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Synthesis of an iberiotoxin derivative by chemical ligation: A method for improved yields of cysteine-rich scorpion toxin peptides

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Abstract

Automated and manual solid phase peptide synthesis techniques were combined with chemical ligation to produce a 37-residue peptide toxin derivative of iberiotoxin which contained: (i) substitution of Val¹⁶ to Ala, to facilitate kinetic feasibility of native chemical ligation, and; (ii) substitution of Asp¹⁹ to orthogonally protected Cys-4-MeOBzl for chemical conjugate derivatization following peptide folding and oxidation. This peptide ligation approach increased synthetic yields approximately 12-fold compared to standard linear peptide synthesis. In a functional inhibition assay, the ligated scorpion toxin derivative, iberiotoxin V16A/D19-Cys-4-MeOBzl, exhibited ‘native-like’ affinity ($K_d = 1.9$ nM) and specificity towards the BK Ca²⁺-activated K⁺ Channel (K_{Ca}1.1). This was characterized by the rapid association and slow dissociation rates ($k_{on} = 4.59 \times 10^5$ M⁻¹ s⁻¹; $k_{off} = 8.65 \times 10^{-4}$ s⁻¹) as determined by inhibition of macroscopic whole-cell currents of cloned human K_{Ca}1.1 channel. These results illustrate the successful application of peptide chemical ligation to improve yield of cysteine-rich peptide toxins over traditional solid phase peptide synthesis. Native chemical ligation is a promising method for improving production of biologically active disulfide containing peptide toxins, which have diverse applications in studies of ion-channel function.

Keywords

Peptide synthesis; Chemical ligation; Toxin; Scorpion; Iberiotoxin; BK Ca²⁺-activated K⁺ channel; K_{Ca}1.1

1. Introduction

Native chemical ligation is a strategic tool for the assembly of poor yielding peptide and poly-peptide sequences. This simple chemical approach makes possible the complete

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solution-phase condensation of two individual fully deprotected peptide moieties of varying lengths to form a site-directed native-state peptide bond [3,4,6]. This approach enables sequential 'block peptide' elongation in a selective and controlled manner. Native chemical ligation deviates from the traditional convergent solid phase peptide synthesis (SPPS) strategies, with the latter incorporating the coupling of a fully orthogonally protected peptide fragment onto an existing peptide-resin support, an approach typically directed by extending normal amino acid coupling procedures [1,10].

Extensions of native chemical ligation now include the chemical and semi-synthesis of large functional proteins (>10 kDa). This includes ion channels [4,14,15,25,39] and the incorporation of small synthetic peptide fragments into larger recombinant proteins [17], which enables the introduction of various non-native amino acids [23,38], fluoro-derivatives (see Ref. [25]) and affinity labels [36]. These represent unique products to the native chemical ligation strategy, with most unachieved via convergent strategies.

Cysteine-rich peptide toxins provide ideal models for chemical synthesis via native chemical ligation, as many are difficult to synthesize or produce in high yields using standard linear SPPS techniques due to their length, hydrophobic nature and multi-cysteine content [37]. The requirements for native chemical ligation peptide candidacy are: (i) an introduced C-terminal thiol ester in the lead N-terminal peptide segment, and (ii) an N-terminal Cysteine in the following C-terminal peptide segment (see Fig. 1). The N-terminal cysteine of (ii) can be easily accommodated by the relevant abundance and sequential positioning of cysteine moieties throughout the length of many classes of peptide toxins. We envisaged that synthesis of longer (>40 amino acid) peptide toxins by native chemical ligation of ~20 amino acid fragments would offer potential advantages in yields due to: (a) reduction of inherent cumulative stepwise SPPS deletions, (b) relative ease of synthesizing and purifying shorter peptides, and (c) avoiding problems of side reactions, disulfide cross-linking and hydrophobic aggregation encountered during acid cleavage of longer, hydrophobic, multiple cysteine-containing peptides.

An important consideration in native chemical ligation synthesis is the kinetic dependence of the ligation reaction on the side chain of the C-terminal amino acid thiol ester of the N-terminal peptide segment (see ref. [16]). The success of ligation is dependent on its selection. Some amino acids at this position (e.g., Leu, Thr, Val, Ile, Pro) have slow reaction or coupling rates and lead to poor yields of the ligated target species. In some cases, substitution at this position may be required to give reasonable yields of the ligated product. However, a single amino acid substitution may also have significant consequences on a toxin's specificity, targeting and overall pharmacological behavior, in some cases rendering a toxin candidate biologically inactive (see Refs. [33,34]). Here, careful design based on comparative structural and pharmacological identity of similar peptide toxin sequences is an important aspect of successful peptide toxin bioengineering.

In this paper, we illustrate the design and synthesis of a 37 amino acid scorpion toxin derivative, iberiotoxin V16A/D19-Cys-4-MeOBzl, via native chemical ligation. The derivative's retention of pharmacological specificity and selectivity to the BK Ca^{2+} -activated K^{+} channel ($\text{K}_{\text{Ca}} 1.1$) is verified by electrophysiological assay. The native chemical ligation approach to increased synthetic yields offers an alternative route to bioengineering cysteine stabilized $\alpha\beta$ motif containing scorpion toxins, which have numerous applications for biological studies of ion channels such as $\text{K}_{\text{Ca}} 1.1$.

