Chemistry of Natural Compounds, Vol. 33, No. 4, 1997

SYNTHESIS OF TWO PEPTIDES OF α-BUNGAROTOXIN AND THE PARTICIPATION OF THE AMINO ACID RESIDUE Trp-28 OF THE NEUROTOXIN IN THE ANTIGENICITY OF THE MOLECULE

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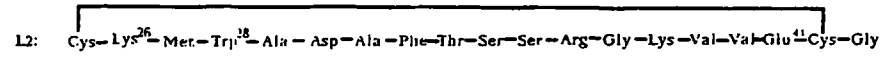
UDC 547.993.3

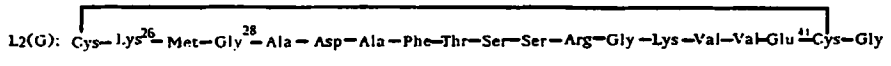
Using the method of solid-phase peptide synthesis, two peptides have been synthesized, one of which corresponds to the central ring structure of α-bungarotoxin (α-BTX), while the second has in position 28 a Gly residue in place of the Trp in the first peptide, and their interrelationship with antitoxin antibodies has been investigated. It has been shown that the amino acid residue Trp-28 of α-BTX, which is the contact residue in binding with the acetylcholine receptor, also participates directly in binding with the active centers of antibodies to α-BTX.

α-Bungarotoxin — the main toxic component of the venom of the snake *Bungarus multicinctus* — belongs to the class of long α-neurotoxins and consists of 74 amino acid residues with five intramolecular disulfide bonds. Its specific interaction with the α-subunit of the nicotinic acetylcholine receptor has been investigated, and the molecular region of the toxin and the receptors involved in binding have been determined [1]. It has been shown that the first, second, and third looped sections of α-BTX contain amino acid residues directly contacting the receptor [2]. The trytophan residue in position 28 of the polypeptide chain of α-BTX, which is one of the invariant residues in the overwhelming majority of long and short α-toxins, is included among these amino acid residues, i.e., it participates in the manifestation of the neurotoxic activity of α-BTX [3].

The aim of the present work was to synthesize two peptides reflecting the central loop structure of α-BTX and to investigate the possible role of the amino acid residue Trp-28, present in this region, in the antigenicity of the α-BTX molecule.

We made use of the concept of surface-simulation synthesis [4] and, by employing solid-phase peptide synthesis, we obtained two nineteen-membered peptides. The first of them, designated L2, synthetically reflected the second loop structure of α-BTX and comprised amino acid residues 26-43, while peptide L2(G) contained the same amino acid residues as L2 in the same sequence, apart from position 28, where the tryptophan residue had been replaced by glycine. The structures of the peptides that we had synthesized are as follows:





The synthesis was carried out with the aid of Wang resin (*p*-benzyloxybenzyl alcohol resin) and N-α-9-fluorenylmethoxycarbonyl-substituted derivatives of *L*-amino acids by the Merrifield method [5]. Since peptide L2 contained a tryptophan residue, to prevent modification of its indole ring during the synthesis and the splitting out of the peptide from the resin we used its *tert*-butoxycarbonyl-protected derivative. To prevent oxidation of the methionine residues in both peptides

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0009-3130/97/3304-0485$18.00

TABLE 1. Amino Acid Analyses of the Synthetic Peptides L2 and L2(G) of α-Bungarotoxin[a]

Peptide	Asx	Thr[b]	Ser[b]	Glx	Gly	Ala	Cys[c]	Val	Met	Phe	Lys	Trp[d]	Arg
L2	1.04 (1)	0.91 (1)	2.10 (2)	1.00 (1)	2.11 (2)	1.90 (2)	1.91 (2)	2.04 (2)	0.90 (1)	1.12 (1)	2.11 (2)	0.89 (1)	1.09 (1)
L2(G)	1.08 (1)	1.10 (1)	1.91 (2)	1.07 (1)	2.94 (3)	2.11 (2)	1.87 (2)	1.93 (2)	0.94 (1)	1.07 (1)	2.01 (2)	–	1.04 (1)

[a]The values expected from the amino acid composition are given in parentheses.
[b]Because of degradation during acid hydrolysis, the results obtained were extrapolated to the initial time of hydrolysis.
[c]Determined as cysteic acid after oxidation with performic acid.
[d]Determined after hydrolysis with 3N *para*-toluenesulfonic acid containing 0.2% of 3-(2-aminoethyl)indole.

TABLE 2. Binding of Various Antibodies to α-BTX with Synthetic Peptides of α-BTX

Peptide	Bound antibodies (Net CPM)			
	C57BV6	Balb/c	SJL	ICR
L2	4107	9036	7416	5707
L2(G)	783	5443	5830	1469

*The results are the mean values of three experiments carried out independently (variation not more than ±1.5%) and have been corrected for the nonspecific binding shown by BSA.

during their detachment from the resin we added thioanisole, which is used for this purpose in various reaction systems. After detachment from the resin, the peptides were desalted on a column of Sephadex G-15, and acid hydrolysates of them were analyzed for their amino acid compositions (Table 1).

