Biochem. J. (1974) **141**, 389–400
Printed in Great Britain

The Primary Structure of the Toxin Laticauda semifasciata III, a Weak and Reversibly Acting Neurotoxin from the Venom of a Sea Snake, *Laticauda semifasciata*

By NOBUYO MAEDA and NOBUO TAMIYA
Department of Chemistry, Tohoku University, Sendai, Japan

(*Received* 21 *December* 1973)

A weak and reversibly acting neurotoxic protein of *Laticauda semifasciata* venom, Laticauda semifasciata III (component LsIII), was sequenced. Component LsIII consists of 66 amino acid residues and has five disulphide bridges, one of which was located between residues 26 and 30. The weak and reversible neurotoxicity of component LsIII is discussed in relation to its structure, which falls between those of the neuro- and cardiotoxins of sea snakes and Elapidae snakes isolated and sequenced so far.

Erabutoxins a, b and c are the neurotoxic proteins from the venom of a sea snake, *Laticauda semifasciata*. Each of them consists of 62 amino acid residues and their amino acid sequences have been elucidated (Tamiya & Arai, 1966; Sato & Tamiya, 1971; Endo *et al.*, 1971; Tamiya & Abe, 1972). The venom also contains a weak and easily reversible neurotoxic component named Laticauda semifasciata III (component LsIII), the isolation and properties of which have been reported by Maeda *et al.* (1974). It is noteworthy that the neurotoxicity of component LsIII is about one-eighth that of erabutoxins and that component LsIII has no cardiotoxicity. The amino acid composition of component LsIII is quite different from those of erabutoxins.

All the neurotoxins for which amino acid sequences have been determined, are classified into two groups, namely the short-chain toxins, containing 60–62 amino acid residues with four disulphide bridges and the long-chain toxins, containing 71–74 amino acid residues with five disulphide bridges (Strydom, 1973). Component LsIII is a new type of venom protein consisting of 66 amino acid residues with five disulphide bridges.

The present paper describes the amino acid sequence and the position of the additional disulphide bridge of component LsIII.

Experimental

Laticauda semifasciata III

Component LsIII was prepared as described by Maeda *et al.* (1974).

Proteinases

Trypsin (twice crystallized, containing 50% of $MgCl_2$), pepsin (twice crystallized), and α-chymotrypsin (three times crystallized) were the products of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxypeptidase A (treated with di-isopropyl phosphofluoridate; from bovine pancreas) and leucine aminopeptidase (from hog kidney) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pronase E was from Kaken Kagaku, Tokyo, Japan.

Reagents

Phenyl isothiocyanate, heptafluorobutyric acid and 1-chlorobutane for the sequence analyser were the 'sequenal' grade of Wako Pure Chemical Industries Ltd., Osaka, Japan. Dimethylallylamine was synthesized from allyl chloride and dimethylamide (Ahlroth, 1965).

Monoiodoacetic acid was recrystallized from chloroform; 2-mercaptoethanol was redistilled under reduced pressure before use; benzene and ethyl acetate were redistilled and other solvents and reagents were used without further purification.

Preparation of reduced and S-carboxymethylated component LsIII

The reduction and *S*-carboxymethylation of component LsIII were performed as described by Crestfield *et al.* (1963). Urea and excess of reagents were removed by gel filtration on a column (1.8cm×65cm) of Sephadex G-25 (coarse grade) in 0.1M-pyridine. The reduced and *S*-carboxymethylated component LsIII eluted at the void volume was freeze-dried. From 106mg of component LsIII about 115mg of reduced and *S*-carboxymethylated component LsIII was obtained.

Citraconylation and tryptic digestion of reduced and S-carboxymethylated component LsIII

Citraconylation was carried out as described by Gibbons & Perham (1970), with slight modifications. To reduced and *S*-carboxymethylated component LsIII (102mg, 14.2μmol) in 2.0% (w/v) $NaHCO_3$ (4.0ml) was added dropwise 1.0M-methylmaleic anhydride in dioxan (1.0ml). The pH of the reaction mixture was kept at 8–9 with 1M-NaOH under continuous stirring at 4°C. After 2h at 25°C, trypsin solution (1mg of enzyme in 0.05M-Tris–HCl buffer, pH8.2; 0.1ml) was added to the mixture, which was left at 37°C for 16h.