2. Materials and methods

2.1. Synthesis of 3-Mercapto-Propionic Acid-Leucine (MPAL) base resin

4-Methylbenzhydrylamine [MBHA] resin ($0.79 \text{ mmole g}^{-1}$; Peptides International) was pre-swollen overnight in dimethylformamide (DMF) and 650 μL *N,N*-diisopropylethylamine (DIEA). After washing the swollen resin with DMF, Boc-Leu (2 mmole) was activated with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.5 M in DMF, 4 mL) and 347 μL of DIEA and allowed to couple for 40 min. After both DMF and dichloromethane (DCM) washes (each 40 mL), the Boc group was removed with 50% (v/v) TFA/DCM (2 min $\times$ 5 min). 3,3'-dithiopropionic acid (2 mmole) dissolved in HBTU (0.5 M in DMF, 8 mL) was then mixed with sufficient DIEA to both activate and neutralize the resin and allowed to couple for 40 min. The resin was then washed with DMF and then treated with 650 μL ethanolamine and 200 μL DIEA in 5 mL DMF for 40 min to cap resin-bound-OBt esters. A DMF wash was followed by reduction of the resin-bound disulfide with 2-mercaptoethanol (650 μL 2-mercaptoethanol, 100 μL DIEA in 5 mL DMF) for 1 h. Following another wash with DMF, Boc-Ala (2 mmole) was activated with HBTU, as above, and immediately coupled for 20 min.

2.2. Boc SPPS of N-terminal portion of Iberitoxin

Peptide A (ZFTDVDCSVSKECWSA¹⁶-[MPAL]) was manually assembled via Boc SPPS using *in-situ* neutralization with 2 mmole Boc-amino acid per 10 min single coupling, as described by Schnölzer et al. [31], using the above described [MPAL]-loaded resin. After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay [30] and maintained above 99.5 % or end-capped with Ac₂O (100 μL in 5 mL DMF) if yield was not achieved. Side chain protecting groups were Asp(cyclohexyl), Cys(4-methylbenzyl), Glu(cyclohexyl), Lys(2-chlorobenzyloxycarbonyl), Ser(benzyl) and Thr(benzyl). Tryptophan was unprotected. Pyroglutamic acid (Z) was coupled in the same way as the other residues. Upon completion of the synthesis, the resin was washed with DMF followed by DCM and then dried under N₂.

2.2.1. HF cleavage—Peptide-[MPAL]-resin was stirred with 20 mL HF and 1 mL *p*-cresol for one hour at 0 °C. HF was removed *in vacuo* and the residue treated with 100 mL of cold methyl *tert*-butyl-ether to precipitate the peptide. The solids were collected onto a glass sinter, washed twice with 50 mL methyl *tert*-butyl-ether, and the peptide dissolved in 100 mL 50/50 (v/v) acetonitrile (MeCN)/H₂O containing 0.1% (v/v) TFA. The solution was lyophilized and stored at -20 °C until required.

2.3. Preparative RP-HPLC/UV of N-terminal portion of Iberitoxin: Peptide A (ZFTDVDCSVSKECWSA¹⁶-[MPAL])

The crude peptide was dissolved in 50 mL unbuffered GnHCl (pH 4) and loaded onto a Phenomenex 21.2 mm $\times$ 250 mm C₄ column (300 Å, 10–15 μm) equipped with a 21.2 mm $\times$ 60 mm guard column packed with the same material. The initial mobile phase was 100% Solvent A. The GnHCl was eluted from the column using Solvent A at 10 mL min⁻¹, followed by elution of the peptide using a two step gradient (0–20% Solvent B 0–20 min, 20–45% Solvent B 20–80 min). Fractions were collected and analyzed by infusion MS. Those containing the target peptide were pooled and lyophilized.

2.4. Fmoc SPPS of C-terminal portion of Iberitoxin

Peptide B [¹⁷CKC(4-*MeOBzl*)LFGVDRGKCMGKKRCRYQ³⁷], 100 μM scale was assembled automatically via solid phase Fmoc coupling, at Keck Peptide Facility (Yale University) in the following manner: Peg-Resin (0.18 mmol g^{-1}) was washed three times

with DMF (2.5 mL), deprotected twice with 20% (v/v) piperidine (2.5 mL, 2 × 0.5 min), and then washed six more times with DMF (2.5 mL). Fmoc-amino acids (0.5 mmole) were activated by 1.5 mL 0.4 M HBTU/DMF/*N*-methyl-Morpholine and double coupled (20 min.). Upon coupling completion, the peptide-resin was washed with DMF (2.5 mL) three times. Side chain protecting groups were: Cys(4-MeOBzl) [position 19 only], Cys(Trt), Lys(Boc) Asp(tBu), Arg(Pbf), and Gln(Trt). On completion of the synthesis the resin was washed with DMF followed by DCM and then dried under N₂. The peptide was cleaved and reverse-phase high performance liquid chromatography/ultraviolet detection (RP-HPLC/UV) purified using a semi-preparative Vydac C₁₈ column (300 Å, 5 µm, 7.8 × 250 mm). Samples were eluted using a linear 1% min⁻¹ gradient of organic (90/10 MeCN/0.08%, v/v TFA aq.; Solvent B) against 0.1% (v/v) TFA aq. (Solvent A) at a flow rate of 1 mL min⁻¹.

