Isolation, properties and amino acid sequence of a long-chain neurotoxin, *Acanthophis antarcticus* b, from the venom of an Australian snake (the common death adder, *Acanthophis antarcticus*)

Hyung Suk KIM and Nobuo TAMIYA

Department of Chemistry, Tohoku University, Sendai, Japan

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The venom of an Australian elapid snake, the common death adder (*Acanthophis antarcticus*), was chromatographed on a CM-cellulose CM52 column. One of the neurotoxic components, *Acanthophis antarcticus* b (toxin Aa b) was isolated in about 9.4% (A_{280}) yield. The complete amino acid sequence of toxin Aa b was elucidated. Toxin Aa b is composed of 73 amino acid residues, with ten half-cystine residues, and has a formula weight of 8135. Toxin Aa b has no histidine or methionine residue in its sequence. The amino acid sequence of toxin Aa b is homologous with those of other neurotoxins with known sequences, although it is novel in having a valine residue at its *N*-terminus and an arginine residue at position-23, where a lysine residue is found in almost all the so-far-known neurotoxins. Irrespective of the latter replacement, the toxin Aa b is fully active, with an LD₅₀ value (in mice) of 0.13 µg/g body weight on intramuscular injection.

The Australian snake *Acanthophis antarcticus* (the common death adder) is distributed throughout the Australian continent except some desert or wetter regions. It is known as one of the most dangerous snakes in Australia and in the world (Cogger, 1979).

More than 60 post-synaptically acting neurotoxins from the venoms of elapid and hydropid snakes have already been sequenced. However, the amino acid sequence has been reported for only one Australian-elapid-snake neurotoxin, by Halpert *et al.* (1979). The isolation of a short-chain neurotoxin from the same venom of *Acanthophis antarcticus* was reported by Sheumack *et al.* (1979).

The present paper describes the isolation, properties and the complete amino acid sequence of a long-chain neurotoxin, *Acanthophis antarcticus* b (toxin Aa b).

Materials and methods

Snake venom

The snakes, *A. antarcticus*, were collected at Armidale, New South Wales, Whyalla, South Australia, and Townsville, Queensland, Australia. The venom was 'milked', diluted with two volumes

of 0.15M-acetic acid and brought back to the laboratory.

Enzymes and chemicals

Trypsin (twice-crystallized and salt-free), α -chymotrypsin (three-times-crystallized) and pepsin (twice-crystallized) were the products of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Staphylococcal proteinase (from *Staphylococcus aureus* V8) was purchased from Miles Laboratories, Elkhart, IN, U.S.A. Thermolysin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and carboxypeptidase Y (from yeast) was the product of Oriental Yeast Co., Suita, Osaka, Japan.

Phenyl isothiocyanate, trifluoroacetic acid, dimethylallylamine, benzene and ethyl acetate (all Sequenal grade) were from Wako Pure Chemical Industries, Osaka, Japan.

Moniodoacetic acid was recrystallized from chloroform/light petroleum (b.p. 30–70°C), and other solvents and reagents were used without further purification.

Disc electrophoresis

The disc electrophoresis of the venom component was performed at pH 4.0 (Reisfeld *et al.*, 1962) with a constant current of 4 mA per gel for 2.5 h.

Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis (Weber & Osborn, 1969) was performed in 10% and 15% (w/v) polyacrylamide

Abbreviation used: toxin Aa b, neurotoxin *Acanthophis antarcticus* b (note that toxins are indicated by Roman, not italic, Latin names).

gel. Sample protein (1.0 mg) was incubated overnight at 37°C with 1% sodium dodecyl sulphate in 50% (v/v) glycerol in 0.01 M-sodium phosphate buffer, pH 7.2, containing 1% (v/v) 2-mercaptoethanol (0.1 ml). The sample solution was applied (50 µl/gel) and electrophoresis was performed at a constant current of 8 mA per gel for 5 h.

