Biochem. J. (1981) **199**, 211–218
Printed in Great Britain

The amino acid sequence and position of the free thiol group of a short-chain neurotoxin from common-death-adder (*Acanthophis antarcticus*) venom

Hyung Suk KIM and Nobuo TAMIYA
Department of Chemistry, Tohoku University, Aobayama, Sendai, Japan

(*Received 21 April 1981/Accepted 5 June 1981*)

The amino acid sequence of a short-chain neurotoxin Acanthophis antarcticus c (toxin Aa c) from the venom of an Australian elapid snake, the common death adder (*Acanthophis antarcticus*, subfamily Acanthophiinae) was elucidated. Toxin Aa c is composed of 62 amino acid residues, including eight half-cystine residues and a cysteine residue. The amino acid sequence of toxin Aa c is homologous with those of other short-chain neurotoxins found in snakes of the family Elapidae, especially with those from snakes of the subfamily Hydrophiinae. The single cysteine residue was located in position 4. Toxin Aa c has a lethal dose (LD_{50}) of 0.08 μg/g body weight of mouse on intramuscular injection.

The venom of an Australian elapid snake, *Acanthophis antarcticus* (the common death adder; family Elapidae, subfamily Acanthophiinae) contains several postsynaptically acting neurotoxins. The amino acid sequence of one of them, a long-chain neurotoxin, Acanthophis antarcticus b (toxin Aa b), was elucidated (Kim & Tamiya, 1981).

The present paper reports the amino acid sequence of a short-chain neurotoxin, Aa c, from *Acanthophis antarcticus* venom. This is the first short-chain neurotoxin ever sequenced from the venoms of Australian elapid snakes (subfamily Acanthophiinae of Dowling & Duellman, 1978). The single cysteine residue of toxin Aa c was located at the fourth position from the *N*-terminus.

Materials and methods

The sources of the snake venom, proteinases and chemicals were the same as described previously (Kim & Tamiya, 1981). The methods of amino acid analysis, disc-gel or paper electrophoresis, lethal-activity measurement, reduction and *S*-carboxymethylation of the preparations, enzymic digestion and separation of the digestion fragments and Edman degradation were also the same as described previously (Kim & Tamiya, 1981).

Abbreviations used: toxin Aa c, neurotoxin Acanthophis antarcticus c; NbS_2, 5,5'-dithiobis(2-nitrobenzoic acid); Py-Et-Cys, *S*-β-(4-pyridylethyl)-L-cysteine; Py-Et-Aa cI(etc.), pyridylethylated component Aa cI (etc.); h.p.l.c., high-pressure liquid chromatography; CmCys (in sequences), carboxymethylcysteine.

Determination of the free thiol group

The free thiol content of the native toxin was determined as described by Ellman (1959) and Kortt & Liu (1973) by treatment with NbS_2 (Wako Pure Chemical Industries, Osaka, Japan). The toxin (60 nmol) was dissolved in 2 ml of 0.05 M-Tris/HCl buffer, pH 8.0, containing 5 or 8 M-urea, and 0.01 M-NbS_2 in 30 μl of 0.05 M-phosphate buffer, pH 7.0 was added to the solution. The release of 2-nitro-5-mercaptobenzoic acid was monitored by ΔA_{412} at 25°C for 140 min, assuming a molar absorption coefficient of 13600 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} (Ellman, 1959).

H.p.l.c.

The phenylthiohydantoin derivatives of amino acids were identified and determined by using a Hitachi HLC-633A high-speed liquid chromatograph equipped with a column of LiChrosorb RP-18 (Merck, Darmstadt, Germany) together with t.l.c. The column was equilibrated and eluted with 0.01 M-sodium acetate buffer, pH 4.5, containing 45% (v/v) acetonitrile at a flow rate of 0.5 ml/min (Omichi *et al.* 1980).

Results

Purification of toxin Aa c

The preliminary separation of *Acanthophis antarcticus* venom components by ion-exchange chromatography was described previously (Kim & Tamiya, 1981).

The components (58.2 A_{280} units) in fraction X were desalted and rechromatographed on a column

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(2.2 cm × 27 cm) of CM-cellulose CM52 by using a linear concentration gradient of 0–0.5 M-NaCl in 0.01 M-phosphate buffer, pH 5.9, in a total volume of 2.0 litres. The main component-containing part of the eluate (1200–1300 ml) was concentrated to about 10 ml by ultrafiltration in a Diaflo apparatus with a UM-2 membrane. The component was desalted by gel filtration on a column (1.8 cm × 123 cm) of Sephadex G-25 (Coarse grade) in 0.1 M-acetic acid, and freeze-dried (yield 48.2 mg).