2.4.1. TFA cleavage—Resulting Fmoc peptides were subjected to a double cleavage with 96% (v/v) TFA in the presence of Anisole (2%, v/v) and H₂O (2%, v/v), acting as protecting group scavengers, for 2 h at 24 °C. The resulting cleaved peptide material was recovered as previously described and stored at -20 °C as lyophilized powder until required.

2.5. Analytical RP-HPLC/UV

Synthetic peptides were separated/quantified either by C₁₈ or C₄ analytical RP-HPLC/UV (Vydac; 5 µm, 300 Å, 4.6 × 250 mm), using the described conditions above. Eluants were monitored at 223 or 280 nm.

2.6. Native ligation

Typically N-terminal-peptide-[MPAL] (Peptide A) and 4-MeOBzl containing C-terminal peptide (peptide B), in equal molar ratios (~30 µM), were dissolved in 4 mL 6 M GnHCl, 100 mM sodium phosphate pH 7.9 and mixed with 30 µL each of thiophenol and benzylmercaptan then allowed to stand at 40 °C. After 18 h a sample was analyzed by LC/MS to confirm the reaction had taken place. After 21 h the solution was diluted with 50 mL unbuffered 6 M GnHCl and extracted with three times 40 mL methyl *tert*-butyl-ether. After removal of a sample for LC/MS analysis, the ligated IbTx-V16A/D19C-4-MeOBzl (Lig-IbTx-MeOBzl) was immediately purified by RP-HPLC (Vydac C₄ 10 mm × 250 mm column, gradient 0–25% Solvent B 0–25 min, 25–37% Solvent B 25–85 min). Fractions were analyzed by infusion MS, pooled and lyophilized.

2.7. Fmoc SPPS of IbTx-D19C-Acm, IbTx-D19K-LC-biotin and linear IbTx-V16A/D19C-4-MeOBzl

Three IbTx analogues were linearly synthesized (0.5 mmole scale), all using the same automated Fmoc synthesizer and cleavage protocols as described in Section 2.4. Here, the standard Fmoc-amino acid and corresponding protecting groups were as previously indicated, except for the use of Fmoc-Cys-Acm in position 19 for IbTx-D19C-Acm [*ZFTDVDCSVSKECWSVCKC(-Acm)LFGVDRGKCMGKKCRCYQ*], Fmoc-Lys(biotin-LC)-OH [N- α -Fmoc-N- ϵ -(D-biotin-6-amidocaproate)-L-lysine; Anaspec Inc.] in position 19 for IbTx-D19K-LC-biotin [*ZFTDVDCSVSKECWSVCKK(-LC-biotin)LFGVDRGKCMGKKCRCYQ*], and Fmoc-Cys(4-MeOBzl)-OH in position 19 for linear IbTx-V16A/D19C-4-MeOBzl [*ZFTD-VDCSVSKECWSACKC(-4-MeOBzl)LFGVDRGKCMGKKCRCYQ*].

2.8. Oxidation of IbTx analogues

Lig-IbTx-MeOBzl, IbTx-D19C-Acm and IbTx-D19K-LC-biotin (10 mg) were sonicated in 20 mL of oxidation buffer of 2 M urea, 0.1 M NaCl, 0.1 M glycine, pH 7.8 in an effort to aid peptide solubility and decrease peptide aggregation potential during oxidation. Peptides

were allowed to oxidize in air by stirring at 22 °C for 18 h. Slow oxidation of linear IbTx-V16A/D19C-4-MeOBzl (10 mg) was aided with the introduction of 50% (v/v) 2-propanol in to the above N₂ purged oxidation buffer (20 mL) and allowed to stir at 22 °C for 36 h. Peptides were immediately acidified (neat TFA), filtered (0.22 µm) and subjected to preparative RP-HPLC/UV purification, as described in Section 2.3.

2.9. Purification and characterization of folded peptides

Purification of the folded toxin derivatives was achieved by RP-HPLC/UV—first on a semi-preparative column, followed by narrow-bore analysis, peptide co-elution and quantification using Vydac C₁₈ columns both subjected to a linear 1% min⁻¹ gradient of 0.1% (v/v) TFA aq. against 90% (v/v) MeCN in 0.08% (v/v) TFA aq. over 60 min. UV absorption was monitored at 223 nm. Electro-spray mass spectroscopy of the folded and purified peptides was performed on a LQC Thermo Finnigan spectrometer.

2.10. Peptide quantification

Peptide concentrations were determined by RP-HPLC/UV integration of peptide samples [Peptide A and B; IbTx-D19K-LC-biotin, standard linear IbTx-V16A/D19C-4-MeOBzl, Lig-IbTx-MeOBzl and IbTx-D19C-Acm], which had previously undergone quantitative amino acid hydrolysis and PTH derivatization and quantification (Keck Peptide Facility, Yale University).

2.11. MALDI TOF/TOF analysis

RP-HPLC/UV purified peptides fractions in aqueous 0.1% TFA were mixed 1:1 with matrix solution (2,5-dihydroxybenzoic acid [DHB] in 1:1 0.1% TFA:MeCN) and spotted onto a thin layer of dried matrix (DHB) saturated in methanol on the target plate. The spots were dried under a stream of N₂ gas. Parent ions were identified on the Ultraflex III MALDI TOF/TOF (Bruker Daltonics), controlled by the Compass 1.2 SR1 software package (Bruker), in positive reflector mode. Parent ions were further selected in the LIFT-MS/MS mode. Peptide II Calibration Mix (Bruker) was used for external calibration.