The digestion was stopped by adjusting the pH to 2.2 with 1M-HCl and the mixture incubated at 25°C for 24h to remove the citraconyl groups. The peptides were separated by Sephadex G-50 (fine grade) column (1.6cm × 74cm) chromatography.

Further enzymic digestion of peptides

Digestion with trypsin and α-chymotrypsin was performed in 0.1M-NH_4HCO_3 buffer, pH7.8, and with pepsin in 0.01M-HCl, for 16h at 37°C with a substrate concentration of about 5mg/ml. The enzyme/substrate ratio was 1:50–100 (w/w).

Paper electrophoresis

The paper electrophoresis was carried out on no. 50 filter paper (Toyo Roshi Co., Tokyo, Japan) with pyridine–acetic acid–water (8:5.5:986.5, by vol., for pH4.8; 25:1:225, by vol., for pH6.5). A voltage gradient of 40V/cm was applied for 90min. Peptides were located on the paper by spraying with 0.2% ninhydrin in acetone, Ehrlich reagent (Smith, 1953), Pauli reagent (Mann & Leone, 1953) and Sakaguchi reagent (Jepson & Smith, 1953).

For preparative electrophoresis, the paper was dried in air and the parts carrying peptides were cut out by the aid of guide strips, which were sprayed with ninhydrin. The peptides were eluted from the paper with 0.2M-pyridine or with 0.2M-acetic acid.

Amino acid analysis

The peptides were hydrolysed with 6M-HCl at 105°C for 20h in evacuated sealed glass tubes. The hydrolysates were analysed with an automatic amino acid analyser (type JLC-5AH, Japan Electron Optics Ltd., Tokyo, Japan).

Amino acid sequence analysis

Edman degradation. The amino acid sequences of small peptides were determined by the direct phenylthiohydantoin method of Iwanaga *et al.* (1969). The concentration of phenylthiohydantoin-amino acid in the organic phase was estimated from the absorbance at 269nm. To identify the ethyl acetate-soluble phenylthiohydantoin derivatives by means of t.l.c., solvent systems II and V were used (Jeppsson & Sjöquist, 1967). Phenylthiohydantoin-arginine and phenylthiohydantoin-histidine in the water phase were detected by spot test on filter paper with Sagaguchi reagent or Pauli reagent respectively. In some cases the amino acid released was identified subtractively by amino acid analysis of a portion of the peptide remaining after a round of Edman degradation.

Automatic sequence analysis. The *N*-terminal sequences of the reduced and *S*-carboxymethylated component LsIII and other relatively large peptide fragments were analysed by Edman degradation by using a sequence analyser (type JAS 47K, Japan Electron Optics Laboratory Co., Tokyo, Japan) by the method described in the manual (cf. Table 1).

Carboxypeptidase A or leucine aminopeptidase digestion. The digestion with carboxypeptidase A was carried out in 0.05M-Tris–HCl buffer, pH8.0, or in 0.1M-NH_4HCO_3, pH7.8, at 37°C for 16h. Leucine aminopeptidase digestion was carried out in 0.05M-Tris–HCl buffer containing 0.0025M-$MgCl_2$. The substrate concentration was about 1mg/ml. Enzymes were used at the enzyme/substrate ratio 1:20–50 (w/w). The digests were applied direct to the amino acid analyser. Asparagine and glutamine appeared superimposed on serine and threonine respectively.

