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t-Boc synthesis of Huwentoxin-I through Native Chemical Ligation incorporating a Trifluoromethanesulfonic acid cleavage strategy

Parashar Thapa^{1,†}, Chino C. Cabalteja¹, Edwin E. Philips III¹, Michael J. Espiritu¹, Steve Peigneur², Bea G. Mille², Jan Tytgat², Theodore R. Cummins^{3,4}, and Jon-Paul Bingham^{1,†,*}

¹Department of Molecular Biosciences and Bioengineering, College of Tropical Agriculture and Human Resources, University of Hawai'i, Honolulu, HI, 96822, USA

²Toxicology and Pharmacology, University of Leuven (KU Leuven), Campus Gasthuisberg O&N II, Leuven 3000, Belgium

³Department of Pharmacology and Toxicology, Indiana University, Indianapolis, IN, USA

⁴Stark Neurosciences Research Institute, 320 West 25th Street, NB-414F, Indianapolis, IN, 46202-2266, USA

Abstract

Tert-butyloxycarbonyl (*t*-Boc) based Native Chemical Ligation (NCL) techniques commonly employ hydrogen fluoride (HF) to create the thioester fragment required for the ligation process. Our research aimed to assess the replacement of HF with Trifluoromethanesulfonic acid (TFMSA). Here we examined a 33 amino acid test peptide, Huwentoxin-I (HwTx-I) as a novel candidate for our TFMSA cleavage protocol. Structurally HwTx-I has an X-Cys¹⁶-Cys¹⁷-X sequence mid-region, which makes it an ideal candidate for NCL. Experiments determined that the best yields (16.8%) obtained for 50 mg of a thioester support resin were achieved with a TFMSA volume of 100 μ L with a 0.5 hour incubation on ice, followed by 2.0 hours at room temperature. RP-HPLC/UV and mass spectra indicated the appropriate parent mass and retention of the cleaved HwTx-I *N*-terminal thioester fragment (Ala¹-Cys¹⁶), which was used in preparation for NCL. The resulting chemically ligated HwTx-I was oxidized/folded, purified, and then assessed for pharmacological target selectivity. Native-like HwTx-I produced by this method yielded an EC₅₀ value of 340.5 ± 26.8 nM for Na_v1.2 and an EC₅₀ value of 504.1 ± 81.3 nM for Na_v1.3, this being similar to previous literature results using native material. This paper represents the first NCL based synthesis of this potent sodium channel blocker. Our illustrated approach removes potential restrictions in the advancement of NCL as a common peptide laboratory technique with minimal investment, and removes the hazards associated with HF usage.

*Corresponding Author: Dr. Jon-Paul Bingham, jbingham@hawaii.edu, Fax: (808) 965-3542, Department of Molecular Biosciences and Bioengineering, College of Tropical Agriculture and Human Resources, University of Hawaii, HI, 96822, USA.

[†]Contributed equally to this work

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Keywords

Peptide Cleavage; Native Chemical ligation; Trifluoromethanesulfonic acid; Peptide toxin; Sodium Channels

1.0 Introduction

Linear solid phase peptide synthesis (SPPS) presents limitations in the production of peptides >50 amino acids [1,2]. Convergent synthetic strategies through linking of orthogonally protected peptide fragments have demonstrated advantages in overcoming this constraint. Yet this approach is often faced with difficulties regarding the peptide fragment's complexity in production [1,3] and potential lack of solubility [4–6]. Recently alternative strategies have been investigated incorporating chemo-selective ligation techniques [7,8]. These varying solution phase approaches have already demonstrated their merit and worth in chemical biology with the synthesis of functional proteins [9,10] semi-synthetic proteins [11,12] (a combination of SPPS products with recombinant materials), and their utilization in overcoming difficult syntheses of certain peptides [13].

The strategy of Native Chemical Ligation (NCL), aims to produce a native-like peptide bond at the point of conjugation, using two unprotected peptide fragments [7]. Design and construction of these strategic ligation positions require consideration of kinetic principles regarding chemo-selective rates for the specific and neighboring amino acids involved [13], and their influence on structure-activity relationships, particularly if amino acid substitutions are made to enhance the ligation kinetics [14,15]. Therefore these influences should be considered not only as they apply to their respective peptide fragments, but for their consequences within the ligated product as a whole.

NCL entails the use of a carboxyl end thioester mediated reaction, which is both highly selective and spontaneous in the formation of a native peptide bond with an opposing α -N-amino Cysteine peptide fragment [4,16]. This cysteine requirement is a potential limitation with some sequences, although in many peptide toxins the sequence dispersal of cysteines, which are often used for the maintenance of three-dimensional structure, offers significant advantage for the route of ligation by NCL [17,18]. In addition this strategy has also been instrumental in the design and construction of disulfide containing cyclic backbone peptides [19].

Fmoc SPPS chemistry has become a dominant synthetic strategy in peptide production due to its simplified assembly and cleavage procedures. However, this particular approach has presented hurdles in the implementation of NCL, as repeated exposure to a strong base such as piperidine during α -N-amino deprotection can impact the stability of the thioester resin [20,21]. In recent years, advances like the removal of Fmoc using less nucleophilic bases [22,23], introduction of a thioester after peptide assembly, and on-resin conversion of peptide acids into peptide thioesters have alleviated the problems associated with the generation of a thioester using Fmoc chemistry [24,25]. However generating a thioester using Boc chemistry is still considered an effective approach and here we report a technology that complements the existing methods in NCL peptide construction [22,24,26].

Boc SPPS chemistry has seen great success in NCL through the incorporation of hydrogen fluoride (HF) in the cleavage of peptides from thioester peptidyl-resins. However, this poses limitations, as HF is extremely toxic and corrosive in nature. Unlike other acids, HF can also cause systemic poisoning in addition to burns, due to its ability to chelate Ca^{2+} ions from soft tissue and bones thereby causing a hypocalcemia, which may even be fatal [3,27]. Specialized Teflon cleavage equipment and handling procedures must be used due to HF's incompatibility with glassware, which create additional considerations to an already hazardous workplace. Thus management and execution of HF cleavage protocols requires a high level of experience and represents the principle drawback to the larger incorporation of NCL in peptide synthesis as a whole.