The purified component gave a single band on polyacrylamide-disc-gel electrophoresis at pH 4.0. The component was named 'toxin Acanthophis antarcticus c' (Aa c).

Some properties of toxin Aa c

The lethal activity (LD_{50}) of toxin Aa c by intramuscular injection to mice was 0.08 μg/g body weight.

The molecular weight was estimated to be 7500 by sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis and 7000 by gel filtration through Sephadex G-50 (Fine grade) in 0.1 M-acetic acid. The amino acid composition of toxin Aa c is given in Table 1.

Toxin Aa c consists of 62 amino acid residues with eight half-cystine residues and a cysteine residue among them. Toxin Aa c is devoid of alanine and phenylalanine residues. Fig. 1 shows the results of the free-thiol-group determination of toxin Aa c.

The maximum value of 0.86 thiol group/mol was obtained in the presence of 8 M-urea; the value was smaller, however, in 5 M-urea.

N-Terminal amino acid sequence

The fifteen amino acid residues from the *N*-terminus of reduced and S-carboxymethylated toxin Aa c were determined by manual Edman degradation to be:

1 5
Met-Gln-CmCys-CmCys-Asn-Gln-Gln-Ser-Ser-

10 15
Gln-Pro-Lys-Thr-Thr-Thr-

Separation of tryptic peptides and determination of their amino acid sequences

The reduced and *S*-carboxymethylated toxin Aa c (3.0 μmol) was digested with trypsin in 3 ml of 0.05 M-Tris/HCl buffer, pH 8.2, at an enzyme/substrate ratio of 1:100 (w/w) for 16 h at 37°C. The digest was applied on a column (1.2 cm × 250 cm) of Sephadex G-25 (Fine grade) in 0.1 M-acetic acid and the eluate was monitored at A_{230} and A_{280}. Fractions T-I, T-II, T-III and T-IV were eluted at 165–213 ml, 216–267 ml, 270–297 ml and 321–366 ml respectively. Two peptides, which were retained on the Sephadex column, and eluted in fractions T-III and T-IV, were pure on the basis of paper electrophoresis at pH 4.8.

Fraction T-I gave five peptides, T-Ia–T-Ie, on

Table 1. *Amino acid composition of toxin Aa c*

Amino acid	Composition (mol/mol of toxin)	No. of residues
Trp*	1.01	1
Lys	5.12	5
His	0.92	1
Arg	4.95	5
Asp	4.97	5
Thr†	6.67	7
Ser	3.43	4
Glu	5.71	6
Pro	4.07	4
Gly	5.98	6
Ala	—	0
½Cys‡	7.95	9
Val	2.16	2
Met	1.00	1
Ile†	3.69	4
Leu	0.91	1
Tyr	1.16	1
Phe	—	0
Total		62
Free thiol group§		1
Mol.wt. (formula)		6898

* Measured by hydrolysis with 4 M-methanesulphonic acid.

† Values for 72 h hydrolysates are given. Threonine gives smaller values after shorter periods of hydrolysis, probably because its successive presence at positions 13–16 (see Fig. 2).

‡ Cystine residues tend to give smaller values. Eight half-cystine residues and one cysteine residue were found by sequence study.

§ Determined by the method of Ellman (1959).

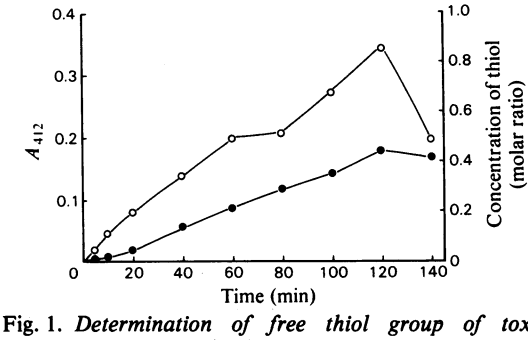

Fig. 1. *Determination of free thiol group of toxin Acanthophis antarcticus c*

To a solution of toxin (60 nmol) in 2.0 ml of 0.05 M-Tris/HCl buffer, pH 8.0, containing 5 M-urea (●) or 8 M-urea (○), 0.01 M-NbS_2 (30 μl) in 0.05 M-phosphate buffer, pH 7.0, was added. A_{412} was monitored at 25°C.

chromatography through a DEAE-cellulose DE52 column (1.2cm × 21cm) equilibrated with 0.01M-NH_4HCO_3, pH8.2. The elution was performed with 0.01M, 0.07M, 0.13M, 0.16M and 0.34M buffers for peptides T-Ia, T-Ib, T-Ic, T-Id and T-Ie respectively. The chromatography of fraction T-II on the same column gave T-IIa, T-IIb, T-IIc, T-IId and T-IIe with starting, 0.05M, 0.15M, 0.21M and 0.36M buffers respectively.

