) and Fmoc-[26–32]-OH (**23**) were synthesized from Fmoc-Gly-Wang resin. After cleavage from the resin, the three segments were separately converted to the thioesters. Segment H-[33–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**24**) was synthesized from H-Thr(*t*-Bu)-2-chloro trityl resin, and was then cleaved from the resin. 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.

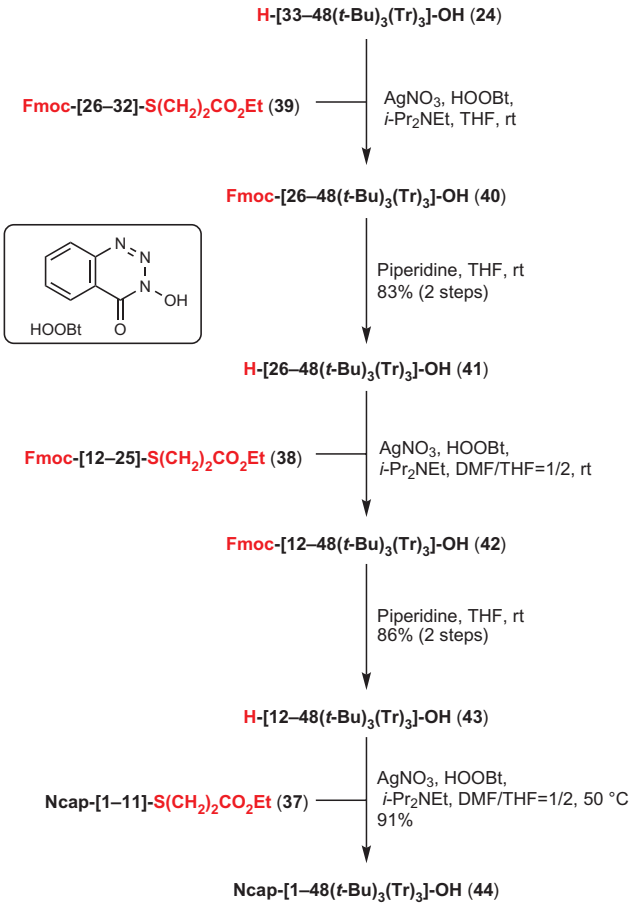

Figure 5 | Convergent assembly of the four peptide segments. The protected polytheonamide **44** was synthesized by the three Ag^+-mediated couplings between the amines and the thioesters. HOOBt = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine.

stereochemistry of polytheonamides A and B, it was decided to synthesize the *R*-sulfoxide diastereomer of **20** of residue 44 in a stereoselective fashion. Figure 3a summarizes preparation of **33**, the Fmoc-protected version of **20** with the *R*-sulfoxide, from the known aspartate **25**. The steric bulk of the 9-phenylfluorenyl (PhFl) group[21] attached to the amine of **25** effectively controlled the first two chemoselective transformations by preventing abstraction of the Cα-proton and reduction of the *t*-butyl ester. Specifically, treatment of **25** with potassium bis(trimethylsilyl)amide and methyl iodide enabled the bis-substitution at the Cβ of **25** to generate β,β-dimethylated **26** (ref. 22), the side-chain ester of which was then selectively reduced using diisobutylaluminium hydride (DIBAL) to produce primary alcohol **27**. Alteration of the protecting group of **27** from PhFl to Boc was performed by means of hydrogenolysis and $(Boc)_2O$ treatment in the presence of Et_3N, leading to **28**. The less nucleophilic nature of the carbamate of **28** compared to the tertiary amine of **27** was particularly important for preventing undesired intramolecular cyclization in the subsequent step. Alcohol **28** was converted into the corresponding triflate by the action of trifluoromethanesulfonic anhydride (Tf_2O) and 2,6-lutidine, and the leaving group was *in situ* displaced by sodium methanethiolate in an intermolecular fashion to produce methyl sulfide **29**. The Boc group was in turn replaced by an Fmoc group by the standard two-step procedure (**29** → **30**).

Stereoselective synthesis of the sulfoxide from sulfide **30** and its stereochemical assignment were not simple tasks. Many chiral reagents for sulfide oxidation were found to exhibit no diastereoselectivity. Gratifyingly, it was found that the Katsuki conditions promoted highly diastereoselective oxidation of sulfide **30** to sulfoxide **32** (ref. 23). Treatment of **30** with a catalytic amount of *S*-**31** in the presence of urea hydrogen peroxide in methanol led to **32** in 85% de (96% de after the second SiO_2 chromatography purification). Finally, removal of the *t*-Bu groups from **32** using trifluoroacetic acid (TFA) in CH_2Cl_2 gave rise to the requisite Fmoc-protected amino acid **33**. The stereochemistry of the sulfoxide of **33** was then unambiguously established using the NMR-based methodology developed by Kusumi[24]. After the three-step reactions