Amino acid analysis

Sample proteins or peptides (10–50 nmol) were hydrolysed in sealed tubes *in vacuo* at 110°C with 6 M-HCl (0.5 ml), usually for 24 h. For the determination of tryptophan content, the toxin (50 nmol) was hydrolysed in a sealed glass tube *in vacuo* at 110°C with 4 M-methanesulphonic acid (0.3 ml) containing 3-(2-aminoethyl)indole (0.2%, w/w; Pierce Chemical Co., Rockford, IL, U.S.A.) for 48 h. The hydrolysate was neutralized with 3.5 M-NaOH (0.3 ml), mixed with 0.2 M-sodium citrate buffer (1.6 ml), pH 2.2, and applied to an automatic amino acid analyser (JLC-8AH; JEOL, Tokyo, Japan). The tryptophan content was determined spectrophotometrically, as described by Edelhoch (1967).

Reduction and S-carboxymethylation of toxin Aa b

Toxin Aa b (4.8 µmol) was dissolved in 8 M-urea (5 ml) containing 1.4 M-Tris/HCl buffer, pH 8.6, and 0.2% (w/w) EDTA, and incubated at 37°C for 16 h (Crestfield *et al.*, 1963). The toxin was reduced by the addition of β-mercaptoethanol (0.1 ml), while the solution was flushed with N₂ at 37°C for 4 h. Carboxymethylation of the reduced toxin was performed in the dark at room temperature for 15 min after the addition of monoiodoacetic acid (0.239 g) in 1 M-NaOH (1 ml). Reduced and S-carboxymethylated toxin Aa b was recovered from the reaction mixture by gel filtration on a column (2.6 cm × 43 cm) of Sephadex G-25 (Fine grade) in 0.1 M-pyridine.

Enzymic digestions of reduced and S-carboxymethylated toxin Aa b and its fragments

The reduced and S-carboxymethylated toxin Aa b (3.0 µmol) was digested with trypsin, which had been treated with diphenylcarbamoyl chloride (Erlanger *et al.*, 1966), in 0.05 M-Tris/HCl buffer (3 ml), pH 8.2, at 37°C for 16 h. The enzyme/substrate ratio was 1:100 (w/w).

The reduced and S-carboxymethylated toxin Aa b (2.4 µmol) was dissolved in 0.05 M-NH₄HCO₃ (2 ml), pH 7.9, and digested with staphylococcal proteinase at an enzyme/substrate ratio of 1:100 (w/w) at 37°C for 16 h.

Some of the peptides from trypsin or staphylococcal-proteinase digest were further cleaved with α-chymotrypsin, pepsin or thermolysin. Digestion of peptides (0.3–0.5 µmol) with α-chymotrypsin was performed in 0.1 M-ammonium acetate (1 ml),

pH 7.8, at an enzyme/substrate ratio of 1:100 (w/w) at 37°C for 30 min. Peptic digestion of the fragment peptides (0.5–0.6 µmol) was done in 0.01 M-HCl (1 ml) at an enzyme/substrate ratio of 1:100 (w/w) at 37°C for 30 min. Digestion with thermolysin of the fragment peptide (0.5 µmol) was performed in 0.1 M-ammonium acetate (1 ml), pH 7.8, at an enzyme/substrate ratio of 1:50 (w/w) at 37°C for 2.5 h.

The reduced and S-carboxymethylated toxin Aa b or its fragment peptide (50 nmol) was treated with carboxypeptidase Y in 0.1 M-phosphate buffer (0.2 ml), pH 6.5, at an enzyme/substrate ratio of 1:50 (w/w) at 37°C, usually for 2, 4 and 6 h. The digests were directly subjected to amino acid analyses.

Manual Edman degradation

The amino acid sequences of the peptides and of reduced and S-carboxymethylated toxin Aa b were determined by the direct phenylthiohydantoin method of Iwanaga *et al.* (1969). The ethyl acetate-soluble phenylthiohydantoin derivatives were identified by t.l.c. (Iwanaga *et al.*, 1969; for arginine, Inagami, 1973) and, sometimes, by regeneration of amino acids from phenylthiohydantoin as described by Mendez & Lai (1975).