Here we investigate a technique that further enhances the replacement of HF with Trifluoromethanesulfonic acid (TFMSA), by specifically focusing on its compatibility with the standard Boc chemistry used to produce thioesters, which are an essential requirement for NCL. The replacement of HF with TFMSA is an idea that has produced several protocols as early as the 90s [26], however here we have optimized the conditions for Huwentoxin-I (HwTx-I), a 33 amino acid spider toxin. TFMSA is a strong acid that is comparatively less hazardous than HF. It also requires no specialized or dedicated equipment/facilities for handling, and is fully compatible with common laboratory glassware [3]. Though TFMSA must still be managed with care, its chemistry provides a safer alternative that is readily adaptable to Fmoc peptide laboratories.

In this paper we provide specific details illustrating a highly accessible, non-specialized, approach in the construction and cleavage of thioester containing peptide fragments for NCL using TFMSA. We illustrate this method by using a 33 amino acid spider toxin, Huwentoxin-I (HwTx-I), as a peptide candidate. Here we compare native, full linear synthesized, and ligated peptides cleaved with TFMSA to demonstrate the suitability of this synthetic strategy in producing native-like peptide toxins. We specifically provide their representative synthetic yields and determine the biological selectivity of the native-like isomer to the sodium channel isoforms: $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$ and $\text{Na}_v1.5$.

2.0 Methods

2.1 Fmoc Synthesis of the C-terminal (CTN) portion of Huwentoxin I

A manual 0.5 mM scale Fmoc SPPS of the C-terminal fragment (CTN), amino acids 17 to 33 (Fig. 1), was performed on Rink amide resin (0.44 meq/g; Peptides International). The resin was swelled for 8–10 hours in Dimethylformamide (DMF; 25 mL; Fischer Scientific), with *N,N*-Diisopropylethylamine added (DIEA; 500 μL ; Alfa Aesar) to increase resin stability. The swollen resin was Fmoc deprotected via flow washing with DMF (1 min., $\times 2$), followed by 50/50% (v/v) piperidine/DMF (1 min., $\times 2$; Alfa Aesar), and then rewashed with DMF (1 min., $\times 2$). 2 mM Fmoc-L-amino acids (Peptides International) were activated *in-situ* using HBTU (4 mL; 0.5M in DMF; HCTU used for Cys residues; Peptides International), with DIEA added as a proton scavenger (347 μL ; 2 mMol; Alfa Aesar). The activated amino acids were then added to the resin and coupled for 20 min. Upon completion a ninhydrin test was performed to ensure coupling yields reached 99.5% [28]. On passing yield verification, peptidyl-resin was subjected to repeated deprotection and amino acid

activation-coupling cycles as above, ensuring adequate DMF flow washings between consecutive steps. If amino acid coupling yield fell $\geq 99.5\%$, the same amino acid was activated and recoupled. Side chain protecting groups included: Cys(Trt), Lys(Boc), Arg(Pbf), Trp(Boc), Asn(Trt), Asp(OBzl) (Peptides International). Upon completion of synthesis the peptidyl-resin was washed with DMF (5 mL, $\times 2$) followed by Dichloromethane (DCM; 10 mL; Fisher Scientific) and dried under N_2 .

2.2 Fmoc cleavage

Assembled peptides were cleaved using a modified Reagent K mixture [TFA (82.5% v/v), phenol (5% v/v; Fisher Scientific), water (5% v/v), thioanisole (5% v/v; Alfa Aesar), and triisopropylsilane (TIPS; Alfa Aesar) (2.5% v/v)]. 40 mL of cleavage mixture per gram of peptidyl-resin was stirred for 2 hours at 24°C . Cleaved slurry was vacuum filtered directly into liquid N_2 -chilled tert-butyl methyl ether (Fisher Scientific). Peptide precipitate was pelleted by centrifugation (3000g, 10 min.) and washed twice with chilled tert-butyl methyl ether. The resulting peptide pellet was suspended in 25% v/v acetic acid, then freeze-dried to form a powder and stored at -20°C until required.

2.3 Preparation of the MPAL resin—MBHA resin (0.79 meq/g; Peptides International) was swelled in DMF (8–10 mL) with DIEA (650 μL). This was then coupled with Boc-Leu (2 mMol; 4 mL 0.5 M HBTU; 347 μL DIEA; 40 min.), washed with DMF then DCM. Peptidyl-resin Boc deprotection was achieved with 100% Trifluoroacetic acid (TFA; 5 min., $\times 2$; Fisher Scientific), then flow-washed with DMF (1 min., $\times 2$), followed by coupling with 3,3'-dithiodipronic acid (2 mMol; 8 mL of 0.5 M HBTU in DMF; 1 mL of DIEA; 40 min.). To counteract ester formation the resin-linker was washed with DMF and treated with ethanolamine (650 μL ; Alfa Aesar) and DIEA (200 μL ; 5 mL; in DMF; 40 min.). This was then flow-washed with DMF (1 min., $\times 2$) and treated with 2-mercaptoethanol (Sigma-Aldrich) (650 μL ; 5 mL of DMF; 100 μL DIEA; 60 min.). Upon peptidyl-resin reduction, the resin was flow-washed with DMF (1 min., $\times 2$) and coupled with Boc-Cys(4-MeOBzl) (40 min.), as described above. The resulting pre-loaded Boc-Cys(4-MeOBzl) thioester linker resin (Boc-Cys(4-MeOBzl)-MPAL resin) was then used in the production of *N*-terminal peptide fragments for NCL at approximately 0.5 mMole scale [29].