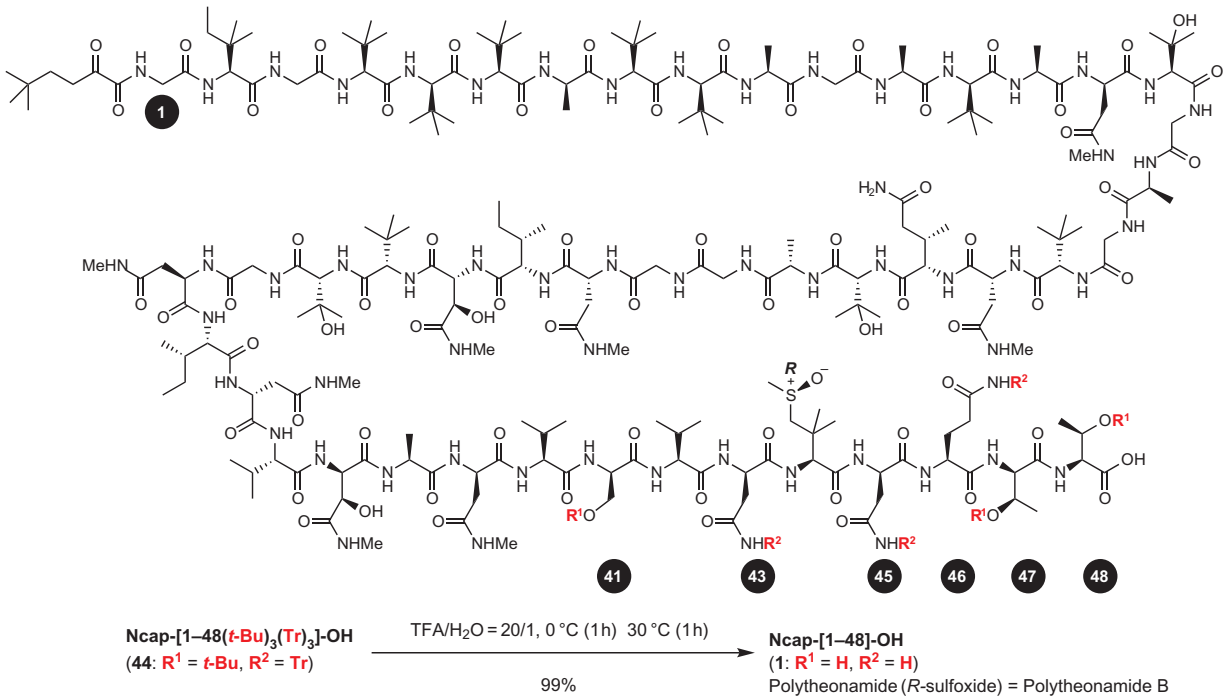

Figure 6 | Completion of the total synthesis of polytheonamide B. The three *t*-Bu and three Tr groups were simultaneously removed under acidic conditions.

from **33**, *O*-mesitylsulfonylhydroxyamine transformed sulfoxide **34** to sulfoximine **35** with the retention of chirality at the sulfur atom[25]. Imine **35** was conjugated with the chiral anisotropic agents (*S*)- and (*R*)-methoxyphenylacetic acids to generate *S*-**36** and *R*-**36**, respectively. The differences in the ^{1}H-chemical shifts ($\Delta\delta$) between *S*-**36** and *R*-**36** were calculated to determine the indicated stereochemistry of the sulfoximine (Fig. 3b). This structural assignment indicated that the catalyst *S*-**31** installed the *R*-stereocentre at the sulfur atom in the reaction from **30** to **32**. Accordingly, **33** was used for the total synthesis of polytheonamide with the *R*-sulfoxide.

Having synthesized **33** and the other non-proteinogenic amino acids as protected forms, the second stage of the total synthesis was to prepare the four requisite peptide segments using an automated solid-phase peptide synthesizer. Segments Ncap-[1–11]-OH (**21**), Fmoc-[12–25]-OH (**22**) and Fmoc-[26–32]-OH (**23**) were synthesized from commercially available Fmoc-Gly-Wang resin[26] using standard Fmoc-based SPPS coupling conditions with *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) activation (Fig. 4). After cleavage from the Wang resin by treatment with 95% aqueous TFA, the three segments were separately converted to the corresponding thioesters using the reagent combination of $HS(CH_2)_2CO_2Et$, HOBt and *N*,*N*′-dialkylcarbodiimide[27]. The crude peptides were then purified using high-performance liquid chromatography (HPLC) to obtain Ncap-[1–11]-$S(CH_2)_2CO_2Et$ (**37**), Fmoc-[12–25]-$S(CH_2)_2CO_2Et$ (**38**) and Fmoc-[26–32]-$S(CH_2)_2CO_2Et$ (**39**) in overall yields of 13%, 11% and 9%, respectively, from the Fmoc-Gly-Wang resin. The synthesis of the C-terminus segment, H-[33–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**24**), began with H-Thr(*t*-Bu)-2-chloro trityl resin using *O*-*t*-Bu-protected Fmoc amino acids for residues 41 and 47, and *N*-Tr-protected Fmoc amino acids for residues 43, 45 and 46 (ref. 28). A more reactive reagent system (*O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt)[29]) was applied for the SPPS coupling, 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 mild acidic conditions (1,1,1,3,3,3-hexafluoro-2-propanol and CH_2Cl_2)[30] without deprotection of the peptide side chains. HPLC purification of the crude mixture resulted in H-[33–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**24**) with an overall yield of 7%.