Result

Isolation of neurotoxin

Dried *A. antarcticus* venom (1.0 g) was dissolved in 0.01 M-sodium/potassium phosphate buffer (30 ml), pH 6.4, and applied to a column (2.6 cm × 40 cm) of CM52 cellulose (Brown Co., Berlin, NH, U.S.A.; capacity 0.70 mequiv./g) equilibrated with the same buffer. The elution pattern monitored at 280 nm is shown in Fig. 1.

The yields of the main thirteen fractions (I–XIII) were 9.2, 19.5, 4.1, 3.6, 5.8, 5.1, 6.1, 13.5, 9.4, 4.4, 2.1, 3.0 and 7.9% respectively, as measured by A₂₈₀. The total recovery was 93.7% as measured by the absorbance. Seven fractions (VII–XIII) showed lethal activity when injected into the hind-leg muscles of mice as solutions in 0.85% (w/v) NaCl (Tamiya & Arai, 1966). Strong neurotoxic activity, with symptoms of dyspnoea and paralysis of the hind legs similar to that of erabutoxins (Tamiya & Arai, 1966), was recovered in fractions VIII, IX, X and XIII.

The component (132.6 A₂₈₀ units) in fraction IX was desalted with 0.01 M-phosphate buffer, pH 7.2, in a Diaflo apparatus with a UM-2 membrane (Amicon Corp., Lexington, MA, U.S.A.) and rechromatographed on a column (2.2 cm × 27 cm) of CM52-cellulose in the same buffer, with a linear gradient of 0–0.3 M-NaCl in a total volume of 1.6 litre. The component in the main

Fig. 1. CM52 cellulose column chromatography of *Acanthophis antarcticus* venom

The dried venom (1.0g) was dissolved in 0.01M-phosphate buffer (30ml), pH 6.4, and loaded on to a column (2.6 cm $\times$ 40 cm) of CM52 cellulose equilibrated with the same buffer. The elution was performed with a linear 0–0.5 M-NaCl gradient in the buffer (2 litres in all) (arrow 1), then with 0.5 M-NaCl in the buffer (arrow 2). The flow rate was 80 ml/h. The protein fractions (I–XIII) underlined were collected separately. O, A_{280} ; ----, [NaCl].

protein peak (1170–1380 ml) was desalted in a Diaflo apparatus in 0.1 M-acetic acid and freeze-dried (yield 112 mg). The freeze-dried preparation was dissolved in 0.01 M-phosphate buffer (10 ml), pH 5.9, and again chromatographed on a CM52-cellulose column (2.6 cm $\times$ 40 cm) equilibrated with the same buffer. The elution was performed with a linear 0–0.5 M-NaCl gradient in a total volume of 2.0 litre of the buffer. The main component, IX, was desalted in 0.1 M-acetic acid and freeze-dried (yield 48.5 mg).

The purified component in fraction IX gave a single band on disc electrophoresis at pH 4.0. The component was named toxin *Acanthophis antarcticus* b (Aa b).

Lethal activity

The LD₅₀ value of toxin Aa b was determined as described previously (Tamiya & Arai, 1966) by injection into the hind-leg muscles of mice weighing 18–22 g and found to be 0.13 μ g/g body weight. The crude venom had an approximate LD₅₀ value of 0.2 μ g/g body weight.

Molecular weight and amino acid composition

Molecular weight of toxin Aa b was estimated to be 8500 by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis and to be 8300 by gel filtration through Sephadex G-50 (Fine grade) in

0.1 M-acetic acid (Andrews, 1965). The amino acid compositions of toxin Aa b and its fragment peptides (see below) are given in Table 1. Toxin Aa b consists of 73 amino acid residues with ten half-cystine residues. The molecular weight calculated from the amino acid composition is 8135.

Terminal amino acid sequence of toxin Aa b

Eighteen amino acids from the *N*-terminus of reduced and *S*-carboxymethylated toxin Aa b were determined by manual Edman degradation to be:

Val-Ile-CmCys-Tyr-Arg-Gly-Tyr-Asn-Asn-Pro-Gln-Thr-CmCys-Pro-Pro-Gly-Glu-Asn-

(CmCys is carboxymethylcysteine). Ordinary carboxypeptidase Y digestion of reduced and *S*-carboxymethylated toxin Aa b gave no C-terminal amino acids.