Table 1. *Programme for sequence analyser*

Operation	Reagent (R) or solvent (S)	Volume (ml)	Operation time (min)
Coupling	R1 Phenyl isothiocyanate (5% in heptane)	0.4	3
	R2 0.4M-Dimethylallylamine buffer	1.0	25
	R2 0.4M-Dimethylallylamine buffer	1.0	
N_2 drying			2
Vacuum drying			10
Washing	S1 Benzene	16	15
	S2 Ethyl acetate*	21	14
N_2 drying			2
Vacuum drying			10
Cleavage	R3 Heptafluorobutyric acid	1.4	4
Vacuum drying			17
Extraction	S3 1-Chlorobutane	12	12
N_2 drying			3
Vacuum drying			10

* This step was added for longer-chain peptides.

Results

N-Terminal sequence of reduced and S-carboxymethylated component LsIII

The *N*-terminal sequence of reduced and *S*-carboxymethylated component LsIII was determined by Edman degradation by the sequence analyser. The programme shown in Table 1 was used.

The phenylthiohydantoin-amino acids identified by the t.l.c. patterns are given by the one-letter IUPAC codes,* underlined by the solid arrows. The amino acids in parentheses were identified after further fragmentation as described below.

Fig. 1 shows, as an example, the yields of phenylthiohydantoin-amino acids in the ethyl acetate layer, calculated from the extinction at 269nm and the t.l.c. patterns developed with solvent V.

Further fragmentation and amino acid sequence of peptide A

The tryptic digest of peptide A (20mg) was chromatographed on a column (1.2cm×14cm) of DEAE-cellulose, which has been equilibrated to 0.02M-NH_4HCO_3 buffer, pH7.8. After the first peak (A-1) had been eluted with the buffer (50ml), a linear gradient was applied with 0.02M-NH_4HCO_3 buffer, pH7.8 (100ml) in the mixing chamber and 0.2M-NH_4HCO_3 buffer, pH7.8 (100ml) in the reservoir. Two peaks, A-2 and A-3, were obtained. Elution with an additional 50ml of the 0.2M-buffer gave the fourth peak, A-4. The final linear gradient elution from 0.2M- (100ml) to 1.0M-(100ml) NH_4HCO_3 buffer, pH7.8, gave the fifth peak, A-5, in 50ml. The flow rate was 1ml/min. On paper electrophoresis at pH4.8, peaks A-1, A-2 and A-4 gave single spots, of which the latter two were Pauli-positive. Peak A-3 gave two spots, A-3a and A-3b, which migrated towards the anode. A-5 gave no clear spots on paper electrophoresis. The amino acid compositions of the fragments are given in Table 3.

R E C Y L N (P) H D T (Q) T (C P S) G (Q E) I -----

Fragmentation of component LsIII

To make good use of the sequence analyser, it is desirable to obtain relatively large fragments, which may be digested further when necessary. The whole scheme of fragmentation is summarized in Scheme 1.

The tryptic digest of citraconylated reduced and *S*-carboxymethylated component LsIII (see the Experimental section) was desalted in a Diaflo apparatus with UM-2 filter (Amicon Corp., Lexington, Mass., U.S.A.) in 0.1M-acetic acid, and applied to a column (1.6cm×74cm) of Sephadex G-50 (fine grade) and eluted with 0.1M-acetic acid. Two peptides, A and B, were detected by extinction at 280nm at the elution volumes of 110 and 120ml respectively. Each peptide was rechromatographed on the same column with 0.1M-ammonium acetate buffer, pH7.8, and freeze-dried. The peptides A and B accounted for the total amino acid composition of component LsIII (Table 2).

Peptide A contained no arginine and was assumed to be the latter half of component LsIII.