2.4 Boc SPPS of the *N*-terminal (NTN) portion of Huwentoxin I

N-terminal segment (NTN) of Huwentoxin I, amino acids 1 to 16 (Fig. 1), was manually synthesized using Boc chemistry, this being a simple modification of the approach detailed in Section 2.1. Boc-Cys(4-MeOBzl)-MPAL resin (0.5 mM) was swelled 8–10 hours in DMF (25 mL). The peptidyl-linker-resin was washed with DMF (20 mL; $\times 3$), deprotected twice with 100% TFA (5 mL, 1 min. $\times 2$), and then re-washed with DMF (40 mL, $\times 2$). Boc amino acids were activated *in-situ* using HCTU/DMF (0.4 M, 2 mL) in 4-fold excess (2 mMol) and added to the drained and activated resin and then shaken. After 20 min. of coupling a ninhydrin test was performed. If the coupling percentage yield was $\geq 99.5\%$ the *N*-terminus was deprotected with 100% TFA, DMF washed (5 mL DMF, $\times 2$) and the next sequential amino acid was activated and coupled, as previously described. If the coupling was $\geq 99.5\%$, the same amino acid was activated and recoupled. Side chain protecting groups included:

Cys(4-MeOBzl), Glu(OBzl), Asn(Xan), Lys(Cl-z), Thr(Bzl), Asp(OBzl) (Peptides International).

2.5 Cleavage of the *N*-terminal fragment (NTN) to generate the thioester containing peptide fragment

The assembled *N*-terminal fragment was cleaved using TFMSA to generate a thioester containing peptide in the following manner: 50 mg Peptidyl-MPAL resin was stirred with thioanisole (200 μ L) and EDT (100 μ L) for 10 min. at 0°C°. 100% TFA (1000 μ L) was added to the slurry and maintained at 0°C. At 20 min. TFMSA (100 μ L; Sigma-Aldrich) was slowly added drop-wise and allowed to stir for additional 10 min. while being maintained at 0°C°. At 30 min. the reaction mixture was removed from the ice bath and allowed to stir at room temperature for 2 hours. Cleaved slurry was vacuum filtered directly into liquid N₂ chilled tert-butyl methyl ether. Peptide precipitate was pelleted by centrifugation (3000g, 10 min.) and washed twice with chilled tert-butyl methyl ether. The resulting peptide pellet was suspended in 25% v/v acetic acid, then freeze-dried to form a powder and stored at -20°C until required.

2.6 RP-HPLC/UV - Peptide purification

Peptides were separated and purified using a C18 Narrow-bore RP-HPLC column (Vydac; 5 μ m, 300 Å, 2.1 $\times$ 250 mm) and later quantified using a capillary bore RP-HPLC column (Phenomenex; 5 μ m, 300 Å, 1.0 $\times$ 250 mm). A Waters 2695 Alliance HPLC System interfaced with a 996 Waters Photo Diode Array (PDA) Detector was used for automated sample analysis and detection. Data was acquired and analyzed using Waters Millennium32 (v3.2) software. Samples were eluted using a standard linear 1% min.⁻¹ gradient of acetonitrile (HPLC grade, Fisher Scientific; MeCN; 90/10 MeCN/0.08% v/v aq. TFA; Solvent B) against 0.1% v/v aqueous trifluoroacetic acid (Spectrophotometric grade, Sigma-Aldrich; aq. TFA; Solvent A) at a flow rate of 250 μ L min.⁻¹ (narrow-bore) or 100 μ L min.⁻¹ (capillary-bore) for a period of 65 min., as shown in Fig. 2 and 3. RP-HPLC column was pre-equilibrated with 5% solvent B, prior to sample injection. Elutant profiles were extracted at 214 nm. Samples for later amino acid quantification were fractionated manually, and subjected to repeated RP-HPLC/UV purification when necessary.

2.7 Electrospray Ionization Mass Spectrometry (ESI-MS)

Speed-Vac dried RP-HPLC/UV purified peptides were re-suspended in 0.1% v/v Formic acid/aqueous (LC/MS grade, Sigma-Aldrich). Samples were delivered to the ionization source of an API 3000 Mass Spectrometer (Applied Biosystems/MDS Sciex) via a Rheodyne® 8125 Injector (20 μ L external loop; Rheodyne®) and infused with carrier solvent (50% MeCN/0.1% v/v Formic acid/Aq.; 50 μ L min.⁻¹) as provided by an ABI 140B Dual Syringe Pump. Full-scan single MS experiments were typically obtained by scanning quadrupole-1 (Q-1) from 400–2200 *m/z* in 2–3 s with a scan step size of 0.1–0.5 Da. Data was acquired using Analyst Software (v.1.4.1) (Applied Biosystems/MDS Sciex). The ESI-MS system was calibrated manually in positive mode with PPG 3000/Mass Standards Kit (Applied Biosystems/MDS Sciex), to achieve <5-ppm mass accuracy, as per the manufacturer's protocol.

2.8 Native chemical ligation

The ligation buffer was prepared by adding TCEP HCl (28.7 mg; 0.1 mmol; Pierce) and 4-Mercaptophenylacetic Acid (MPAA; 42.1 mg; 0.26 mmol; Sigma-Aldrich) to a solution of 6 M GnHCl and 0.1 M Sodium phosphate (5 mL). The pH of the ligation buffer was adjusted to 7.0 by adding NaOH or HCl drop-wise. Approximately equimolar concentrations of *N*-terminal thioester peptide fragment (3.0 mg; 1.6 μ mol) and *C*-terminal peptide fragment (3.5 mg; 1.6 μ mol) were added to the ligation buffer. The pH was re-adjusted to 7, and allowed to react for 3 hours at 25°C (Fig. 1). Once complete, the reaction was terminated by diluting the reaction mixture 6 fold with 0.1 % v/v TFA/Aq. (30 mL), and immediately desalted via preparative C18 RP-HPLC/UV [14].