Isolation and amino acid sequences of fragment peptides

The tryptic fragments of reduced and *S*-carboxymethylated toxin Aa b were resolved into fractions T-I, T-II and T-III by gel filtration on a Sephadex G-25 (Fine grade) column (1.2 cm $\times$ 250 cm) in 0.1 M-acetic acid. Fractions T-I, T-II and T-III were eluted at 162–188 ml, 193–224 ml and 232–272 ml respectively.

Fraction T-I gave two peptides, T-Ib and T-Ia, on

Table 1. *Amino acid compositions of toxin Aa b and its tryptic peptides*
The composition of each peptide is given as the molar ratio of amino acids determined.

Amino acid	Toxin Aa b	Peptide (residue nos.) ...	T-IIIa (1-5)	T-IIa (6-23)	T-IIIb (24-33)	T-IIIc (34-35)	T-Ia (36-49)	T-IIId (50-55)	T-IIb (56-70)	T-IIId (71-73)	T-Ib (62-73)
Lys	4.02 (4)					0.98 (1)	1.00 (1)	1.02 (1)	1.07 (1)		
Arg	5.56 (6)		1.00 (1)	0.94 (1)	0.90 (1)				1.79 (2)	1.00 (1)	3.01 (3)
Asp*	6.99 (7)			3.00 (3)	0.94 (1)			1.00 (1)	2.00 (2)		1.00 (1)
Thr	4.85 (5)			1.74 (2)	0.84 (1)		0.87 (1)		1.13 (1)		
Ser	3.71 (4)				1.79 (2)			0.75 (1)	0.87 (1)		
Glu*	3.98 (4)			1.91 (2)			0.85 (1)	1.08 (1)			
Pro	9.02 (9)			3.25 (3)			1.09 (1)		2.92 (3)	1.75 (2)	5.19 (5)
Gly	3.88 (4)			1.88 (2)		1.02 (1)	1.13 (1)				
Ala*	3.00 (3)				1.07 (1)		2.05 (2)				
½Cys†	8.81 (10)		0.83 (1)	1.57 (2)	1.65 (2)		1.50 (2)		2.63 (3)		0.67 (1)
Val	7.36 (7)‡		0.85 (1)	1.05 (1)			2.87 (3)	1.08 (1)	0.92 (1)		1.09 (1)
Ile	1.82 (2)‡		0.65 (1)				0.71 (1)				
Leu*	1.01 (1)						0.86 (1)				
Tyr	2.95 (3)		0.92 (1)	0.98 (1)				0.84 (1)			
Phe	3.01 (3)			0.92 (1)	0.93 (1)				0.83 (1)		0.76 (1)
Trp§	0.95 (1)				(1)						
Total residues	73		5	18	10	2	14	6	15	3	12
Yield (%)			32	27	23	16	22	13	23	5	8
N-Terminus	Val		Val	Gly	Thr	Gly	Val	Ser	Cys	Arg	Cys

* The amino acid contents of the peptides were calculated by taking the total molar contents of these amino acids as round numbers.

† Cystine residues tend to give smaller values, and are determined as *S*-carboxymethylcysteine in peptides.

‡ Values for 72 h hydrolysates are given.

§ Measured from hydrolysis with 4 M-methanesulphonic acid.

chromatography through a DE52 cellulose (Brown Co.; capacity 0.84 mequiv./g) column (1.2 cm × 15 cm) equilibrated with 0.01 M-NH₄HCO₃, pH 8.2. The elution was performed with the starting buffer for peptide T-Ib and with 0.10 M-buffer for peptide T-Ia. The chromatography of fraction T-II on the same column gave peptide T-IIa with 0.08 M-buffer and T-IIb with 0.15 M-buffer. The chromatography of fraction T-III gave a mixture of peptides T-IIIc and T-IIId, a mixture of peptides T-IIIa and T-IIId, and peptide T-IIb with the starting, 0.03 M and 0.20 M buffers respectively.