In view of the fact that the peptides were intended for the synthetic reflection of the central ring structure of α-BTX, their cyclic monomeric forms were obtained by forming a disulfide bond between the two cysteine residues. Cyclization was achieved by a negative Ellman reaction with 5,5′-dithiobis-2-nitrobenzoic acid. The monomeric fraction of the peptide was purified by liquid chromatography with Sephadex G-50 F and, finally, by reversed-phase HPLC. The yield of monomer was 2.3-6.4% of the amount of peptide taken for cyclization.

Antibodies against α-BTX were obtained in three lines (C57B1/6, Balb/c, and SJL) and in random-bred mice using sublethal amounts of α-BTX. Recognition of the synthesized cyclic peptides by antibodies against intact α-BTX was investigated by double-antibody radioimmune analysis (Table 2).

From the spatial orientation of the exposed second loop structure of α-BTX [6], including amino acid residues 23-44 of the polypeptide chain, its participation in the antigenicity of the α-BTX molecule was assumed, and this has been demonstrated [7]. The results that we have obtained show that after the glycine replacement of the invariant Trp-28 the total capacity of the region of α-BTX represented by peptide L2 for binding with antitoxin antibodies fell to 21.4% for the SJL antibodies and to 80.9% for the C57B1/6 antibodies.

Thus, we have synthesized two peptides of α-BTX using the 9-fluorenylmethoxycarbonyl strategy of solid-phase peptide synthesis. The first of them synthetically reflected the central loop structure of the α-BTX molecule, which contains one of the invariant amino acid residues found in the overwhelming majority of α-neurotoxins — Trp-28. In the second peptide, this residue had been replaced by a glycine residue. These peptides were investigated for their ability to bind with antibodies against α-BTX by means of double-antibody radioimmune assay. It was shown that the Trp-28 amino acid residue in the central loop structure of the α-BTX molecule, which exhibits the neurotoxic properties of α-BTX, also participates directly in the antigenicity of the molecule, and this is of fundamental importance for the synthesis of an artificial antigen based on α-BTX with the aim of creating a peptide vaccine against the powerful neurotoxic action of α-BTX.

EXPERIMENTAL

α-BTX from the venom of the snake *Bungarus multicinctus* was obtained from Miami Serpentarium Laboratories (Punta Gora, FL, USA) and its homogeneity was confirmed by reversed-phase HPLC with a linear gradient of acetonitrile from 0 to 90% on a Vydac 218TP54 analytical column (5 μ, C18, 4.6 × 250 mm). The synthesis of the peptides was carried out on a Coupler 250 Peptide Synthesizer semiautomatic synthesizer (Vega Biotechnologies, Tucson, AZ, USA) or by the manual method, using *L*-amino acids protected by the N-α-9-fluorenylmethoxycarbonyl group. The protective groups for the side-chains were: for arginine, 4-methoxy-2,3,6-trimethylbenzylsulfonyl; for aspartic and glutamic acids, *tert*-butyl esters; for cysteine, triphenylmethyl; for lysine, *tert*-butoxycarbonyl; for serine and threonine, *tert*-butyl. All the organic solutions used for the synthesis of the peptides had synthetic or spectrophotometric purity.

Production of Antisera. Mouse antisera were obtained to the following lines: C57B1/6, SJL, Balb/c, and random-bred mice obtained from the National Cancer Institute and Lackson Laboratory (Bar Harbor, ME, USA). A solution of 4 μg of α-BTX in 50 μl of 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2 (PBS) was mixed with an equal volume of complete Freund's adjuvant, and mice were immunized by injection of the mixture into the pads of their rear paws. Reimmunization was performed with the same amount of α-BTX three times at two-week intervals (incomplete Freund's adjuvant was used, in accordance with the NIH requirements for immunizing animals). The antisera used in this work were obtained on the 73rd day after the initial immunization.

Solid-Phase Radioimmune Assay. The titers of the antibodies in the antisera were determined by solid-phase radioimmune assay in binding with α-BTX before they were used in the experiments for binding peptides L2 and L2(G). α-BTX (0.5 μg in 50 μl of PBS) or each of the peptides (2.5 μg in 50 μl of PBS) was bound (4°C, 17 h) with the wells of 96-well polyvinyl plates (Falcon, Lincoln Park, NY, USA), after which the wells were washed with PBS, blocked (37°C, 1 h) with 100 ml of 1% BSA/PBS, and washed five times with PBS. Antisera diluted 1:1000 in 0.1% BSA/PBS was added to the wells (50 μl each) and, after incubation at 4°C for 12 h, they were washed five times with PBS and were reacted with rabbit antimouse (IgG+IgM) antibodies (Accurate Chemical and Scientific Co., Westbury, NY, USA) (dilution 1:2000, v/v, in 0.1% BSA/PBS at 37°C for 2 h. After washing, the immune complex was revealed with an excess of ^{125}I-labeled protein (2×10^5 CPM in 50 μl of 0.1% BCA/PBS) at room temperature for 2 h, washed with PBS, and transferred to individual test tubes for the measurement of included radioactivity with the aid of a gamma counter (Gamma Master, LKB). The assays were performed in triplicate and the results were corrected for nonspecific binding in control wells that were not coated with α-BTX or the peptides but were blocked with BSA.

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