2.9 Peptide oxidation

The RP-HPLC/UV purified and ligated peptide (1 mg mL⁻¹) was air oxidized using 100 mM NH₄HCO₃ pH 8 and stirred for 5 days at 2°C. The oxidized material was membrane filtered (0.45 μ m) prior to semi-preparative C18 RP-HPLC/UV fractionation.

2.10 Peptide quantification

Amino acid analyses of the RP-HPLC/UV purified peptides were performed at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Samples were hydrolyzed *in-vacuo* with 6 N HCl/0.2% v/v phenol for 16 hours at 115°C. Analysis was undertaken using a Hitachi L-8900 PH amino acid analyzer (ion-exchange separation, post-column derivatization with ninhydrin for detection at 570 nm and 440 nm). Data was collected using EZChrom Elite for Hitachi software and tabulated in a Microsoft Excel spreadsheet.

2.11 Expression of voltage-gated ion channels in *Xenopus laevis* oocytes

For the expression of Na_v channels (rNa_v1.2, rNa_v1.3, rNa_v1.4, hNa_v1.5, the auxiliary subunits β 1, β 1) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mACHINE transcription kit (Ambion®). The harvesting of stage V–VI oocytes from anaesthetized female *X. laevis* frog was previously described [30]. Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng/nL using a micro-injector (Drummond Scientific®). The oocytes were incubated in a solution containing: 96 mM NaCl; 2 mM KCl; 1.8 mM CaCl₂; 2 mM MgCl₂ and 5 mM HEPES (pH 7.4), supplemented with 50 mg/L gentamycin sulfate.

2.12 Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed at room temperature (18–22°C) using a Geneclamp 500 amplifier (Molecular Devices®) controlled by a pClamp data acquisition system (Axon Instruments®). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was: 96 mM NaCl; 2 mM KCl; 1.8 mM CaCl₂; 2 mM MgCl₂, and 5 mM HEPES (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.8 and 1.5 M Ω . The elicited currents were filtered at 1 kHz and sampled at 20 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. In order to avoid

overestimation of a potential toxin-induced shift in the current–voltage relationships of inadequate voltage control when measuring large sodium currents in oocytes, only data obtained from cells exhibiting currents with peak amplitude below 2 μ A were considered for analysis. For the electrophysiological analysis of toxins a number of protocols were applied from a holding potential of –90 mV with a start-to-start interval of 0.2 Hz. Sodium current traces were evoked by 100 ms depolarizations to V_{max} (the voltage corresponding to maximal sodium current in control conditions). To assess the concentration–response relationships, data were fitted with the Hill equation: $y = 100/[1 + (EC_{50}/[toxin])^h]$, where y is the amplitude of the toxin-induced effect, EC_{50} is the toxin concentration at half maximal efficacy, $[toxin]$ is the toxin concentration and h is the Hill coefficient. All data are presented as mean $\pm$ standard error (S.E.M) of at least 5 independent experiments ($n = 5$). All data were tested for normality using a D’Agostino Pearson omnibus normality test. All data were tested for variance using Bonferroni test or Dunn’s test. Data following a Gaussian distribution were analyzed for significance using one-way ANOVA. Non-parametric data were analyzed for significance using the Kruskal–Wallis test. Differences were considered significant if the probability that their difference stemmed from chance was 55% ($p < 0.05$). All data was analyzed using pClamp Clampfit 10.4 (Molecular Devices®) and Origin 7.5 software (Originlab®).

3.0 Results

3.1 Synthesis of the fragments

Solid phase peptide synthesis of HwTx-I *N*-terminal (NTN) and HwTx-I *C*-terminal (CTN) peptide fragments were undertaken using ‘in-house’ made MPAL and commercially available acid resins respectively. Figures 2A and B illustrates the resulting RP-HPLC/UV and ESI-MS spectra of the target peptide materials. Also shown is the chromatographic purity of the products with the respective retention time (R_t) of 36.0 and 36.6 min. (Fig. 2A and B, insert). Observed m/z for the *N*-terminal and *C*-terminal fragments were aligned with expected calculated values.

3.2 Cleavage of the NTN (Thioester)

Different protocols were undertaken to maximize the yield of the NTN fragment containing the thioester (Tables 1–3). An initial set of experiments A, B, C and D (Table 1) were conducted to determine the optimal TFMSA volume per resin weight. During this initial set of conditions other reagents involved in the cleavage were kept constant. Upon completion of the first set of experiments the desired volume for the cleavage was determined to be 100 μ L for 50 mg of resin. The next set of experiments E, F and G (Table 2) were conducted to determine the optimal incubation time at room temperature. The second set of experiments were conducted with a TFMSA volume of 100 μ L. Finally a third set of experiments H, I and J (Table 3) were conducted to evaluate the implication of ice incubation on the final target yield. The third set of experiments was performed using a TFMSA volume of 100 μ L. After completion of the three sets of experiments we have found that the best yields for 50 mg of resin were achieved with a TFMSA volume of 100 μ L and a total incubation of 2.5 hours, which includes 0.5 hours of incubation on ice. After optimization, a 50 mg cleavage yielded approximately 11 mg of peptidic material, of which approximately 16% was the

target peak as determined by RP-HPLC/UV. The RP-HPLC/UV profiles of the best and worst cleavage conditions are shown as supplementary information for comparison [Fig. S1].

3.3 Ligation of the Peptide Fragments

Approximately equimolar concentrations of NTN (3.0 mg; 1.6 μ mol) and CTN (3.5 mg; 1.6 μ mol) were used for the ligation of the peptide fragment. Peptide fragments, MPAL, and TCEP were added to 5 mL of ligation buffer. The mixture was allowed to incubate at pH 7.1 for 4 hours. Subsequently, the reaction was terminated by diluting the reaction mixture with 30 mL of 0.1 % v/v TFA/Aq. The diluted material was subjected to preparative RP-HPLC/UV to remove GnHCl and MPAA. Figure 2C illustrates the resulting RP-HPLC/UV chromatogram and ESI-MS spectrum of the target material, demonstrating its purity and respective R_t of 38.6 min. The observed m/z and calculated m/z for the ligated and reduced fragment confirm the correct assembly. Ligation yields were determined to be approximately 27%, with 1.8 mg of target material recovered.