Two components, T-IIa1 and T-IIa2, were obtained from T-IIa by passing it through a column (1.2cm × 142cm) of Sephadex G-25 (Fine grade) in 0.05M-NH_4HCO_3, pH8.2, at the elution volumes of 126ml and 147ml respectively. The chromatography of T-IIb on the same column gave two peptides, T-IIb1 and T-IIb2, at 117ml and 144ml respectively.

The amino acid compositions and some properties of the tryptic peptides are given in Table 2. The amino acid analysis showed that peptides T-Ia and T-IIa1 were identical.

Each peptide (0.4–0.8 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 c.

Peptide T-Ib (0.6 μmol) was further cleaved with thermolysin in 1ml of 0.1M-ammonium acetate, pH7.8, at an enzyme/substrate ratio of 1:50 (w/w) at 37°C for 3h. The digest was freeze-dried and loaded on a column (1.6cm × 13cm) of DEAE-cellulose DE52 equilibrated with 0.01M-NH_4HCO_3, pH8.2. Two peptides, T-IbTh1 and T-IbTh2, were eluted with starting and 0.12M buffers respectively.

The amino acid analysis showed that peptides T-Ib, T-III and T-Ie were the products of incomplete digestion with trypsin.

Alignment of fragment peptides

The alignment of tryptic peptides was deduced as in Fig. 2 by the analysis of α-chymotryptic fragments of reduced and *S*-carboxymethylated toxin Aa c. Reduced and *S*-carboxymethylated toxin Aa c (0.5 μmol) was digested with α-chymotrypsin in 1ml of 0.1M-ammonium acetate, pH7.8, at an enzyme/substrate ratio of 1:100 (w/w) at 37°C for 40min. The digest was applied to a column (1.2cm × 19cm) of DEAE-cellulose DE52 in 0.01M-NH_4HCO_3, pH8.2. By increasing the buffer concentration, fractions C-I, C-II, C-III and C-IV were eluted with the starting, 0.24M, 0.33M and 0.40M buffers respectively.

Fraction C-I was separated into two peptides, C-Ia and C-Ib, which were eluted from a column (1.6cm × 75cm) of Sephadex G-25 (Fine grade) in 0.01M-NH_4HCO_3, pH8.2, at 75ml and 123ml respectively. The carboxypeptidase Y digestion of peptide C-Ia released only leucine in 1h, and leucine, arginine and isoleucine in a molar ratio of 1:0.63:0.38 in 3h. These results established the alignment of peptides T-Ia and T-Ie.

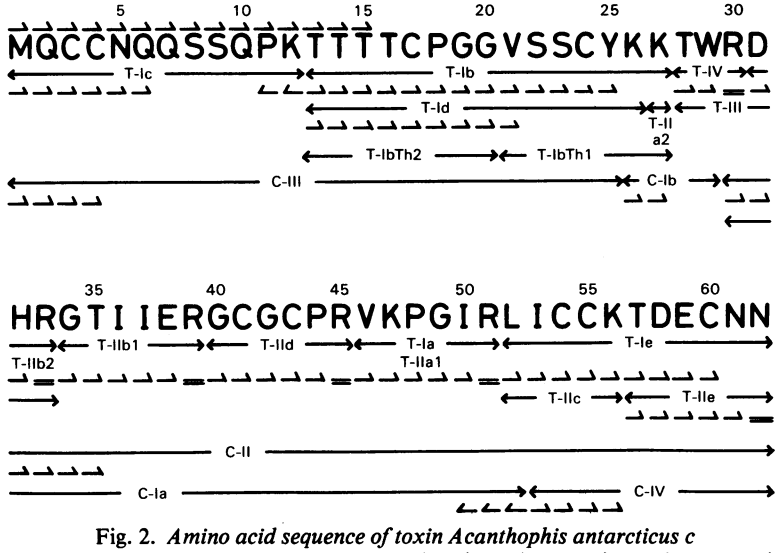

Fig. 2. *Amino acid sequence of toxin Acanthophis antarcticus c*

The prefixes T, C, and Th refer to peptides produced by digestion with trypsin, α-chymotrypsin and thermolysin respectively. Arrows to the right and left indicate amino acid residues detected by Edman degradation and carboxypeptidase Y digestion respectively. The double-underlined residues were confirmed by the amino acid analysis without hydrolysis after the removal of preceding residues by Edman degradation. The one-letter notation for amino acids is given in *Biochem. J.* (1969) **113**, 1–4

Table 2. *Tryptic peptides from reduced and S-carboxymethylated toxin A a c*

The composition (mol/mol of peptide) of each peptide is given, with the nearest integer (≡no. of residues) in parentheses.