Two peptides, T-IIIa and T-IIId, were separated from each other on a column (1.5 cm × 52 cm) of Sephadex G-25 (Fine grade) in 0.05 M-NH₄HCO₃, pH 8.2, at a flow rate of 18 ml/h. Peptides, T-IIIa and T-IIId were eluted at 75 ml and 85 ml respectively. Peptides T-IIIc and T-IIId were separated from each other by paper electrophoresis in 0.1 M-pyridine/acetic acid buffer, pH 4.8, at 40 V/cm for 90 min with no. 50 filter paper (Toyo Roshi Co., Tokyo, Japan). After electrophoresis, the paper was dried, and the spots carrying the peptides were cut out with the aid of guide strips and extracted with 0.1 M-pyridine.

All the peptides isolated were pure on the basis of paper electrophoresis at pH 4.8. The amino acid

compositions, the yields and *N*-terminal amino acids as determined by Edman degradation of purified tryptic peptides are given in Table 1.

Each peptide (0.2–0.5 μmol) was subjected to manual Edman degradation. The results are summarized in Fig. 2, together with the results of direct Edman degradation of reduced and *S*-carboxymethylated toxin Aa b.

The sequence of peptide T-IIIa agrees with the *N*-terminal sequence 1–5 of reduced and *S*-carboxymethylated toxin Aa b. Peptide T-IIa was further cleaved with thermolysin. Two peptides, T-IIaThi and T-IIaThii, were eluted at 114 ml and 177 ml respectively by gel filtration on a Sephadex G-25 (Fine grade) column (1.6 cm × 118 cm) in 0.02 M-ammonium acetate, pH 8.2. The carboxypeptidase Y digestion of peptide T-IIa gave the C-terminal sequence:

-Val-CmCys-Phe-Thr-Arg

The peptic digestion of peptide T-IIb afforded peptides, T-IIbPii and T-IIbPi, which were eluted from a Sephadex G-25 column (1.6 cm × 118 cm) in 0.05 M-NH₄HCO₃, pH 8.2, at 122 ml and 157 ml respectively. No cleavage was observed between Phe-65 and Pro-66. The carboxypeptidase Y diges-

Fig. 2. Amino acid sequence of *Acanthophis antarcticus* b

The prefixes T, S, C, P and Th refer to peptides produced by digestion with trypsin, staphylococcal proteinase, chymotrypsin, pepsin and thermolysin respectively. Arrows to the right indicate amino acids detected by Edman degradation. Arrows to the left indicate amino acids detected by carboxypeptidase Y digestion. The isolation of the chymotryptic peptides, S-IIICa, b and c, from peptide S-III is not described in the text. Key to the one-letter notation for amino acids; A, alanine; C, half-cystine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

tion of peptide T-IIb released arginine and proline in a molar ratio of 1:0.84.

Ten cycles of Edman degradation on peptide T-Ib gave the sequence:

CmCys-Asn-Pro-Phe-Pro-Val-Arg-Pro-Arg-Arg-

leaving only two proline residues, which were reasonably assigned to the C-terminal sequence of reduced and S-carboxymethylated toxin Aa b from its resistance to carboxypeptidase Y digestion and non-lysine or -arginine terminus. The detection of a peptide T-IIIe also supports the finding. These sequences established the alignment of peptides T-IIb and T-IIIe at the C-terminus.

Alignment of fragment peptides

The alignment of tryptic peptides was deduced as shown in Fig. 2 from the analysis of fragments of reduced and S-carboxymethylated toxin Aa b produced by digestion with staphylococcal proteinase. The fragments produced by staphylococcal proteinase digestion were applied to a column (1.2 cm × 21 cm) of DE52-cellulose in 0.01 M-NH₄HCO₃, pH 8.2. By increasing the buffer concentration, peptides S-I, S-II, S-III and S-IV were

eluted with the starting, 0.15 M, 0.20 M and 0.25 M buffers respectively.

Chymotryptic digestion of peptide S-IV gave peptides S-IVCd (CmCys-30-Glu-38), a mixture of two peptides, S-IVCa and S-IVCc, and S-IVCb (Thr-22-Trp-25), which were eluted from a Sephadex G-25 column (1.6 cm × 118 cm) with 0.02 M-ammonium acetate, pH 8.2, at 130 ml, 172 ml and 209 ml respectively. Peptides S-IVCa (Asn-18-Phe-21) and S-IVCc (CmCys-26-Phe-29) were eluted separately on a DE52-cellulose column (1.2 cm × 15 cm) with 0.10 M- and 0.20 M-NH₄HCO₃ buffers, pH 8.2, respectively.