3.4 Oxidation of HwTx I

Linear ligated HwTx-I was oxidized using 0.1 M NH_4HCO_3 at pH 7.8. Oxidation was conducted at 4°C for 120 hours. Lowering the pH to 4, by the addition of acetic acid drop-wise terminated the oxidation reaction. Figure 2D illustrates the ESI-MS spectrum and RP-HPLC/UV chromatogram of the ligated, folded, and active disulfide isomer of HwTx-I. Also shown is the respective R_t of the target material of 36.5 min (Fig. 2D). Final confirmation is shown with Figure 3 which illustrates both peptide identity and folding with a 1:2 (native:synthetic) RP-HPLC/UV co-elution of the native-like ligated-oxidized HwTx-I and isolated native HwTx-I (Fig. 3C), together with the whole native *Ornithoctonus huwena* venom (Fig. 3A and B). Continuation of the HwTx-I oxidation and purification strategy, lead to the production of ~7 nMoles of the target peptide.

3.5 Electrophysiological recordings

Using the *Xenopus* oocyte expression system, all ligated HwTx-I disulfide isomers were investigated for their activity against $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$ and $\text{Na}_v1.5$ (Data not shown). Only one isomer was found to show significant activity against these Na_v channel isoforms. 1 μ M HwTx-I inhibited $\text{Na}_v1.2$ channels with $84.4 \pm 2.3\%$ while the current through $\text{Na}_v1.3$ channels was reduced with $71.3 \pm 3.2\%$. The same concentration inhibited $\text{Na}_v1.4$ and $\text{Na}_v1.5$ channels with $17.6 \pm 1.9\%$ and $15.7 \pm 2.8\%$, respectively (Fig. 4). In order to assess the concentration dependence of the toxin induced inhibition, concentration-response curves were constructed for $\text{Na}_v1.2$ and $\text{Na}_v1.3$ channels. For $\text{Na}_v1.2$, EC_{50} values yielded 340.5 ± 26.8 nM. For $\text{Na}_v1.3$, EC_{50} values were determined at 504.1 ± 81.3 nM (Fig. 5) which are similar to the expected literature values [31].

4.0 Discussion

Like many venomous secretions isolated from cone snails, snakes, and scorpions, the spider toxins have received burgeoning interest due to their bioactivity and flexibility to undergo various forms of peptide bioengineering. Huwentoxin-I (HwTx-I), a 33 amino acid

neurotoxin isolated from the venom extract of *Ornithoctonus huwena* represents a valuable resource as a potential molecular probe due to its biological activity [32]. Initially purported to be an N-type Ca^{2+} inhibitor [33], Huwentoxin-I bioactivity has expanded its application in various assays. Recently, Wang *et al.* [31] reported HwTx-I inhibition of particular tetrodotoxin sensitive voltage gated sodium channel (TTX-S VGSC) subtypes. Although complete structural determination of this toxin has yet to be undertaken its ability to block the normal function of ion channels is likely attributed to its inhibitor cysteine knot motif (ICK), a structural element common among peptide toxins [32,34].

HwTx-I asserts its mechanism of action on Na_v channels by binding to an amino acid stretch known as the S3–S4 linker [31]. A typical Na_v channel is comprised of two subunits: an α subunit and a β auxiliary subunit. The α subunit is the target for all known peptide toxins and consists of four domains with six transmembrane helices. HwTx-I binding occurs on the extracellular region near the voltage-sensitive S4 segment, rendering S4 in a closed position thereby inactivating the flow of ions through the channel. Voltage gated sodium channels play important physiological roles in neuronal signaling and their deregulation is implicated in various pathological states such as: epilepsy and migraines [35]. Their control has also been associated with pain management. As such, investigation of HwTx-I and its receptor-ligand interactions is of particular pharmaceutical interest.

Given the limited nature of venom extractions from *O. huwena*, investigations focusing on HwTx-I, including analogues, variants or non-native-like bioengineered sequences, are best carried out through synthetic production. However, due to the sequence length of the peptide (33 amino acids), continuous solid phase synthesis could be a chemically challenging approach [26]. Therefore, chemical synthesis of HwTx-I greatly benefits from the use of the native chemical ligation (NCL) technique as shown in this study. The cysteine residues at position 16 and 17 provide an ideal site for the ligation of the 16-mer (NTN) and 17-mer (CTN) fragments, which can both be easily be synthesized using SPPS.

The essential requirement for NCL is the presence of the thioester at the *C*-terminus of the NTN peptide fragment, which leads to the formation of a native-like peptide bond with its adjoining *N*-terminal cysteine-containing CTN fragment. Due to the susceptibility of thioesters to bases, such as piperidine, Boc synthesis has been the method of choice to generate the thioester. However Boc synthesis faces its own challenges. The major challenge in Boc synthesis is the use HF during peptide-resin cleavage. HF is highly toxic and requires specialized, dedicated and expensive laboratory apparatus for transferring and handling. Furthermore, due to its toxic nature, HF should be only be handled by specially trained individuals. These requirements often result in the exclusion of routine HF usage in the laboratory.