2.12. Planar bilayer assay of Lig-IbTx-MeOBzl

Single-channel recording of K_{Ca}1.1 channels from rat skeletal muscle in planar lipid bilayers was performed as previously described [7]. The recording solution was 200 mM KCl, 10 mM Mops-KOH, pH 7.4 on both sides of the bilayer, with 0.2 mM CaCl₂ on the intracellular side and 0.1 mM EDTA plus 0.1 mg mL⁻¹ bovine serum albumin on the extracellular side as defined by native asymmetry of the channel.

2.13. Electrophysiological assay of Lig-IbTx-MeOBzl

Lig-IbTx-MeOBzl was tested for blocking activity against human K_{Ca}1.1 channels. An N-terminal Flag-BK version of the human K_{Ca}1.1 channel (HSlo) was stably expressed in a human cell line (HEK293) as previously described [2]. Channel activity was monitored by the whole-cell patch clamp electrophysiological technique [18] using an Axon 200B amplifier and Clampex software (Axon Instruments) for voltage pulse programming and data acquisition. The pipette (intracellular) solution containing 0.5 µM free Ca²⁺ [35] was 100 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1.3 mM EGTA, 10 mM Hepes-KOH, pH 7.2. The standard bath (extracellular) solution was 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes-KOH, pH 7.4. Briefly, macroscopic HSlo currents were recorded from single HEK293-HSlo cells in whole-cell mode by stepping the membrane voltage from a holding potential of -80 mV to a series of consecutive test potentials delivered in increments of +10 mV up to +100 mV for 100 ms. Current records, low-pass filtered at 5 kHz, were corrected for capacitance transients and leak current using a negative P/5 pulse

protocol. Synthetic scorpion toxins and paxilline (Sigma–Aldrich) were applied to cells by continuous gravity perfusion of the bath solution (0.5 mL min^{-1}) into a low volume cell recording chamber (RC24E, Warner Instruments).

3. Results

Stepwise linear SPPS approaches failed to provide a free ‘spinster thiol’ D19C derivative of IbTx. Failure is suspected as a result of incorrect disulfide pairing or intra-molecular disulfide bonding. Possible confirmation is provided by IbTx-D19C-Acm, an orthogonally protected ‘spinster thiol’ derivative. This oxidized biologically active derivative (Obs. MH^+ 4265.4 Da, Calc. MH^+ 4265.8 Da) demonstrated a K_d of 1.6 nM via lipid bilayer (Table 1), and demonstrated characteristic discrete long-lived blocked states upon assay by single BK_{Ca} channels as observed in other αKTx1 toxins (see [11,2]; data not shown). Synthetic yields of IbTx-D19C-Acm were ~2% per 100 mg peptide oxidized. Selective thiol deprotection using AgBF_4 , as described by Yoshida et al. [40], resulted in a biologically inactive form of IbTx-D19C. Molecular mass analysis indicated the presence of methionine sulfoxide (a difference of +16 Da; Obs. MH^+ 4211.2 Da, Calc. MH^+ 4210.8 Da) that accounted for biological inactivity by a structural modification of Met²⁹ (see Ref. [32]). With these results, alternative approaches were examined to construct an IbTx orthogonally protected ‘spinster thiol’ template derivative in high yields.

3.1. Peptide assembly

Synthesis of IbTx-D19K-LC-biotin (as discussed in Section 2) provided 183 mg g^{-1} of resin, with the target peptide representing 24% of total RP-HPLC/UV profile at 223 nm of the crude cleaved material. Typically upon oxidation and purification, 2.8–3.3 mg of folded bioactive material was recovered for each 100 mg oxidized (2.8–3.3% yield).

Linearly synthesized IbTxV16A/D19C-4-MeOBzl provided 212 mg of material per gram of resin via double cleavage, with the target peptide representing 15% of total RP-HPLC/UV profile at 223 nm. Upon oxidation and purification, 1 mg of folded material was recovered for each 100 mg oxidized (~1% yield).

For native chemical ligation, Peptide A, 16 amino acids in length; IbTx [1–16]V16A-COSR (Obs. MH^+ 1988.2 Da, Calc. MH^+ 1988.7 Da; Fig. 1), was manually assembled via Boc SPPS on the described [MPAL] base resin. Typically single HF cleavage of one gram of resin provided 260 mg of peptide material, with the target peptide representing 37% of total RP-HPLC/UV profile at 223 nm. Upon purification of the reduced material, <99% purity, 14 mg was recovered for each 100 mg of crude peptide (~14% yield).

Peptide B, 21 amino acids in length (Fig. 1), was assembled via automated Fmoc SPPS. Single acid cleavage of Peptide B was undertaken with 180 mg of peptide recovered from one gram of resin. RP-HPLC/UV analysis indicated the presence of a dominant peak (62% of the total RP-HPLC/UV profile) correlating to the target thiol-protected material. Molecular mass analysis confirmed its presence (Calc. MH^+ 2546.2 Da and Obs. MH^+ 2546.7 Da, see Fig. 1). Upon purification of the reduced orthogonally protected material, <99% purity, 17.9 mg was recovered for each 100 mg of crude peptide (~18% yield).