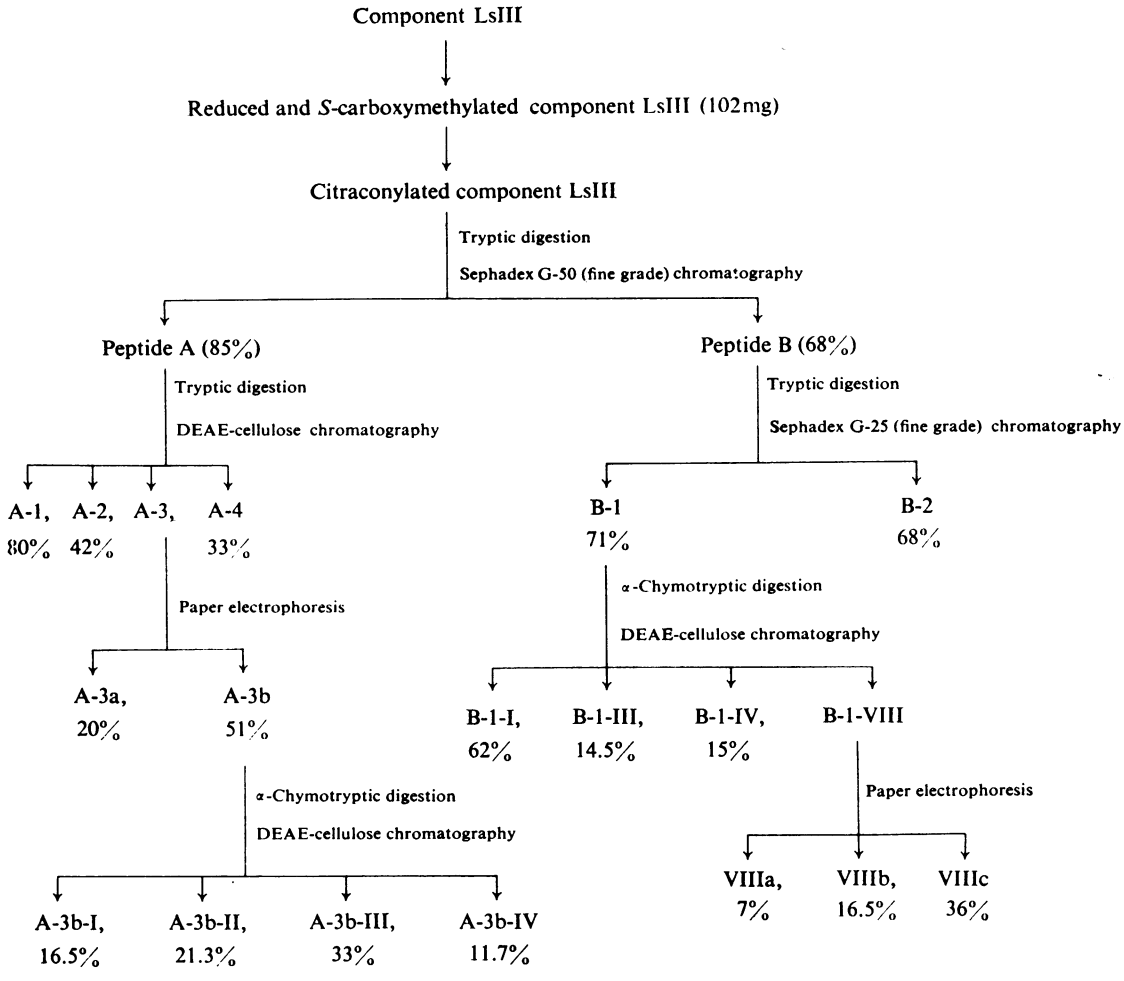
N-Terminal sequence of peptide A

The peptide A, consisting of 33 amino acid residues, was applied to the sequence analyser. The washing step with ethyl acetate (washing S2, Table 1) was added to minimize the amount of by-products.

The amino acid sequence of peptide A-4 was determined by the sequence analyser (Fig. 2). The direct and subtractive manual Edman degradation of the peptides A-2 and A-3a (Fig. 2) confirmed the results and suggested that the peptide A-4 was partially hydrolysed to peptides A-3a and A-2. The incomplete tryptic digestion may be due to the acidic amino acids at both sides of the lysine residue at the middle of peptide A-4. With proline at the *C*-terminus, peptide A-4 is evidently the *C*-terminal part of the peptide A or component LsIII. Peptide A-1 is the *N*-terminal part of peptide A.