Here we illustrate the use of TFMSA instead of HF to generate a thioester at the *C*-terminus of the NTN. Several reaction conditions were tested to assess optimal TFMSA cleavage parameters specific to our peptide sequence. (Tables 1–3). Variables such as TFMSA volume, and incubation on ice versus incubation at room temperature were taken into consideration. It was found that 50 mg of resin with 100 μL of TFMSA in conjunction with the scavenging reagents yielded the greatest concentration of target peptide, as determined

by RP-HPLC/UV. We have found that increasing the acid volume or incubation time on ice did not produce favorable results (Tables 1 and 3). As TFMSA cleaves the peptide from the solid-phase support resin, determining the appropriate TFMSA volume is critical to maximize peptide yields. TFMSA used in low volumes demonstrates an inability to effectively cleave resin bound peptide, which results in low peptide yields. In addition when used in large volumes, TFMSA being a strong acid, has the ability to degrade the cleaved peptide thereby reducing the final yield of target material. Specifically for our peptide when the TFMSA volume was kept at 100 μ L the optimal incubation time at room temperature was 2 hours, producing a target peak at 16.69 % of the total peak area, as determined by RP-HPLC/UV. When the incubation time was 1.5 hours the target peak was 12.29 % of the total peak area. This reduction potentially reflects incomplete cleavage of the resin bound peptide due to the shorter incubation time. When the incubation time was increased to 3 hours the target peak area was determined to be 7.62 %, a possible effect now seen with degradation of the cleaved peptide.

TFMSA being a strong acid gives off heat during the cleavage reaction. To minimize the heat generated during cleavage and to limit the potential for accelerated peptide degradation, a drop-wise addition of TFMSA into the reaction mixture is done in an ice bath. Keeping all of the other reagents constant, the cleavage was conducted at varying ice bath incubations of 30, 60 and 120 minutes. The highest product yield was achieved at 30 minutes of incubation. A decreased yield at longer incubation times in an ice bath might be due to the increased exposure time to TFMSA, which underlines the importance of controlled timing with TFMSA exposure during the cleavage process.

Such experimental control and cleavage testing is emphasized with the use of TFMSA, as seen by Swerdloff *et.al.* [36] who reported *N*- to *O*- acyl rearrangement of serine and threonine residues during a two-step cleavage from a PAM resin using TFMSA. This procedure however has the potential of forming α -amino succinimide derivatives in aspartyl containing peptides on MBHA resins when they are exposed to TFMSA, although these side products can be suppressed by employing low temperature cleavage conditions, as employed here [37]. It is advised to keep these and other potential side reactions in mind while performing cleavages using TFMSA, and may necessitate the adaption of cleavage conditions mentioned in this paper to better suit the target sequence.

Random oxidation of the ligated HwTx-I produced 10 out of the 16 possible disulfide isomers [38] (Supplemental Fig. S2). Our goal here was to produce as many isomers as possible and to examine the potential for alternative structures that could have demonstrated bioactivity. Out of these 10 only one isomer displayed activity at the Na_v isoforms 1.2 and 1.3 and minimal activity on 1.4 and 1.5 which corresponds to previous reports of activity of HwTx-I [31]. This active isomer is thought to have adopted the complex cysteine knot motif that exists in the native form of HwTx-I, as indicated by peptide co-elution (Fig. 3C). This provides an additional level of validation for producing native-like peptide toxins by NCL, this time using an alternative cleavage process. This endeavor compliments and expands upon the previously reported linear Fmoc synthesis [39], as well as the recombinant production of HwTx-I [40] and now provides a potential route to explore structure activity relationships through a more convenient and rapid synthetic approach employing NCL.

As illustrated, TFMSA cleavage could serve as a technique to generate thioester peptide containing peptide fragments for those laboratories unable to perform HF cleavages due to the lack of technical skills or specialized apparatus. This provides the opportunity to expand into NCL strategies without HF. As a qualifying statement, laboratories with existing infrastructure to handle HF might find it still beneficial to continue using their current protocols due to the expected higher yields in comparison to our method. Yet the ease and accessibility of using TFMSA for NCL and NTN fragment production, as well as its potential cost benefit if specialized HF lab equipment has not already been purchased also merits consideration.

5.0 Conclusion

NCL is an invaluable process that has improved the syntheses of many once thought too lengthy and difficult sequences. The most popular approach to achieve thioester fragment production uses the acid labile deprotection strategies incorporated into Boc SPPS, which when generating a thioester calls for the use of a solid-phase support resin that requires HF for the cleavage process. HF is extremely hazardous, which limits its common use. Here we have shown that TFMSA (although still hazardous as a strong acid), can be used as a safer alternative to HF as a cleavage reagent in production of NTN thioester containing peptide fragments. Our aim is to add to the growing repertoire of TFMSA cleavage strategies available, and to provide avenues for undertaking NCL and Boc SPPS in laboratories that have previously viewed these techniques as unapproachable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Arg(Pbf)	<i>N</i> -alpha-9-Fluorenylmethoxycarbonyl- <i>N</i> -gamma-2,2,4,6,7-Pentamethyldihydrobenzofuran-5-Sulfonyl-L-Arginine
Asn(Trt)	<i>N</i> -alpha-9-Fluorenylmethoxycarbonyl- <i>N</i> -beta-Trityl-L-Asparagine
CTN	<i>C</i> -terminal fragment
Cys(Trt)	<i>N</i> -alpha-9-Fluorenylmethoxycarbonyl-S-Trityl-L-Cysteine
Da	Daltons
DCM	Dichloromethane

DIEA	Diisopropylethylamine
DMF	Dimethylformamide
EC₅₀	toxin concentration at half maximal efficacy
EDT	1,2 Ethane Dithiol
ESI-MS	Electrospray Ionization Mass Spectrometry
Fmoc	9-Fluorenylmethoxycarbonyl
HBTU	N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uraniumhexafluorophosphate
HCTU	1H-Benzotriazolium-1-[bis(Dimethylamino)Methylene]-5-Chloro-Hexafluorophosphate-(1-),3-Oxide
HF	Hydrofluoric Acid
HwTx-I	Huwentoxin-I
LC/MS	Liquid Chromatography interfaced Mass Spectrometry
Lys(Boc)	N-alpha-9-Fluorenylmethoxycarbonyl-N-ε-Boc-L-lysine
MBHA	(4-Methyl)benzhydrylamine
MeCN	Methylcyanide
MH⁺	Monoisotopic molecular mass
MPAA	4-Mercaptophenylacetic Acid
Nav	voltage gated sodium channel
NTN	N-terminal fragment
PDA	Photo Diode
RP-HPLC/UV	Reverse phase - High performance liquid chromatography interfaced Ultra-violet detection
Rt	retention time
TCEP	Tris(2-carboxyethyl)phosphine
TFA/aq	Trifluoroacetic acid/aqueous
TFMSA	Trifluoromethanesulfonic Acid
TIPS	Triisopropylsilane