Amino acid	Peptide ...	T-Ic	T-Ib	T-Id	T-IIa2	T-IV	T-IIb2	T-III	T-IIb1	T-IId	T-Ia (T-IIa1)	T-IIe	T-IIc	T-IIe
	Residues nos. ...	1–12	13–27	13–26	27	28–30	31–33	28–33	34–39	40–45	46–51	52–62	52–56	57–62
Lys		1.09 (1)	2.00 (2)	1.00 (1)	1.00 (1)						1.04 (1)	1.00 (1)	1.00 (1)	
His							0.90 (1)	1.01 (1)						
Arg						1.00 (1)	0.94 (1)	2.01 (2)	1.02 (1)	0.92 (1)	1.07 (1)			
CmCys		1.94 (2)	1.55 (2)	1.67 (2)						1.72 (2)		2.56 (3)	1.66 (2)	0.60 (1)
Asp		1.00 (1)					1.00 (1)	1.00 (1)				3.02 (3)		3.00 (3)
Thr			3.39 (4)	3.38 (4)		0.99 (1)		1.00 (1)	1.09 (1)			1.04 (1)		0.96 (1)
Ser		1.68 (2)	2.03 (2)	1.81 (2)										
Glu		3.96 (4)							1.00 (1)			0.88 (1)		0.97 (1)
Pro		0.91 (1)	0.85 (1)	0.96 (1)						0.99 (1)	1.08 (1)			
Gly			2.17 (2)	1.74 (2)					1.04 (1)	2.00 (2)	1.00 (1)			
Val			1.25 (1)	1.26 (1)							0.87 (1)			
Met		0.90 (1)												
Ile									1.58 (2)		0.92 (1)	0.78 (1)	0.62 (1)	
Leu												0.91 (1)	0.82 (1)	
Tyr			0.97 (1)	1.03 (1)										
Trp						(1)		(1)						
Total residues		12	14	14	1	3	3	6	6	6	6	11	5	6
Yield (%)		78.7	58.7	20.7	10.3	53.0	59.0	9.5	57.0	70.7	68.7	60.0	6.3	7.3
N-Terminus		Met	Thr	Thr	Lys	Thr	Asp	Thr	Gly	Gly	Val	Leu	Leu	Thr

Fraction C-II contained a single component peptide, C-II, as shown by paper electrophoresis at pH 4.8. Six cycles of Edman degradation of peptide C-II gave the sequence:

Arg-Asp-His-Arg-Gly-Thr-

which established the alignment of peptides T-IV, T-IIb2 and T-IIb1.

Location of the free thiol group

The toxin Aa c was *S*-pyridylethylated with 4-vinylpyridine [Wako; stabilized with 0.02% (w/v) *p*-t-butylcatechol] as described by Hermodson *et al.* (1973). The toxin (0.7 μmol) was dissolved in 2 ml of 0.13 M-Tris/HCl buffer, pH 7.6, containing 8 M-urea and 0.01% (w/v) EDTA, and 4-vinylpyridine (0.1 ml) was added to the solution. The mixture was kept under N_2 in a stoppered vessel at room temperature with gentle stirring for 2 h and applied to a column (1.2 cm × 55 cm) of Sephadex G-25 (Fine grade) in 0.1 M-acetic acid. The protein-containing part of the eluate was freeze-dried (yield 110%, w/w).

The preparation (50 nmol) was hydrolysed with 6 M-HCl (0.5 ml) in an evacuated and sealed glass tube at 105°C and subjected to automatic amino acid analysis. The Py-Et-Cys content of the preparation was determined by amino acid analysis through a column (0.8 cm × 10 cm) of JEOL JC-R2 resin at 70°C with 0.35 M-sodium citrate buffer, pH 5.22. Authentic Py-Et-Cys, synthesized as described by Cavins & Friedman (1970), gave the following elementary analysis. Found: C, 52.85; H, 6.32; N, 12.32; S, 14.13; theoretical values: C, 53.08; H, 6.24; N, 12.38; S, 14.17%.