Peptide A-3b (6mg) was digested further with α-chymotrypsin. The digest was subjected to DEAE-cellulose column (1.2cm×14cm) chromatography with 0.01M-NH_4HCO_3 buffer, pH7.8. After the first peak, A-3b-I, had been eluted with the buffer in 25ml, a linear gradient was applied with 0.01M-NH_4HCO_3 buffer, pH7.8 (100ml), in the mixer and 0.1M-NH_4HCO_3 buffer, pH7.8 (100ml), in the reservoir. Three peaks, A-3b-II, A-3b-III, and A-3b-IV,

* Key to one-letter notation for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.



Scheme 1. *Enzymic fragmentation of reduced and S-carboxymethylated component LsIII*

The percentage values are the yields of the peptides at each digestion step. For further details of the peptides see the text.

were eluted successively in 100ml. Assuming that the lysine residue is at the *C*-terminus of the tryptic peptide A-3b, the peptide A-3b-I is presumed to be the *C*-terminal fragment of peptide A-3b. The amino acid analysis and four steps of manual Edman degradation of peptide A-3b-I indicate a sequence of T-G-T-E-I-K (Fig. 2). The glutamic acid residue was confirmed also by leucine aminopeptidase digestion. Peptide A-3b-II was revealed to be the *N*-terminal part of peptide A-3b by its amino acid composition (Fig. 2). Carboxypeptidase A digestion of peptide A-3b-IV for 4h gave asparagine and valine in a molar ratio of 1.0:0.75. After nine steps' cleavage of peptide A-3b by the sequence analyser (shown by dotted arrows in Fig. 2) the remaining peptide was washed out from the reaction cup and analysed for the sequence manually, both directly and subtractively. The amino acid sequence of the peptide A concluded from the above results is given in Fig. 2.

Further fragmentation and amino acid sequence of peptide B

On tryptic digestion, peptide B (20mg) was split into two fragments, B-1 and B-2, the former of which was eluted at the void volume from a column (1.4cm×58cm) of Sephadex G-25 (fine grade) with 0.1M-acetic acid, and the latter was retarded on the column by an adsorption effect (Porath, 1960). The amino acid compositions of the peptides are given in Table 4.

Peptide B-2 was Ehrlich-positive and was ascribed to the *C*-terminal region of peptide B, since it contained arginine and no lysine. Leucine aminopeptidase digestion of peptide B-2 for 20h produced tryptophan, arginine, *S*-carboxymethylcysteine, serine

Table 2. *Amino acid composition of tryptic peptides from citraconylated reduced and S-carboxymethylated component LsIII*

The results are expressed in molar proportions of the amino acids. Values in parentheses give the nearest whole numbers.

	Reduced and *S*-carboxymethylated component LsIII	Peptide A	Peptide B
Lysine	3.95 (4)	2.81 (3)	1.03 (1)
Histidine	1.02 (1)	—	0.83 (1)
Arginine	1.81 (2)	—	2.42 (2)
CM-cysteine*	8.00 (10)*	3.50 (5)*	3.41 (5)*
Aspartic acid	6.18 (6)	3.05 (3)	3.01 (3)
Threonine	5.79 (6)	3.51 (4)	1.82 (2)
Serine	5.70 (6)	1.93 (2)	3.37 (4)
Glutamic acid	6.16 (6)	2.23 (2)	3.67 (4)
Proline	3.65 (4)	1.92 (2)	1.23 (2)
Glycine	4.15 (4)	3.10 (3)	1.17 (1)
Alanine	3.88 (4)	2.95 (3)	1.05 (1)
Valine	2.91 (3)	2.06 (2)	1.16 (1)
Isoleucine	1.88 (2)	0.90 (1)	0.83 (1)
Leucine	1.95 (2)	0.95 (1)	1.04 (1)
Tyrosine	2.76 (3)	0.85 (1)	1.68 (2)
Phenylalanine	0.91 (1)	0.93 (1)	—
Tryptophan	2† (2)	(–)‡	(+)‡ (2)
Total	66	33	33

* *S*-Carboxymethylcysteine tends to give low values.