The resulting peptides gave single spots on paper electrophoresis at pH 4.8. The C-terminal sequence of peptide S-IV was determined to be:

-Lys-Val-Val-Glu

by digestion with carboxypeptidase Y. These results establish the alignment of T-IIb, T-IIIc and T-Ia.

Peptide S-I, which was rich in arginine (three residues) and proline (five residues), was subjected to Edman degradation (Fig. 2). Tryptic peptides T-IIIb and T-IIb can be aligned from the results of ten cycles of the degradation. The amino acid analysis

Fig. 3. Comparison of the primary structures of *Acanthophis antarcticus* b and three other long-chain neurotoxins. The positions of the invariant amino acids are 'boxed' and (----) indicates a deletion. The numbers of residues are shown by homology with toxin Aa b. (1) *Acanthophis antarcticus* b (toxin Aa b, present paper); (2) *Notechis scutatus* scutatus III-4 (Halpert *et al.*, 1979); (3) *Astrotia stokesii* c (Maeda & Tamiya, 1978); (4) *Naja melanoleuca* b (Botes, 1972).

of peptide S-I and of the remaining fragment of peptide S-I after ten cycles of the degradation also confirmed the C-terminal alignment.

Discussion

A neurotoxin, Aa b, was isolated and sequenced from the venom of the Australian snake *Acanth-*

Fig. 4. Structure of *Acanthophis antarcticus* b

The picture is drawn schematically by following the three-dimensional structure of *Naja naja siamensis* 3 (Walkinshaw *et al.*, 1980). The one-letter notation for amino acids is explained in Fig. 2. Heavy-lined circles show the invariant residues in the long-chain neurotoxins with known sequences, and double-lined circles indicate the amino acid residues discussed in the present paper.

ophis antarcticus. The complete amino acid sequence of toxin Aa b is shown in Fig. 2. Toxin Aa b belongs to the long-chain type, consisting of 73 amino acid residues with ten half-cystine residues among them.

In Fig. 3, the primary structure of toxin Aa b is compared with those of three other long-chain neurotoxins, namely toxin *Notechis* III-4 (Halpert *et al.*, 1979), toxin *Astrotia stokesii* c (Maeda & Tamiya, 1978) and toxin *Naja melanoleuca* b (Botes, 1972) from the Australian elapid *Notechis scutatus scutatus*, sea-snake *Astrotia stokesii* and African elapid *Naja melanoleuca* respectively.

The amino acid sequence of toxin Aa b is homologous with other long-chain neurotoxins. The homology is the highest with the sequence of Australian-elapid toxin, *Notechis* III-4, accounting for 76% of the residues.

The three-dimensional structure of toxin *Naja naja siamensis* 3 (from Thailand cobra), one of the long-chain neurotoxins, has been elucidated by Walkinshaw *et al.* (1980). The structure of toxin Aa b is drawn schematically in Fig. 4, by following that described for *N. n. siamensis* 3.

Toxin Aa b is devoid of histidine and methionine

residues, as in the cases of three other long-chain neurotoxins: *Dendroaspis polylepis* polylepis V_N1 (from black mamba) (Strydom, 1972), V_N2 (Strydom & Haylett, 1977) and *Ophiophagus hannah* a (from king cobra) (Joubert, 1973). The valine residue at the *N*-terminus is novel for all the homologous venom components, including neurotoxins, cardiotoxins, cytotoxins, proteinase inhibitors and others.

The three so far absolutely invariant residues for the neurotoxins, namely Trp-25, Arg-33 and Gly-34 are conserved in toxin Aa b. An aromatic residue at position-29 (Phe), which corresponds to Phe-32 of erabutoxin b according to the stereochemical structures of *N. n. siamensis* 3 (Walkinshaw *et al.*, 1980) and erabutoxin b (Low *et al.*, 1976), and a hydrophobic residue (Ile, Leu or Val) at position-54 (Val) are also conserved. Two residues, Asp-27 and Lys(or Arg)-49, which are found in all the fully active neurotoxins, are also present. Toxin Aa b has, however, an arginine residue at position-23, which is usually occupied by a lysine residue in neurotoxins. The replacement preserves the positive charge and can be introduced by a single base replacement in the codon. This has not, however, been observed previously.