The *S*-pyridylethylated toxin Aa c contained 0.59 mol of Py-Et-Cys and 6.47 mol of half-cystine per mol. The contents of other amino acids were the same as those of native toxin. These results suggest that the modification of the free thiol group was incomplete.

The preparation, i.e. a mixture of modified and unmodified toxin Aa c, was reduced and *S*-carboxymethylated as usual (Kim & Tamiya, 1981). The final preparation was applied to a column (1.6 cm × 12 cm) of DEAE-cellulose DE52 equilibrated with 0.01 M-Tris/HCl buffer, pH 8.0. The elution pattern is shown in Fig. 3.

The yields of components, Py-Et-Aa cI and Py-Et-Aa cII (Fig. 3) were 27.2 and 20.4% (w/w) respectively. The results of amino acid analysis of Py-Et-Aa cI and Py-Et-Aa cII (Table 3) show that one residue of Py-Et-Cys is contained only in Py-Et-Aa cI.

Edman degradation was performed on Py-Et-Aa cI (0.14 μmol) to locate the Py-Et-Cys residue. Py-Et-Aa cII (0.10 μmol) was also degraded as a control. The standard Py-Et-Cys phenylthiohydantoin derivative was prepared by using the coupling and conversion steps of the manual Edman method. The Py-Et-Cys phenylthiohydantoin derivative is water-soluble to a similar extent as the arginine and histidine derivatives and can be identified by the elution time (10.4 min) on h.p.l.c. and by R_F value (0.38) on t.l.c. in ethyl acetate.

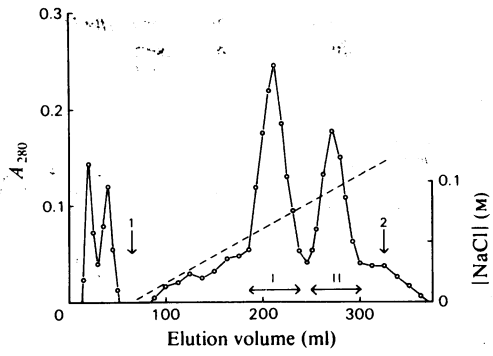

Fig. 3. *DEAE-cellulose DE52 column chromatography of S-pyridylethylated toxin Aa c after reduction and S-carboxymethylation*

Toxin Aa c treated with 4-vinylpyridine was reduced and *S*-carboxymethylated. The preparation (0.7 μmol) was dissolved in 0.01 M-Tris/HCl buffer (3 ml), pH 8.0, and applied to a column (1.6 cm × 21 cm) of DEAE-cellulose DE52 equilibrated with the same buffer. The elution was performed with a linear concentration gradient of NaCl (0–0.1 M) in the buffer (total 400 ml) (arrow 1), then with 0.5 M-NaCl in the buffer (arrow 2). The flow rate was 120 ml/h. Fractions I (Py-Et-Aa cI) and II (Py-Et-Aa cII) were collected separately. O, A_{280}; ----, [NaCl].

Table 3. *Amino acid compositions of reduced and S-carboxymethylated Py-Et-Aa cI and Py-Et-Aa cII*

Amino acid	Composition (mol/mol of peptide) Py-Et-Aa cI	Py-Et-Aa cII
Lys	5.16 (5)	5.03 (5)
His	1.08 (1)	1.09 (1)
Py-Et-Cys	0.93 (1)	0.18 (0)
Arg	4.62 (5)	4.92 (5)
CmCys	6.90 (8)	7.83 (9)
Asp	5.00 (5)	5.00 (5)
Thr	6.46 (7)	6.37 (7)
Ser	3.61 (4)	3.72 (4)
Glu	5.89 (6)	6.11 (6)
Pro	4.10 (4)	4.22 (4)
Gly	6.26 (6)	6.12 (6)
Val	2.48 (2)	2.21 (2)
Met	1.17 (1)	1.05 (1)
Ile	3.43 (4)	3.46 (4)
Leu	1.08 (1)	1.05 (1)
Tyr	1.25 (1)	1.17 (1)

Fig. 4. *Amino acid sequences of the short-chain neurotoxins of snakes belonging to the family Elapidae* 1–12, Hydrophiinae; 13, the present paper (Acanthophiinae); 14–19, Laticaudinae; 20–22, Elapinae (mambas); 23–38, Elapinae (cobras). The definition of the one-letter symbols for amino acids is given in Biochem. J. (1969) **113**, 1–4.