† Estimated spectrophotometrically (Goodwin & Morton, 1946).

‡ Tryptophan was detected by spot test with Ehrlich reagent.

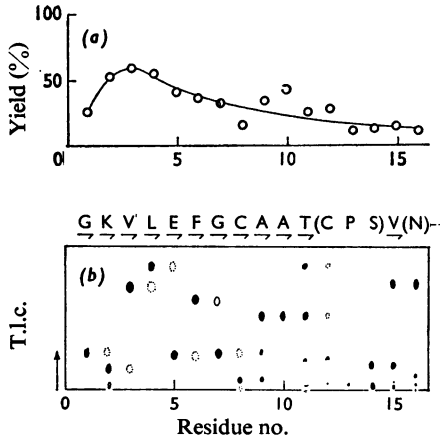


Fig. 1. *N-Terminal sequences of peptide A*

The percentage yields of phenylthiohydantoin-amino acids in each ethyl acetate layer of a 16-step Edman degradation by using a sequence analyser are shown in (*a*). The amino acids identified by t.l.c. patterns (*b*) are given by the one-letter IUPAC code underlined by the solid arrows shown above (*b*). ●, Definite spot; ○, faint spot.

plus asparagine and alanine in the molar proportions 1.65:0.54:1.87:3.49:1.00. No aspartic acid was detected. Manual Edman degradation of peptide B-2 gave the sequence shown in Fig. 2.

Peptide B-1 was treated with α-chymotrypsin and the digest subjected to chromatography on a column (1.2cm×14cm) of DEAE-cellulose. After the first peak, B-1-I, had been eluted with 0.01M-NH_4HCO_3 buffer, pH7.8, in 25ml, a linear gradient was applied with 0.01M-NH_4HCO_3 buffer, pH7.8 (100ml), in the mixing chamber and 0.1M buffer (100ml) in the reservoir, and peptide fractions B-1-II, B-1-III, B-1-IV, B-1-V and B-1-VI were obtained. Elution with the second linear gradient from 0.1M (50ml) to 1.0M (50ml) buffer gave peptide fractions B-1-VII and B-1-VIII. The flow rate was 1ml/min.

The peptide fraction B-1-VIII was further separated into three components B-1-VIIIa, B-1-VIIIb, and B-1-VIIIc by paper electrophoresis at pH4.8 for 90min. Table 4 shows the amino acid compositions of the peptides, which account for that of peptide B-1. The *N*-terminal sequence study of reduced and *S*-carboxymethylated component LsIII described above gives a peptide sequence of B-1-IV–B-1-III–B-1-VIIIb–B-1-I. Peptide B-1-I was placed at the *C*-terminus of peptide B-1 because it contained lysine, which was assumed to be the *C*-terminal residue of both peptides. Carboxypeptidase A digestion of peptides B-1-VIIIb or B-1-VIIIc liberated tyrosine only.

The sequences of peptides B-1-III and B-1-VIIIb were determined by manual Edman degradation (Fig. 2). The distinction between glutamine and glutamic acid at positions 17 and 18 was difficult because of the overlapping of the t.l.c. spots of Edman-degradation products. The position of the amide group was determined as follows. Peptide B-1 (8mg) was digested with pepsin and the digest chromatographed on a column (1.2cm×14cm) of DEAE-cellulose by using a linear gradient from 0.01M-(100ml) to 0.2M- (100ml) NH_4HCO_3 buffer, pH7.8, followed by elution with 0.2M-NH_4HCO_3 buffer, pH7.8. A peptide, the amino acid composition of which corresponded to residues 1–18 of peptide B-1, was eluted in 40ml. Carboxypeptidase A digestion of the fragment 1–18 (0.5mg) at pH7.8 (see the Experimental section) liberated no amino acids. The digestion of the peptide at pH6.4 at an enzyme/substrate ratio of 1:10 (w/w) for 24h produced 0.58mol of glutamic acid and 0.67mol of glutamine/mol of peptide. Pronase E digestion followed by leucine aminopeptidase digestion of peptide B-1-VIIIc (approx. 0.5mg) liberated threonine (with glutamine) and glutamic acid at a molar ratio of 2:1 in addition to *S*-carboxymethylcysteine, glycine, isoleucine, leucine and tyrosine (1 mol of each). From these results, together with the relative electrophoretic mobility of the peptides, the amino acid at position 18 was concluded to be glutamic acid, and that at position 17 to be glutamine.