3.2. Native ligation

Typically Peptide A and B, as observed in Fig. 1, were mixed in approximately equal molar ratio (13 μM ; i.e. 30.4 and 33.7 mg, respectively), as described, with ligation completed after ~21 h. RP-HPLC/UV analysis illustrated a significant decrease in starting materials; Peptide A (Rt 26.1 min) and Peptide B (Rt 35.7 min), together with an increase in a newly abundant peak, A–B (Rt 40.5 min), representing the reduced ligated target (see Fig. 2). LC/

MS illustrated the ligation process by the new ion species $[M + 4H]^{4+}$ 1079.9 and $[M + 3H]^{3+}$ 1439.4 (Fig. 2); Calc. MH^+ 4314.8 Da and Obs. MH^+ 4315.2 Da as illustrated in Fig. 2. Ligation yields were determined by weight to be 32%, providing a purified unfolded ligated product of 20.5 mg. Upon batch-wise oxidation in urea/50% (v/v) 2-propanol buffer, 2.5 mg of folded ligated material was purified to <99% purity, which represents 12% per 100 mg of ligated peptide.

The ligated reduced product demonstrated the same RP-HPLC/UV retention time and molecular mass as the standard linear synthesized IbTx-V16A/D19C-4-MeOBzl. Both oxidized and purified ligated IbTx-V16A/D19C-4-MeOBzl (Lig-IbTx-MeOBzl) and standard linear synthesized oxidized IbTx-V16A/D19C-4-MeOBzl were RP-HPLC/UV co-injected in a 2:1 ratio, respectively, and demonstrated identical retention of 33.3 min as illustrated in Fig. 3. High resolution MALDI TOF/TOF of the final target provided the molecular mass of MH^+ 4308.93 Da (Calc. MH^+ 4308.9 Da), confirming both full oxidation and purity, as illustrated in Fig. 3.

3.3. Biological assay of Lig-IbTx-MeOBzl

To determine whether Lig-IbTx-MeOBzl has biological activity against $K_{Ca1.1}$ channels, we tested its effect on the cloned human $K_{Ca1.1}$ channel (HSlo) expressed in a human cell line. Fig. 4A shows an example of HSlo channel activity recorded as macroscopic current from an HEK293-HSlo cell using the whole-cell patch clamp technique. Depolarization of the cell membrane with 0.5 μM free Ca^{2+} in the intracellular pipette solution gives rise to outward K^+ currents activated at membrane voltage more positive than +30 mV. Perfusion of the cell with bath solution containing 50 nM Lig-IbTx-MeOBzl inhibits HSlo current by 80% or greater depending on the test voltage (Fig. 4B). Paxilline is a tremorgenic fungal compound that has been characterized as a potent inhibitor of mammalian $K_{Ca1.1}$ channels [21,35]. Addition of 1 μM paxilline to the bath solution inhibits greater than 95% of the outward current, demonstrating that most of the K^+ current recorded under these conditions is attributable to paxilline-sensitive $K_{Ca1.1}$ channels.

The kinetics of the interaction of Lig-IbTx-MeOBzl with HSlo channels were also characterized by experiments illustrated in Fig. 4C. Rapid perfusion of bath solution containing Lig-IbTx-MeOBzl resulted in progressive time-dependent inhibition of $K_{Ca1.1}$ current to a steady-state level reached several minutes after onset. The time course of toxin inhibition is a measure of the association rate of the toxin-channel binding reaction, since it is not limited by the fast delivery rate of the solution to the recording chamber. Similarly, slow recovery of K^+ current observed after perfusion with toxin-free bath solution is a measure of the time course of the toxin-channel dissociation reaction. Association and dissociation time courses for Lig-IbTx-MeOBzl, charybdotoxin (ChTx), and IbTx-D19K-LC-biotin from experiments of Fig. 4C were fit to a single exponential and the resulting time constants were used to calculate a second-order association rate constant (k_{on}) and a first order dissociation rate constant (k_{off}) for toxin binding according to the standard kinetic model for one-site binding reaction. Calculated k_{on} values are: $4.6 \times 10^5 \text{ s}^{-1} M^{-1}$ (Lig-IbTx-MeOBzl), $1.3 \times 10^5 \text{ s}^{-1} M^{-1}$ (ChTx), and $9.9 \times 10^4 \text{ s}^{-1} M^{-1}$ (IbTx-D19K-LC-biotin). Corresponding calculated k_{off} values are: $8.7 \times 10^{-4} \text{ s}^{-1}$ (Lig-IbTx-MeOBzl), $2.3 \times 10^{-3} \text{ s}^{-1}$ (ChTx), and $5.1 \times 10^{-4} \text{ s}^{-1}$ (IbTx-D19K-LC-biotin). The equilibrium dissociation constant for binding of the various toxins estimated from the relationship, $K_d = k_{off}/k_{on}$, are: 1.9 nM (Lig-IbTx-MeOBzl), 17.6 nM (ChTx), and 5.1 nM (IbTx-D19K-LC-biotin), see Table 1. The K_d values calculated from the latter kinetic ratio compare favorably with K_I values of 2.9 nM for Lig-IbTx-MeOBzl and 13 nM for ChTx as determined by fitting the concentration dependence of steady-state inhibition to a one-site binding isotherm (Fig. 4D). These results show that synthetic Lig-IbTx-MeOBzl has high affinity for the human $K_{Ca1.1}$

channel and a slower dissociation rate than that of ChTx for mammalian $K_{Ca}1.1$ channels as previously described for the native toxins [5,11,34].