					5					10					15					20					25					30			
1. *Astrotia stokesii* a	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	G	N	S	C	Y	K	K	T	W	S	D	H	R
2. *Hydrophis ornatus* '73 a	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	G	N	S	C	Y	K	K	T	W	S	D	H	R
3. *Hydrophis cyanocinctus* hydrophitoxin a	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
4. b	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
5. *Pelamis platurus* pelamitoxin a	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
6. *Enhydrina schistosa* 5	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
7. 4	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
8. *Lapemis hardwickii* neurotoxin	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	S	D	H	R
9. *Hydrophis ornatus* '75 a	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C			A	E	S	S	C	Y	K	K	T	W	R	D	F	R
10. *Aipysurus laevis* a	L	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	D	C			A	D	N	S	C	Y	K	K	T	W	Q	D	H	R
11. c	L	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	D	C			A	D	N	S	C	Y	K	K	T	W	K	D	H	R
12. b	L	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	D	C			A	D	N	S	C	Y	K	M	T	W	R	D	H	R
13. *Acanthophis antarcticus* c	M	Q	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	T	C	P	G	G	V	S	S	C	Y	K	K	T	W	R	D	H	R
14. *Laticauda semifasciata* erabutoxin c	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	E	S	S	C	Y	H	K	Q	W	S	D	F	R
15. b	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	E	S	S	C	Y	H	K	Q	W	S	D	F	R
16. a	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	E	S	S	C	Y	N	K	Q	W	S	D	F	R
17. *Laticauda colubrina* II	R	R	C	Y	N	Q	Q	S	S	Q	P	K	T	T	K	S	C	P	P	G	E	N	S	C	Y	N	K	Q	W	R	D	H	R
18. *Lacticauda laticaudata* laticotoxin a	R	R	C	F	N	H	P	S	S	Q	P	Q	T	N	K	S	C	P	P	G	E	N	S	C	Y	N	K	Q	W	R	D	H	R
19. a′	R	R	C	F	N	H	P	S	S	Q	P	Q	T	N	K	S	C	P	P	G	E	N	S	C	Y	N	K	Q	W	R	D	H	R
20. *Dendroaspis jamesoni kaimosae* toxin Vn[1] 1	R	I	C	Y	N	H	Q	S	T	T	P	A	T	T	K	S	C			G	E	N	S	C	Y	K	K	T	W	S	D	H	R
21. *Dendroaspis viridis* toxin 4.11.3	R	I	C	Y	N	H	Q	S	T	T	P	A	T	T	K	S	C			G	E	N	S	C	Y	K	K	T	W	S	D	H	R
22. *Dendroaspis polylepis* toxin α	R	I	C	Y	N	H	Q	S	T	T	R	A	T	T	K	S	C			E	E	N	S	C	Y	K	K	Y	W	R	D	H	R
23. *Naja haje annulifera* CM-10	M	I	C	Y	K	Q	Q	S	L	Q	F	P	I	T	T	V	C	P		G	E	K	N	C	Y	K	K	Q	W	S	G	H	R
24. CM-12	M	I	C	Y	K	Q	R	S	L	Q	F	P	I	T	T	V	C	P		G	E	K	N	C	Y	K	K	Q	W	S	G	H	R
25. CM-14	M	I	C	H	N	Q	Q	S	S	Q	P	P	T	I	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D	H	R
26. *Naja haje haje* CM-10a	M	I	C	H	N	Q	Q	S	S	Q	P	P	T	I	K	T	C	P		G	E	T	N	C	Y	K	K	Q	W	R	D	H	R
27. *Naja nivea* toxin *β*	M	I	C	H	N	Q	Q	S	S	Q	R	P	T	I	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D	H	R
28. *δ*	L	Q	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D	H	R
29. *Naja haje* toxin α	L	Q	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D	H	R
30. *Naja melanoleuca* toxin d	M	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	Q	W	S	D	H	R
31. *Naja naja oxiana* toxin II or toxin α	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	S		G	E	T	N	C	Y	K	K	W	W	S	D	H	R
32. *Naja haje haje* CM-6	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D	H	R
33. *Naja nigricollis* toxin α	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	V	W	R	D	H	R
34. *Hemachatus haemachatus* toxin II	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	S	C	P		G	D	T	N	C	Y	N	K	R	W	R	D	H	R
35. IV	L	E	C	H	N	Q	Q	S	S	Q	T	P	T	T	Q	T	C	P		G	E	T	N	C	Y	K	K	Q	W	S	D	H	R
36. *Naja naja atra* cobrotoxin	L	E	C	H	N	Q	Q	S	S	Q	T	P	T	T	T	G	C	S	G	G	E	T	N	C′	Y	K	K	R	W	R	D	H	R
37. *Naja mossambica mossambica* neurotoxin I	L	E	C	H	N	Q	Q	S	S	E	P	P	T	T	T	R	C	S	G	G	E	T	N	C	Y	K	K	R	W	R	D	H	R
38. III	L	N	C	H	N	Q	M	S	A	Q	P	P	T	T	T	R	C	S	R	W	E	T	N	C	Y	K	K	R	W	R	D	H	R