The third stage involved the assembly of the four structurally complex segments. The molecular weight of each segment exceeded 1,000 Da (Fig. 5). After many unsuccessful attempts, Aimoto conditions[20] ($AgNO_3$ and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt), and *N*,*N*-diisopropylethylamine in THF/DMF) were found to be exceptionally effective for coupling Fmoc-[26–32]-$S(CH_2)_2CO_2Et$ (**39**) and H-[33–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**24**). The thioester was transformed *in situ* by the action of silver salt to the corresponding HOOBt ester, which was attacked by the amine, resulting in the adduct Fmoc-[26–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**40**). Piperidine treatment of the product, followed by HPLC purification, gave rise to the half structure of the protected polytheonamide, H-[26–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**41**), with a yield of 83% for two steps. The obtained amine **41** was next coupled with Fmoc-[12–25]-$S(CH_2)_2CO_2Et$ (**38**) under similar reaction conditions, and subsequently deprotected to produce H-[12–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**43**) with a yield of 80% for two steps and the subsequent purification. Finally, the protected polytheonamide Ncap-[1–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**44**) was synthesized by Ag^+-mediated coupling of **43** with Ncap-[1–11]-$S(CH_2)_2CO_2Et$ (**37**), and purified (91% yield). These three high-yield couplings clearly demonstrated the broad applicability of the present coupling protocol for the generation of highly complex molecules with numerous reactive functionalities.

The last stage of the total synthesis was the removal of the six protective groups (Fig. 6). The protected peptide was subjected to carefully controlled acidic conditions. Exposure of Ncap-[1–48(*t*-Bu)$_3$(Tr)$_3$]-OH (**44**) to TFA/H_2O (20:1) mixture for 1 h at 0 °C and an additional 1 h at 30 °C, and subsequent purification by reversed-phase HPLC, delivered polytheonamide with the *R*-sulfoxide in a yield of 99%. Longer reaction times or higher temperatures resulted in lower yields, presumably owing to acid-sensitive functionalities such as the β-tertiary alcohols at residues 16, 23 and 31. Thus, by applying the four described synthetic stages, the largest non-ribosomal peptide was chemically constructed for the first time.

HPLC analyses of the synthetic and natural polytheonamide indicated that the polytheonamide with *R*-sulfoxide is polytheonamide B (**1**). The retention time of synthetic **1** ($t_R = 29.15$ min) matched that of natural polytheonamide B (**1**: $t_R = 29.03$ min), but not that of its sulfoxide epimer polytheonamide A (epi-**1**: $t_R = 24.75$ min) (Inertsil C8, 4.6 × 150 mm, UV 210 nm, *n*-PrOH/H_2O 35:65, 0.5 ml min^{-1}, 45 °C). Detailed NMR data for synthetic **1** were collected (800 MHz), and the assigned signals clarified that synthetic **1** is identical to natural **1** in all respects (^{1}H NMR, DQF-COSY, TOCSY and NOESY: see Supplementary Information for details). Overall, these data unambiguously show that polytheonamides A and B have the *S*-sulfoxide and *R*-sulfoxide, respectively.

A preliminary toxicity study of the natural product and the synthetic compound was carried out using mouse leukaemia P388 cells. Synthetic **1** displayed an EC_{50} value (98 pM) comparable to that of the natural form (79 pM). However, the protected analogue **44** surprisingly did not exhibit detectable toxicity (>1 μM), in spite of it having the same molecular length as parent **1**. Presumably, the bulky and hydrophobic protective groups impede the efficient formation of the membrane-spanning ion channel by masking important polar residues, although the structural basis of this hypothesis awaits clarification.

In summary, the first total synthesis and structural elucidation of polytheonamide B, the largest known non-ribosomal peptide, was achieved through the convergent assembly of four complex peptides. Our versatile and modular strategy should be useful for synthesizing various analogues to obtain structural insights into the conformational behaviour of this peptide, as well as its channel function and potent toxicity. Future investigations will include more detailed studies towards the elucidation of the molecular mode of action of polytheonamide and the rational design of new tailor-made channel peptides.

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Author contributions

M. Inoue conceived and designed the study. N.S. and S.T. performed the total synthesis. N.S. and S.M. contributed the structural analyses. T.T., K.O., H.I., Y.M. and M.Iida performed the fragment syntheses. M.Iida and N.L. performed the bioassay. M.Inoue, N.S. and S.M. co-wrote the paper.

Additional information

The authors declare no competing financial interests. Supplementary information and chemical compound information accompany this paper at www.nature.com/naturechemistry. Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/. Correspondence and requests for materials should be addressed to M.I.

ERRATUM

Total synthesis of the large non-ribosomal peptide polytheonamide B

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In the version of this Article originally published online, an in-house error led to the incorrect representation of stereochemistry in Figs 1, 2 and 6. These have now been corrected in all versions of the Article.