4. Discussion

Chemical technologies of SPPS and bioconjugation facilitate transformation of peptide toxins into biological probes. Investigating alternative methods of peptide assembly may provide avenues for successful probe production in high yields. Our approach using native chemical ligation can improve yields, but requires careful due diligence in its design. The illustrated production of ligated IbTx-V16A/D19C-4-MeOBzl extends from comparative analysis of the structure activity relationships of ChTx (Table 1).

ChTx is a typical member of the α KTx1 family of scorpion toxins characterized by: (i) a short α -helix (typically residues 13–21), (ii) two stranded antiparallel β -sheets (typically residues 25–36; see Ref. [19]), and (iii) a defined channel-toxin interactive surface at amino acid positions Ser¹⁰, Trp¹⁴, Arg²⁵, Lys²⁷, Met²⁹, Asn³⁰, Arg³⁴ and Tyr³⁶ [34,32]. ChTx exhibits broad pharmacological recognition of various K_v channels in contrast to the structural homolog IbTx, which is a highly specific blocker of $K_{Ca}1.1$ ($K_d < 1$ nM; 9). ChTx and IbTx, both 37mers, exhibit 68% sequence identity (Table 1), including all the aforementioned essential toxin surface interactive side chains except for Asn 30 ChTx/Gly 30 IbTx. These structural features are compounded with identical positioning of cysteine moieties that participate in a 7–28, 13–33 and 17–35 disulfide-bridging configuration, providing a common fold of the peptide backbone in the three-dimensional structure (see Ref. [19]). A major difference between these two peptides is the overall number of charged amino acids present, 13 for ChTx and 9 for IbTx, which results in differing net charge of +4 and +2, respectively.

The derivative ChTx-R19C and the various labeled fluorescent [9] and radio-labeled [20] conjugates, coupled via a ‘spinster thiol’, retain K_v channel selectivity, as the R19 modification maps to the ‘backside’ of the toxin with respect to the channel-binding surface. In IbTx-D19K-LC-biotin ($K_d = 2.5/5.1$ nM) and IbTx-D19C-Acm ($K_d = 1.6$ nM), derivatives also retain potency and selectivity towards $K_{Ca}1.1$ (Table 1; 2), indicating functional and structural conservation within the two toxins.

Combining the structure–activity profiles of ChTx [33,34], IbTx [5,8,19], IbTx-chimeras [12], and our previous IbTx derivative studies [2], a suitable thiol-ester ligation construct was designed, containing an orthogonally protected ‘spinster thiol’. Native chemical ligation requires a C-terminal peptide segment (Peptide B) to contain an N-terminal Cys (see Fig. 1). In IbTx six cysteine moieties are present, with position Cys¹⁷ providing a medial sequence position (see Table 1). The next requirement is a kinetically favorable amino acid for coupling at the C-terminal MPAL thioester end of the N-terminal peptide segment (Peptide A, Fig. 1). In IbTx Val¹⁶ provides a kinetic hindrance to the ligation process. Hackeng et al. [16] showed that of all the neutral, aliphatic amino acids, a C-terminal Ala-thioester peptide provided a useful reaction rate in native chemical ligation. Other substitutions were either unreactive or hindered ligation yields (see Ref. [16]). To maximize chemical ligation of IbTx, Val¹⁶ was changed to Ala. Pharmacological consequences of this mutation were considered; substitution of Val¹⁶ by Glu in ChTx showed a two-fold decrease in binding affinity (see Table 1; [34]), indicating the absence of essential channel-toxin interactions. This is compounded by the amino acid 16-position also being on the non-interactive face, neighboring the established 19-position of both toxins ([2], see Fig. 5). Here we hypothesized that a neutral non-polar Val¹⁶ to Ala substitution would not only lead to minimal alteration in channel-binding properties, but would in fact facilitate the production of the target material by native chemical ligation.

Support for our hypothesis comes from the observed changes in dissociation rates: $K_d < 1$ nM (wild type) versus 1.9 nM for Lig-IbTx-MeOBzl as determined by whole-cell patch clamp (Table 1). The use of these opposing faces provides for molecular bi-functionality. We refer to these as ‘Janus-molecules’ since Janus is the Roman god of gates and doors, usually depicted by a double-faced head looking in opposite directions. Additional derivatization at this non-interactive face and resulting changes in pharmacology are illustrated in Table 1. Similar conclusions are reiterated in other α KTx1 ‘Janus’ toxins, such as ChTx-R19C and HgTx-A19C ([32,28] respectively) and reflect the conserved three-dimensional structure observed within the α KTx1 toxin family members [13,24,29]. This potentially provides an indication that these and other α KTx1 toxins, together with their pharmacological resolving dipole moment, as discussed by Frémont et al. [8], can tolerate limited changes in charge state, hydrophobicity and mass additions. The extent of these tolerances at 19-position within IbTx and aforementioned α KTx1 toxins is presently unknown.

