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Communication

# Total synthesis of snake toxin α-bungarotoxin and its analogues by hydrazide-based native chemical ligation

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) play important roles in intercellular communications of nerve cells. α-Bungarotoxins (αBtx) is a moderator for the nAChRs. Chemical synthesis provides a promising way to access αBtx and their analogues. Here, we reported a new method for α-bungarotoxin by combining Fmoc-SPPS and peptide hydrazide based ligation strategy. The two-segment ligation method may enable efficient synthesis of αBtx analogues. These synthetic toxin peptides are useful tools for development of imaging or therapeutic reagents.



Nicotinic acetylcholine receptors (nAChRs) are one of pentameric ligand-gated ion channels, which can respond to the neurotransmitter acetylcholine. nAChRs are widely exist in the central and peripheral nervous system and essential to neurotransmission. They play crucial roles in intercellular communications in nervous system [1]. As one of the best-studies of the ionotropic receptors, many animal venom including α-bungarotoxins (αBtx) were found to regulate the receptors and have been applied for structure-function studies [2].

αBtx can strongly bind to nAChR and cause heart failure or neuro transduction disorder. αBtx contains 74 amino acid residues and 5 pairs of disulfide bonds to keep structural stability and potent bioactivity. The peptide is type II α neurotoxin derived from snake venom. αBtx have been widely used on nAChR-related researches, such as analyzing the amount of muscle nAChRs in myasthenia gravis and detecting corresponding binding nicotinic subunits on Western blots [3]. Meanwhile, αBtx probes were used for monitoring receptor expression changes in neurodegenerative or psychiatric diseases [4]. In the process of studying the structural, functional mechanism or other related researches, adequate amount of homogeneous αBtx toxins or its analogues are urgently required.

In previous accessible methods, αBtx was usually obtained by natural extraction from snake poison gland. But the banded krait-Bungarus Multicinctus contains a number of toxins with polarity and structural similarity. Those impurities are difficult to separate from the final αBtx product even with the most advanced purification technology and then led to the impurity of αBtx [5]. Xu and co-workers reported a recombinant expression approach to produce αBtx with an additional methionine and a glycine at N-terminal [6]. But expression work needed delicate construction of expression vectors, tedious expression and purification steps. Besides, expression method is difficult to provide the αBtx sample with artificial modifications.

Herein, we reported a new total chemical synthesis method for the preparation of toxin peptide αBtx. Our new approach takes advantage of Fmoc solid-phase peptide synthesis (SPPS) for individual peptide segments and peptide-hydrazide-based native chemical ligation (NCL) for segment condensation [7]. Desired toxin peptides were successfully obtained after folding of linear peptides. With the newly developed method, we synthetized natural αBtx and its analogue αBtx(V31). This new method would provide a convenient strategy to produce biophysical probes (such as fluorescent or isotope labeling) for kinetic studies of toxin and related receptor binding process.

In the past decades, one of the most important breakthrough in peptide synthesis was SPPS technique, especially through the Fmoc-based SPPS method [8]. Fmoc-SPPS can introduce functional groups into the peptide at atom level precision, such as non-natural amino acids or post-translational modifications [9]. A series of ligation approaches (Kent's native chemical ligation (NCL) [10], Liu's hydrazide-based NCL [7a,11], Li's Ser/Thr ligation [12] and Bode's KAHA ligation [13], *etc.*) have been reported for the

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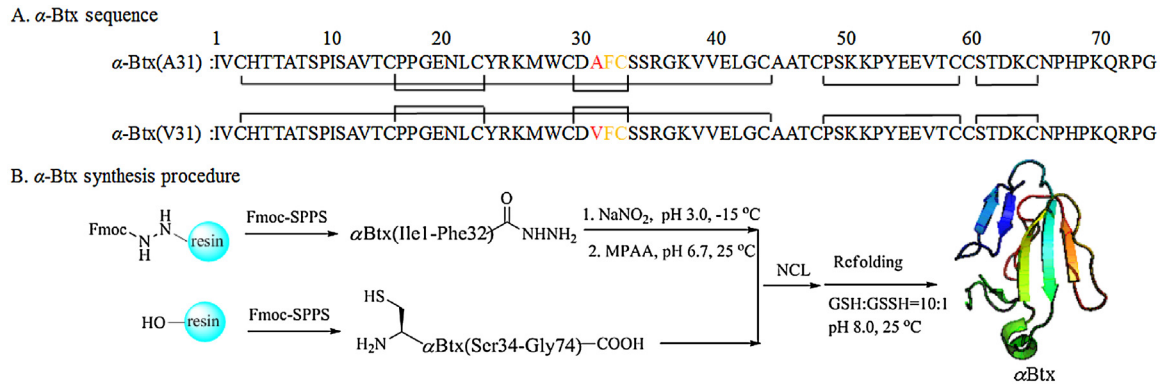


**Scheme 1.** Sequences, synthetic routes and pdb structure of αBtx toxins. A. Sequences and disulfide bonds of αBtx(A31) and αBtx(V31). Different residues are marked in red and ligation sites for NCL are marked in yellow. B. Synthesis route and conditions for αBtx.

synthesis of larger proteins or polypeptides. Those advances have made great contributions to the chemical synthesis of proteins. Even the small-to-medium sized membrane protein could be chemically synthesized by the newly developed removable backbone modification (RBM) strategy [14]. Using chemical synthesis techniques, many toxins such as mambalgin [15], alpha scorpion toxin protein Ts3 [16] had been reported. Those progresses would greatly facilitate the chemical synthesis of αBtx.