		35					40					45					50					55					60			No. of residues: Different from those of Aa c	Total	
1.	G	T	I	I	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	1. Maeda & Tamiya, 1978
2.	G	T	I	I	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	2. N. Maeda & N. Tamiya, unpublished work
3.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	K	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	3. Liu & Blackwell, 1974
4.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	4. Liu & Blackwell, 1974
5.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	5. Wang *et al.*, 1976
6.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N	14	60	6. Fryklund *et al.*, 1972
7.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	H	T	N	E	C	N	N	13	60	7. Fryklund *et al.*, 1972
8.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	H	T	N	E	C	N	N	13	60	8. Fox *et al.*, 1977
9.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	H	T	N	E	C	N	N	13	60	9. N. Maeda & N. Tamiya, unpublished work
10.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	K	T	N	E	C	N	N	14	60	10. Maeda & Tamiya, 1976
11.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	K	T	N	E	C	N	N	14	60	11. Maeda & Tamiya, 1976
12.	G	T	R	I	E	R	G	C	G	C	P	Q	V	K	P	G	I	K	L	E	C	C	K	T	N	E	C	N	N	14	60	12. Maeda & Tamiya, 1976
13.	G	T	I	I	E	R	G	C	G	C	P	R	V	K	P	G	I	R	L	I	C	C	K	T	D	E	C	N	N	0	62	13. The present paper
14.	G	T	I	I	E	R	G	C	G	C	P	T	V	K	P	G	I	N	L	S	C	C	E	S	E	V	C	N	N	19	62	14. Tamiya & Abe, 1972
15.	G	T	I	I	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	S	C	C	E	S	E	V	C	N	N	19	62	15. Sato & Tamiya, 1971
16.	G	T	I	I	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	S	C	C	E	S	E	V	C	N	N	19	62	16. Sato & Tamiya, 1971
17.	G	S	I	T	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	R	C	C	E	S	E	D	C	N	N	19	62	17. A. Sato & N. Tamiya, unpublished work
18.	G	T	I	T	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	T	C	C	Q	S	E	D	C	N	N	22	62	18. A. Sato & N. Tamiya, unpublished work
19.	G	T	I	T	E	R	G	C	G	C	P	Q	V	K	S	G	I	K	L	T	C	C	Q	S	D	D	C	N	N	22	62	19. A. Sato & N. Tamiya, unpublished work
20.	G	T	I	I	E	R	G	C	G	C	P	K	V	K	Q	G	I	H	L	H	C	C	Q	S	D	K	C	N	N	21	60	20. Strydom, 1973
21.	G	T	I	I	E	R	G	C	G	C	P	K	V	K	R	G	V	H	L	H	C	C	Q	S	D	K	C	N	N	22	60	21. Banks *et al.*, 1974
22.	G	T	I	I	E	R	G	C	G	C	P	K	V	K	P	G	V	G	I	H	C	C	Q	S	D	K	C	N	Y	25	60	22. Strydom, 1972
23.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	R	23	61	23. Joubert, 1975
24.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	R	24	61	24. Joubert, 1975
25.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	V	G	I	Y	C	C	K	T	N	K	C	N	R	19	61	25. Joubert, 1975
26.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	V	G	I	Y	C	C	K	T	D	K	C	N	R	18	61	26. Joubert & Taljaard, 1978
27.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	V	G	I	Y	C	C	K	T	D	K	C	N	R	19	61	27. Botes, 1971
28.	G	S	I	T	E	R	G	C	G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	N	18	61	28. Botes *et al.*, 1971
29.	G	S	I	T	E	R	G	C	G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	N	18	61	29. Botes & Strydom, 1969
30.	G	T	I	I	E	R	G	C	G	C	P	S	V	K	K	G	V	K	I	N	C	C	T	T	D	R	C	N	N	18	61	30. Botes, 1972
31.	G	T	I	I	E	R	G	C	G	C	P	K	V	K	P	G	V	N	L	N	C	C	R	T	D	R	C	N	N	18	61	31. Grishin *et al.*, 1973, Arnberg *et al.*, 1974
32.	G	S	I	T	E	R	G	C	G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	N	19	61	32. Joubert & Taljaard, 1978
33.	G	T	I	I	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	N	C	C	T	T	D	K	C	N	N	15	61	33. Eaker & Porath, 1967
34.	G	T	I	I	E	R	G	C	G	C	P	T	V	K	P	G	I	N	L	K	C	C	T	T	D	R	C	N	N	17	61	34. Strydom & Botes, 1971
35.	G	S	R	T	E	R	G	C	G	C	P	T	V	K	P	G	I	K	L	K	C	C	T	T	D	R	C	N	K	21	61	35. Strydom & Botes, 1971
36.	G	Y	R	T	E	R	G	C	G	C	P	S	V	K	N	G	I	E	I	N	C	C	T	T	D	R	C	N	N	21	62	36. Yang *et al.*, 1969
37.	G	Y	R	T	E	R	G	C	G	C	P	T	V	K	K	G	I	E	L	N	C	C	T	T	D	R	C	N	N	20	62	37. Gregoire & Rochat, 1977
38.	G	Y	K	T	E	R	G	C	G	C	P	T	V	K	K	G	I	Q	L	H	C	C	T	S	D	N	C	N	N	24	62	38. Gregoire & Rochat, 1977