Further support for our hypothesis in IbTx bioengineering comes from the 12-fold increase of yields by weight, of the folded ligated target material, in comparison to the standard full linear synthetic route with a post-cleavage oxidation that yields ~1% (see [37]). Increased yields are attributed to (i) enhanced ligated linear production and (ii) improved yield in peptide folding. Initial yields from standard linear synthesis of the 37mer IbTx-A16 V/D19C-4-MeOBzl (31.8 mg of target peptide/gram resin) are poor in comparison to the smaller segmented construction of the 16mer-Peptide A (~96.2 mg of target/gram resin) and 21mer-Peptide B (~111.6 mg of target/resin gram), which are used for native chemical ligation. With ligation production reaching ~32%, a ~100% increase in production of the unoxidized linear target is achievable. Yet the major hurdle in both approaches is the folding of the peptide-toxin. Standard linear synthesis of the IbTx-A16 V/D19C-4-MeOBzl yielded 1%/100 mg, while native chemical ligated oxidized target increased to 12%/100 mg. This increase is related to the quality of linear starting material achievable, and purification steps prior to the ligation reaction. Minimization of thiol containing peptide deletion or N-terminal terminated products limits unwanted peptide polymerization.

Synthesis of a ligated bioactive derivative of IbTx suggests a number of potential research applications. Giangiacomo et al. [12] and Mullmann et al. [27] described the pharmacological properties of α KTx1 toxin family chimeras using both IbTx and ChTx, and later Noxiustoxin as peptide–toxin templates, respectively. The combination of different native-like N- and C-terminal sequence segments resulted in non-native peptide toxins that exhibited unique biological activity [12]. Our approach of segmented ‘block’ style synthesis and native chemical ligation readily lends itself to toxin chimera production. Here, one could readily generate libraries of full-length peptide chimeras combining various N- and C-terminal peptide segments. This same approach may also facilitate investigation of toxin structure–activity relationships. Here an ‘alanine walk’ via ligation of various peptide block segments would mitigate repetitive low-yields and could aid in the generation of multi-point mutations within a single ligated toxin sequence.

5. Conclusion

Native chemical ligation offers a powerful synthetic tool that allows peptide elongation to go beyond the constraints of the standard length of 50–60 amino acids of automated linear SPPS (see Refs. [4,26]). This ligation scheme also opens new and varied opportunities in peptide toxin studies, focusing on smaller peptides 30–40 amino acids in length. These efforts will provide a greater understanding of pharmacological interactions and how these same peptides provides for an architectural foundation for drug design, as articulated by Menez [22].

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Abbreviations

SPPS	solid phase peptide synthesis
BK/K_{Ca}1.1	large conductance Ca ²⁺ -activated K ⁺ channel
K_v	voltage-gated K ⁺ channels
HSlo	human BK channel
ChTx	charybdotoxin
IbTx	iberiotoxin
IbTx-D19K-LC-biotin	iberiotoxin-D19K-LC-biotin
Lig-IbTx-MeOBzl	native chemical ligated iberiotoxin-V16A/D19C-4-MeOBzl
Acm	S-acetamidomethyl-L-cysteine
4-MeOBzl	S-4-methoxybenzyl
MBHA	4-methylbenzhydrylamine
DHB	2,5-dihydroxybenzoic acid
DMF	dimethylformamide
DIEA	<i>N,N</i> -diisopropylethylamine
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
DCM	dichloromethane
HF	hydrogen fluoride
TFA	trifluoroacetic acid
MeCN	acetonitrile
GnHCl	guanidine hydrochloride
AC₂O	acetic anhydride
RP-HPLC/UV	reverse phase high pressure liquid chromatography interfaced UV spectroscopy
LC/MS	reverse phase high pressure liquid chromatography interfaced mass spectroscopy
MS	mass spectroscopy
MH⁺	mono isotopic molecular mass
HEK293	human embryonic kidney cell line
MALDI-TOF	matrix-assisted laser adsorption ionization time of flight

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Fig. 1.

Schematic representation of the chemical native ligation process involving two peptide fragments to produce a bioengineered Iberitoxin derivative. Peptide A, the leading fragment (16mer) contains both the C-terminal thiol ester, as well as the substituted Val¹⁶ to Ala to promote kinetic feasibility during the ligation reaction. Peptide B, the Following Fragment (21mer) contains a free N-terminal cysteine to participate and regenerate its chemical functionality during and after ligation respectively – the latter is required for sequential disulfide bond formation. Peptide B also contains an orthogonally protected Cys-4-MeOBzl at position 19 within the full ligated parent sequence. This specific position has demonstrated to be a point for chemical bioconjugation or derivatization which does not greatly affect the biological activity or specificity of the parent toxin. Sequential molecular masses, as determined by ESMS, illustrate the progressive chemical modifications that occur which result in the full length folded parent toxin of 37 amino acids containing 3 disulfide bonds (oxidation is indicated by curved bar above peptide fragment A-B).

Fig. 2.

Representative RP-HPLC/UV profile obtained from the LC/MS run of the crude ligated products at ~21 h. Peptide fragments A (Rt 26.1 min) and B (Rt 31.7 min) are labeled as the remaining initial starting products, as determined by m/z and RP-HPLC retention, with the reduced ligated product A–B (Rt 40.5 min) presenting the major UV contributor to the RP-HPLC profile (Phenomenex C_4). Molecular mass analysis of starting materials Peptide A and B are shown in their multiple charge states, as indicated, with the final unfolded target Peptide A–B as derived from the LC/MS profile of crude ligated material.