The chemically synthetic route of αBtx is shown in Scheme 1. αBtx was divided into two segments, αBtx(Ile$^{1}$-Ala$^{31}$Phe$^{32}$)-CONHNH$_2$ (**1**) and αBtx(Cys$^{33}$-Gly$^{74}$)-COOH (**2**). The peptides **1** and **2** were first synthesized by Fmoc SPPS and then ligated by peptide-hydrazide-based NCL to afford the full-length sequence αBtx **3**. Finally, αBtx **3** was folded to give the native αBtx **4**.

The preparation of peptide hydrazide **1** began with the coupling of Phe32 to H$_2$NHN-2-Trt-resin, which was prepared from 2-Cl-Trt-resin by previous method [17]. The chain assembly of peptide **1** was performed by a peptide automatic synthesizer (CS-Bio CS136XT) under standard coupling (HCTU/DIEA) and Fmoc deprotection conditions (20% piperidine in DMF). After final cleavage from the resin under standard conditions, the crude peptide was analyzed by analytical high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (MSI-MS). The HPLC result showed that peptide **1** was found as the major peak (Fig. S1 in Supporting information) which was determined by ESI-MS (calcd. 3532.1 Da *vs.* obs. 3530.0 Da). The crude peptide was purified by semi-prepared RP-HPLC and obtained 142 mg of purified **1** in a 20% isolated yield of product (Fig. 1A).

To prepare peptide **2**, the coupling of Gly74 (HBTU/HOBt/DIEA with catalytic amount of DMAP) was firstly carried out on a HO-WANG-resin. The following assembly of peptide **2** was performed by a similar procedure as peptide **1**. However, the result showed that it was inefficient using the normal Fmoc-AA coupling strategy and no desired product was observed by analytical RP-HPLC (data not show). It was speculated that the embedding effect of peptide chain on the resin caused the low coupling efficiency. To address this problem, we use Fmoc-(Dmb)Gly-OH to replace Fmoc-Gly-OH at Gly43 during chain peptide assembly. The backbone modified Dmb group can perturb the secondary structure formation and promoting coupling efficiency [18]. The Dmb group can be completely cleaved under TFA cocktails cleavage conditions. For this time, αBtx(Cys$^{33}$-Gly$^{74}$)-COOH was synthesized in good efficiency. Analysis of the crude peptide by analytical RP-HPLC indicated that peptide **2** (Fig. S3 in Supporting information) to be the major peak as determined by ESI-MS (calcd. 4496.16 Da *vs.* obs. 4498.0 Da). The crude peptide was purified by semi-prepared RP-HPLC and obtained 372 mg of purified **2** in a 21% yield of isolated product (Fig. 1B).

With the purified two peptide segments in hand, we carried out the ligation experiment. In general, peptide hydrazide **1** (1 equiv., 22.9 mg) was dissolved in 6 mol/L Gn·HCl buffer (2.5 mL, pH 3), after cooling to −15 °C by ice-salt bath, 10 equiv. NaNO$_2$ was added and gently stirred for another 15–20 min to obtain peptide hydrazide. After that, 30 equiv. 4-carboxybenzenethio (MPAA) was added to transfer peptide hydrazide into the corresponding thioester **1′** *in vivo*. Then, 0.8 equiv. (22.5 mg) of the peptide **2** with N-terminal Cys residue was added and the pH was adjusted to 6.7 for peptide ligation. The ligation reaction was stirred at room temperature and monitored by RP-HPLC. The reaction traces were shown in Fig. 2A. After 12 h, the reaction was completed and the production was purified by semi-prepared RP-HPLC. The ligation product peptide **3** (calcd. 7994.25 Da *vs.* obs. 7993.97 Da) was confirmed by mass spectrometry (Fig. 2B). The peptide **3** was thus obtained in an isolated yield of 57% (24.3 mg).

After the lyophilization of the linear peptide **3**, a folding step was conducted in aqueous buffer with 1 mmol/L GSH and 0.1 mmol/L GSSG, pH 8.0. The final concentration of linear peptide

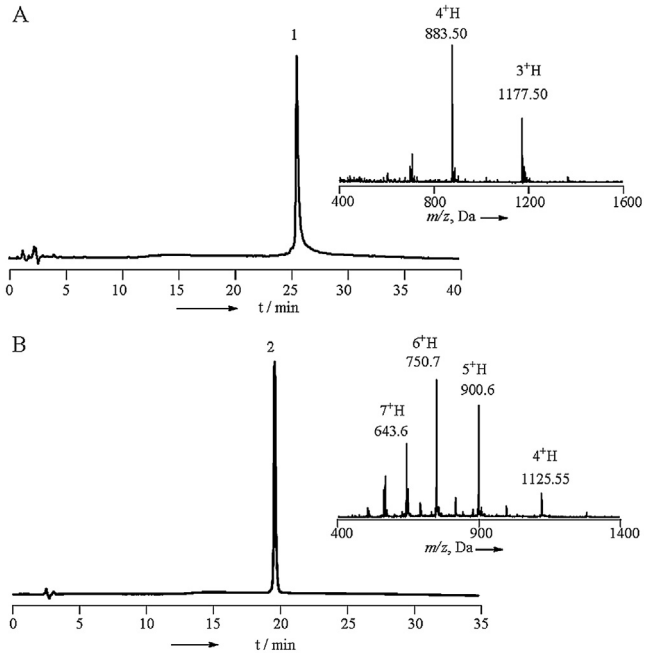


**Fig. 1.** Analytical HPLC (λ = 214 nm) and ESI-MS data of (A) αBtx(Ile$_1$-Ala$_{31}$Phe$_{32}$)-CONHNH$_2$ (peptide **1**) and (B) αBtx(Cys$_{33}$-Gly$_{74}$)-COOH (peptide **2**). HPLC conditions: a linear gradient of 10%-69% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 min.

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