The phenylthiohydantoin derivatives of methionine, glutamine, carboxymethylcysteine and Py-Et-Cys were obtained successively on four cycles of Edman degradation of Py-Et-Aa cI, giving yields of 94, 92, 58 and 48% (mol/mol) respectively, whereas those of methionine, glutamine, carboxymethylcysteine and carboxymethylcysteine were obtained from Py-Et-Aa cII, giving yields of 77, 89, 48 and 45% respectively.

These results indicate that the cysteine residue is present at position 4 in the Aa c molecule.

Discussion

A fully active neurotoxin Aa c was isolated and sequenced from the venom of an Australian elapid snake *Acanthophis antarcticus*. Toxin Aa c belongs to the short-chain type, consisting of 62 amino acid residues with eight half-cystine residues and one cysteine residue. This is the first short-chain neurotoxin ever characterized from the Australian elapid snake (subfamily Acanthophiinae). It is noteworthy that the presence of a cysteine residue has so far been known only among true-sea-snake (subfamily Hydrophiinae) short-chain neurotoxins.

Fig. 4 shows the amino acid sequences of 38 short-chain neurotoxins of family Elapidae.

The amino acid sequence of toxin Aa c is homologous with those of other short-chain neurotoxins of snakes belonging to the family Elapidae, especially to those of subfamily Hydrophiinae. The amino-acid-differences of the sequences of Fig. 4 from the sequence of toxin Aa c show the close relationship of toxin Aa c to Hydrophiinae neurotoxins.

The toxins can be classified into four groups, namely into those of: (1) Hydrophiinae and Acanthophiinae (Aa c) (1–13, Fig. 4); (2) Laticaudinae (14–19, Fig. 4); (3) Elapinae (mambas) (20–22, Fig. 4); and (4) Elapinae (cobras) (23–38, Fig. 4) (Tamiya & Maeda, 1978). More examples of Acanthophiinae neurotoxins are needed to confirm their relationships.

Fryklund *et al.* (1972), who described for the first time Hydrophiinae neurotoxins (toxins nos. 6 and 7, Fig. 4) with a cysteine residue tentatively located it at the third position. The present results on toxin Aa c, however, show that the cysteine residue is at the fourth position. In the present study, the treatment of the toxin with 4-vinylpyridine was performed under mild conditions. The partially modified preparation thus prepared was separated into modified and unmodified toxins after the reduction and carboxymethylation. The Py-Et-Cys was detected only at the fourth position of the modified toxin derivative.

According to the stereochemical structure of erabutoxin b, one of the short-chain neutotoxins, put forward by Low *et al.* (1976), Kimball *et al.* (1979) and Tsernoglou & Petsko (1976, 1977), phenylalanine-4 next to half-cystine-3 is 'buried' in the molecule in close contact with valine-59. The close contact of phenylalanine-4 and valine-59 was also shown by nuclear Overhauser effects between protons of these residues (Inagaki *et al.* 1980). The homology suggests that cysteine-4 of Aa c is also 'buried'. It is noteworthy that valine-59 of erabutoxin b is replaced by glutamic acid residues in all the neurotoxins with a cysteine residue at position 4.

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