Fig. 3. Analysis of the oxidized IbTx derivative. High-resolution molecular mass analysis of the final oxidized target ligated IbTxV16A/D19C-4-MeOBzl as determined by MALDI-TOF/TOF. Insert: RP-HPLC/UV chromatograph (Vydac C₁₈) illustrating the 2:1 co-elution of the fully oxidized target peptide together with its identical non-ligated linear synthesized folded counterpart.

Fig. 4.

Comparison of the blocking effect of synthetic scorpion toxin derivatives on the human $K_{Ca1.1}$ channel. (A) Inhibition of $HSlo$ current by ligated IbTx-A16 V/D19C-4-MeOBzl (Lig-IbTx-OMeBzl) and paxilline. Records of $HSlo$ current evoked by a series of voltage pulses from -80 mV to $+100$ mV in steps of $+10$ mV are shown before (control) and after addition of 50 nM Lig-IbTx-OMeBzl and 1 mM paxilline to the extracellular bath perfusion solution. (B) Current amplitudes measured at the end of the step pulse from experiments in (A) are plotted versus the step voltage. Solid and dotted lines for control (filled circles) and Lig-IbTx-OMeBzl (open circles) data respectively correspond to current values before and after subtraction of values measured in the presence of paxilline (filled triangles). (C) Time course of inhibition and recovery after addition of toxin followed by perfusion with standard toxin-free bath solution. $HSlo$ current was continuously monitored by voltage pulses from -80 to $+50$ mV (50 ms) delivered once every 10 s. Values are normalized to peak current from each cell measured before addition of toxin. Overlaid data for three different experiments indicates current values measured during addition and removal of 50 nM Lig-IbTx-OMeBzl (squares), 100 nM ChTx (upward triangles), and 200 nM IbTx-D19K-LC-biotin (downward triangles). Gray lines correspond to exponential fits of data as described in the text. (D) Concentration-dependence of toxin inhibition. Normalized currents taken after steady-state inhibition by ChTx (filled triangles) or Lig-IbTx-OMeBzl (open triangles) as in (C) are plotted as a function of toxin concentration. Solid curves correspond to fits of the data for each toxin to: $f_N = K_I / (K_I + [Tx])$, where f_N is the fraction of normalized current, $[Tx]$ is the toxin concentration, and K_I is the equilibrium dissociation constant for the toxin. K_I values are 13.0 nM for ChTx and 2.9 nM for Lig-IbTx-OMeBzl.

Fig. 5.

Structural representation of interactive surfaces of native IbTx and its orthogonally protected derivative. Left structure highlights, in green, the amino acids and their relative positions that are essential for toxin-channel binding. The yellow highlights the disulfide bonds deep within the core of the toxin structure which are essential for maintaining biologically active structure/template. The right panel illustrates an 180° rotation of the same toxin. Here the use of the position Asp¹⁹ has been a focal point for peptide bioconjugation and bioengineering, this being away from the interactive toxin-channel surfaces. Here the selective point for native chemical ligation, Val¹⁶ (as indicated in black), lays adjacent to the non-interactive Asp¹⁹, it would be expected that this area, the backside of the molecule, plays little role in the toxin's biological specificity towards K_{Ca}1.1.

Table 1

The sequential development of the bioengineered IbTx.

	Sequence	K_d^a (nM)	Reference ^b
ChTx (WT)	ZFTNVSCCTSKECWSVCQRLHNTSRGKCMNKKCRCYS	8.8	[9]
		17.6	This work
ChTxR19C	ZFTNVSCCTSKECWSVCQCLHNTSRGKCMNKKCRCYS	8.8	[32]
ChTxV16E	ZFTNVSCCTSKECWSECQRLHNTSRGKCMNKKCRCYS	18	[34]
IbTx (WT)	ZFTDVDCSVSKECWSVCKDLFGVDRGKCMGKKCRCYQ	<1	[11]
IbTxD19X	ZFTDVDCSVSKECWSVCKXLFVDRGKCMGKKCRCYQ	1.6 ^c (X = Cys-Acm)	This work
		2.5 ^c (X = K-LC-biotin)	[2]
		5.1 (X = K-LC-biotin)	This work
		2.5 ^c (X = K-LC-biotin-streptavidin)	[2]
IbTxV16A/D19X ₂ (Lig-IbTx)	ZFTDVDCSVSKECWSACKX ₂ LFGVDRGKCMGKKCRCYQ	1.9 (X ₂ = Cys-4-MeOBzl)	This work

Detailed pharmacological examination of both native and mutant forms of ChTx provided key information for the introduction of non-native amino acids into the sequence of IbTx. Initially ChTx – wild type (WT) was pharmacologically compared to two individual peptide mutants, ChTxR19C and ChTxV16E, which illustrated a maximal two-fold decreasing in affinity. Combining both mutations in a single sequence would likely not perturb toxin channel interactions. Given the structural and sequence similarities of ChTx and IbTx, and to test this hypothesis, the final combination of a double point mutation: (i) position 19 – this for later application derivatization, and (ii) position 16 –specifically introduced to promote ligational kinetics to maximize peptide synthesis, supports the sequential development of IbTx bioengineering in providing the biologically active parent template toxin ligated IbTxV16A/D19C-4-MeOBzl.

^a As assigned via whole cell patch clamp.

^b References as given in text.

^c K_d is assigned by single channel analysis via lipid bilayer (see comments in Ref. [21]).