The replacement of the usual Tyr-21 residue by phenylalanine is the third example among neurotoxins that follow the pattern for the sea-snake toxins *Astrotia stokesii* b and c (Maeda & Tamiya, 1978).

The X-ray structures (Low *et al.*, 1976; Tsernoglou & Petsko, 1976, 1977) and an n.m.r. study (Inagaki *et al.*, 1978) suggested that the usual Tyr-21 residue was hydrogen-bonded with glutamic acid-38. In *A. stokesii* b and c, the replacement of residue-21 by phenylalanine is accompanied by the replacement of the glutamic acid residue by isoleucine and valine respectively (Maeda & Tamiya, 1978). For toxin Aa b, however, in spite of the replacement at position-21 by phenylalanine, a glutamic acid residue at position-38 is conserved. It is noteworthy that toxin Aa b has full activity as a neurotoxin, irrespective of these replacements.

Toxin Aa b has a long C-terminal 'tail'. Eleven residues after the last half-cystine-62 was found previously only with Notechis III-4. In toxin Aa b, the third histidine residue from the C-terminus is replaced by an arginine residue; otherwise the sequence of the 'tail' is the same as that of Notechis III-4. These eleven-membered 'tails' have five proline residues, whereas shorter 'tails' of other long-chain neurotoxins have three or less. Maeda & Tamiya (1978) proposed that the C-terminal carboxy groups of long-chain neurotoxins were salt-linked to the amino groups of lysine-35, which were nearly invariant among the long-chain neurotoxins. The high proline content of the long 'tail' may twist the 'tail' to give approximately the same total length. The salt-linkage was not observed in the X-ray structure of *N. n. siamensis* 3 (Walkinshaw *et al.*, 1980), probably because the crystal was grown at pH 2.83.

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References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Botes, D. P. (1972) *J. Biol. Chem.* **247**, 2866–2871
- Cogger, H. G. (1979) *Reptiles and Amphibians of Australia* (rev. edn.), pp. 372–373, A. H. and A. W. Reed, Sydney
- Crestfield, A., Moore, S. & Stein, W. (1963) *J. Biol. Chem.* **238**, 622–627
- Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
- Erlanger, B. F., Cooper, A. G. & Cohen, W. (1966) *Biochemistry* **5**, 190–196
- Halpert, J., Fohlman, J. & Eaker, D. (1979) *Biochimie* **61**, 719–723
- Inagaki, F., Miyazawa, T., Hori, H. & Tamiya, N. (1978) *Eur. J. Biochem.* **89**, 433–442
- Inagami, T. (1973) *Anal. Biochem.* **52**, 318–321
- Iwanaga, S., Wallén, P., Gröndahl, N. J., Henshen, A. & Blombäck, B. (1969) *Eur. J. Biochem.* **8**, 189–199
- Joubert, F. J. (1973) *Biochim. Biophys. Acta* **317**, 85–98
- Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D. & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2991–2994
- Maeda, N. & Tamiya, N. (1978) *Biochem. J.* **175**, 507–517
- Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **68**, 47–53
- Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
- Sheumack, D. D., Howden, M. E. H. & Spence, I. (1979) *Toxicon* **17**, 609–616
- Strydom, D. J. (1972) *J. Biol. Chem.* **247**, 4029–4042
- Strydom, D. J. & Haylett, T. (1977) *S. Afr. J. Chem.* **30**, 40–48
- Tamiya, N. & Arai, H. (1966) *Biochem. J.* **99**, 624–630
- Tsernoglou, D. & Petsko, G. A. (1977) *FEBS Lett.* **68**, 1–4
- Tsernoglou, D. & Petsko, G. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 971–974
- Walkinshaw, M. D., Saenger, W. & Maelicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2400–2404
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412