References

1. Dawson PE, Kent SB. Synthesis of native proteins by chemical ligation. *Annu Rev Biochem.* 2000; 69:923–960. [PubMed: 10966479]
2. Kochendoerfer GG, Kent SB. Chemical protein synthesis. *Curr Opin Chem Biol.* 1999; 3(6):665–671. [PubMed: 10600727]
3. Kent SB. Chemical synthesis of peptides and proteins. *Annu Rev Biochem.* 1988; 57:957–989. [PubMed: 3052294]
4. Kent S. Origin of the chemical ligation concept for the total synthesis of enzymes (proteins). *Biopolymers.* 2010; 94(4):iv–ix. [PubMed: 20593463]
5. Durek T, et al. Convergent chemical synthesis and high-resolution x-ray structure of human lysozyme. *Proceedings of the National Academy of Sciences.* 2007; 104(12):4846–4851.
6. Barlos K, Gatos D. 9-Fluorenylmethoxycarbonyl/tbutyl-based convergent protein synthesis. *Biopolymers.* 1999; 51(4):266–278. [PubMed: 10618595]
7. Dawson P, et al. Synthesis of proteins by native chemical ligation. *Science.* 1994; 266(5186):776–779. [PubMed: 7973629]
8. Dawson PE, et al. Modulation of Reactivity in Native Chemical Ligation through the Use of Thiol Additives. *Journal of the American Chemical Society.* 1997; 119(19):4325–4329.
9. Torbeev VY, Kent SB. Convergent chemical synthesis and crystal structure of a 203 amino acid “covalent dimer” HIV-1 protease enzyme molecule. *Angew Chem Int Ed Engl.* 2007; 46(10):1667–1670. [PubMed: 17397076]
10. Bang D, et al. Total chemical synthesis of crambin. *J Am Chem Soc.* 2004; 126(5):1377–1383. [PubMed: 14759195]
11. Ottesen JJ, et al. Semisynthesis of phosphovariants of Smad2 reveals a substrate preference of the activated T beta RI kinase. *Biochemistry.* 2004; 43(19):5698–5706. [PubMed: 15134444]
12. Muir TW. Studying protein structure and function using semisynthesis. *Biopolymers.* 2008; 90(6):743–750. [PubMed: 18924136]
13. Hackeng TM, et al. Protein synthesis by native chemical ligation: expanded scope by using straightforward methodology. *Proc Natl Acad Sci U S A.* 1999; 96(18):10068–10073. [PubMed: 10468563]
14. Johnson ECB, Kent SBH. Insights into the Mechanism and Catalysis of the Native Chemical Ligation Reaction. *Journal of the American Chemical Society.* 2006; 128(20):6640–6646. [PubMed: 16704265]
15. Bang D, et al. Kinetically controlled ligation for the convergent chemical synthesis of proteins. *Angew Chem Int Ed Engl.* 2006; 45(24):3985–3988. [PubMed: 16639756]
16. Kent S, et al. Through the looking glass—a new world of proteins enabled by chemical synthesis. *J Pept Sci.* 2012; 18(7):428–436. [PubMed: 22674813]
17. Lewis RJ, et al. Drugs from the peptide venoms of marine cone shells. *Australas Biotechnol.* 1994; 4(5):298–300. [PubMed: 7765678]
18. Flinn JP, et al. Role of disulfide bridges in the folding, structure and biological activity of ω -conotoxin GVIA1. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology.* 1999; 1434(1):177–190. [PubMed: 10556572]
19. Clark RJ, et al. The engineering of an orally active conotoxin for the treatment of neuropathic pain. *Angew Chem Int Ed Engl.* 2010; 49(37):6545–6548. [PubMed: 20533477]
20. Kent SBH. Total chemical synthesis of proteins. *Chemical Society Reviews.* 2009; 38(2):338–351. [PubMed: 19169452]
21. Kent S. Novel forms of chemical protein diversity – in nature and in the laboratory. *Curr Opin Biotechnol.* 2004; 15(6):607–614. [PubMed: 15560989]
22. Schnolzer M, et al. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res.* 1992; 40(3–4):180–193. [PubMed: 1478777]
23. Bu X, et al. An improved deblocking agent for direct Fmoc solid-phase synthesis of peptide thioesters. *Tetrahedron Letters.* 2002; 43(13):2419–2422.

24. Blanco-Canosa JB, Dawson PE. An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. *Angew Chem Int Ed Engl*. 2008; 47(36): 6851–6855. [PubMed: 18651678]
25. Mende F, Seitz O. 9-Fluorenylmethoxycarbonyl-Based Solid-Phase Synthesis of Peptide α -Thioesters. *Angewandte Chemie International Edition*. 2011; 50(6):1232–1240.
26. Jubilut GN, et al. Evaluation of the trifluoromethanesulfonic acid/trifluoroacetic acid/thioanisole cleavage procedure for application in solid-phase peptide synthesis. *Chem Pharm Bull (Tokyo)*. 2001; 49(9):1089–1092. [PubMed: 11558592]
27. Ozcan M, et al. Possible hazardous effects of hydrofluoric acid and recommendations for treatment approach: a review. *Clin Oral Investig*. 2012; 16(1):15–23.
28. Sarin VK, et al. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Analytical Biochemistry*. 1981; 117(1):147–157. [PubMed: 7316187]
29. Bingham JP, et al. Synthesis of an iberiotoxin derivative by chemical ligation: A method for improved yields of cysteine-rich scorpion toxin peptides. *Peptides*. 2009; 30(6):1049–1057. [PubMed: 19463736]
30. Liman ER, et al. Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron*. 1992; 9(5):861–871. [PubMed: 1419000]
31. Wang M, et al. The effects of huwentoxin-I on the voltage-gated sodium channels of rat hippocampal and cockroach dorsal unpaired median neurons. *Peptides*. 2012; 34(1):19–25. [PubMed: 22094230]
32. Liang S. An overview of peptide toxins from the venom of the Chinese bird spider *Selenocosmia huwena* Wang [=Ornithoctonus huwena (Wang)]. *Toxicon*. 2004; 43(5):575–585. [PubMed: 15066414]
33. Peng K, et al. The effect of Huwentoxin-I on Ca(2⁺) channels in differentiated NG108–15 cells, a patch-clamp study. *Toxicon*. 2001; 39(4):491–498. [PubMed: 11024489]
34. Zhang D, Liang S. Assignment of the three disulfide bridges of huwentoxin-I, a neurotoxin from the spider *selenocosmia huwena*. *J Protein Chem*. 1993; 12(6):735–740. [PubMed: 8136023]
35. Catterall WA, et al. Inherited Neuronal Ion Channelopathies: New Windows on Complex Neurological Diseases. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2008; 28(46):11768–11777. [PubMed: 19005038]
36. Swerdloff MD, et al. Solid phase synthesis of bioadhesive analogue peptides with trifluoromethanesulfonic acid cleavage from PAM resin. *International Journal of Peptide and Protein Research*. 1989; 33(5):318–327.
37. Thompson PE, et al. Synthesis of peptide amides using Fmoc-based solid-phase procedures on 4-methylbenzhydrylamine resins. *International Journal of Peptide and Protein Research*. 1995; 46(2): 174–180. [PubMed: 8567172]
38. Espiritu MJ, et al. Incorporation of post-translational modified amino acids as an approach to increase both chemical and biological diversity of conotoxins and conopeptides. *Amino Acids*. 2014; 46(1):125–151. [PubMed: 24221351]
39. Liang S, et al. Solid-phase synthesis of huwentoxin-I and its structure and bioactivity analysis. *Science in China Series C: Life Sciences*. 1997; 40(5):449–457. [PubMed: 20229294]
40. Meng E, et al. Functional e of spider neurotoxic peptide huwentoxin-I in *E. coli*. *PLoS One*. 2011; 6(6):e21608. [PubMed: 21731778]

Figure 1.

Methodology and optimized reaction conditions for the generation of the thioester through TFMSA cleavage. After the peptide fragment with the thioester (blue) was produced NCL was used to join the *N*-terminal (blue) and *C*-terminal (green) peptide fragments at the cysteines (red) to form a ligated full sequence of the reduced peptide. Random oxidation of the ligated peptide was undertaken to produce the bioactive HwTx-I sequence.

Figure 2. RP-HPLC/UV chromatogram and ESI-MS of the: (A) N-terminal-Thioester (NTN), (B) C-terminal peptide fragment (CTN), (C) Ligated linear unoxidized peptide, and (D) Ligated linear oxidized peptide.

Figure 3.

(A) Native milked venom of *Ornithoctonus huwena*. (B) Chemically ligated and oxidized HwTx-I showing retention time parity with native HwTx-I (arrow). (C) Co-elution of native and synthetic HwTx-I (1:2 ratio), indicates the production of “native-like” material.

Figure 4.

Comparison of the pharmacological activity of the active isomer and an inactive isomer on mammalian Na_V channels expressed in *X. laevis* oocytes. Panels show superimposed representative current traces illustrating the typical effect on Na_V1.2, Na_V1.3, Na_V1.4 and Na_V1.5 for 1 μM active and inactive isomer. The asterisk indicates the current trace in the presence of toxin; the dotted line indicates zero current level. Each experiment was performed at least 3 times (n = 3). Data are presented as mean ± S.E.

Figure 5.

Concentration-response curves for the active HwTx-I isomer on Na_v1.2 and Na_v1.3 channels. Results are indicated as mean $\pm$ S.E. from experiments performed in triplicate. The calculated EC₅₀ values for Na_v1.2 and Na_v1.3 were determined to be 340.5 ± 26.8 nM and 504.1 ± 81.3 nM respectively.

Table 1

Percent yield of target compound (NTN-thioester) under various reaction conditions. Conditions A, B, C and D shows changes in total peak area due to increasing TFMSA volume. In addition to the stated reagents the cleavage mixture contains: thioanisole 100 μL ; 1,2 EDT 50 μL ; TFA 1000 μL .

Resin weight: 50mg	Cleavage mixture			
	A	B	C	D
TFMSA (μL)	50	100	150	200
Ice bath 0°C (min.)	30	30	30	30
Room Temp (min.)	120	120	120	120
% Area of target peptide ¹	1.60	16.80	12.30	12.20

Table 2

Percent yield of target compound (NTN-thioester) under various reaction conditions. Conditions E, F, and G assessed optimal reaction time at room temperature (25°C) while keeping the TFMSA volume constant at 100 µL. In addition to the stated reagents the cleavage mixture contains: thioanisole 100 µL; 1,2 EDT 50 µL; TFA 1000 µL.

Resin weight: 50mg	Cleavage mixture		
	E	F	G
TFMSA (µL)	100	100	100
Ice bath 0°C (min.)	30	30	30
Room Temp (min.)	90	120	180
% Area of target peptide¹	12.90	16.70	7.60

Table 3

Percent yield of target compound (NTN-thioester) under various reaction conditions. Conditions H, I, and J evaluated various reaction times in an ice bath, with the room temperature and TFMSA volume being kept constant. In addition to the stated reagents the cleavage mixture contains: thioanisole 100 μ L; 1,2 EDT 50 μ L; TFA 1000 μ L.

Resin weight: 50mg	Cleavage mixture		
	H	I	J
TFMSA (μ L)	100	100	100
Ice bath 0°C (min.)	30	90	120
Room Temp (min.)	120	120	120
% Area of target peptide ¹	16.60	5.10	6.20