Table 3. *Amino acid compositions of the fragments from peptide A*

The results are expressed in molar proportions of the amino acids. Values in parentheses give the nearest whole numbers. For details of the fragments and peaks, see the text.

Fragments ...	A-1	A-2	A-3a	A-3b	A-4 (A-2+A-3a)	A-3b-I	A-3b-II	A-3b-III (A-3b-IV+A-3b-I)	A-3b-IV	A
Lysine	1.00 (1)		0.87 (1)	0.82 (1)	0.92 (1)	0.97 (1)		0.81 (1)		3
CM-cysteine*		0.48 (1)	0.5 (2)	0.92 (2)	2.50 (3)			0.94 (2)	0.61 (2)	5
Aspartic acid		1.00† (1)	1.00† (1)	1.00† (1)	2.00† (2)			1.00† (1)	1.00† (1)	3
Threonine		0.94 (1)		2,58 (3)	0.96 (1)	1.67 (2)		2.86 (3)	1.03 (1)	4
Serine			0.79 (1)	0.81 (1)	1.03 (1)			0.73 (1)	0.99 (1)	2
Glutamic acid				1.88 (2)		1.00† (1)	1.00† (1)	1.32 (1)		2
Proline		0.76 (1)		1 (1)	0.97 (1)			0.71 (1)	1.02 (1)	2
Glycine	1.00† (1)			1.91 (2)		1.12 (1)		1.88 (2)	1.62 (1)	3
Alanine			0.91 (1)	2.01 (2)	1.07 (1)			1.96 (2)	2.01 (2)	3
Valine				1.68 (2)			0.94 (1)	1.01 (1)	1.50 (1)	2
Isoleucine				0.66 (1)		0.84 (1)		0.86 (1)		1
Leucine				0.76 (1)			0.84 (1)			1
Tyrosine		0.67 (1)			0.76 (1)					1
Phenylalanine				1 (1)			0.85 (1)			1
Total	2	5	6	20	11	6	4	16	10	33
Yield (%)	80	42	20	51	33	16.5	21.3	33	11.7	

* *S*-Carboxymethylcysteine tends to give low values. The small yield of A-3a (20%) made it difficult to obtain an exact amino acid composition, especially the quantity of *S*-carboxymethylcysteine. The peptide gave, however, two *S*-carboxymethylcysteines on the first two steps of manual Edman degradation.

† The amounts of these amino acids were taken as standards.

Table 4. *Amino acid composition of the fragments from peptide B*

Fragments B-1 and B-2 are the tryptic peptides from peptide B, and B-1-I, -III, -IV, -VIII are the chymotryptic peptides from peptide B-1. The results are expressed in molar proportions of the amino acids. Values in parentheses show nearest whole number.

Fragment ...	B-1-I	B-1-III	B-1-IV (B-1-VIIIa+Arg)	B-1-VIIIa	B-1-VIIIb	B-1-VIIIc (B-1-III+B-1-VIIIb)	B-1	B-2	B
Lysine	1.00† (1)						1.03 (1)		1
Histidine		0.74 (1)				0.91 (1)	0.91 (1)		1
Arginine			0.75 (1)				0.93 (1)	0.5 (1)	2
CM-cysteine*			0.50 (1)	0.4 (1)	1.19 (2)	1.40 (2)	2.06 (3)	0.75 (2)	5
Aspartic acid		2.00† (2)				2.00† (2)	2.13 (2)	1.00† (1)	3
Threonine		0.96 (1)			1.01 (1)	1.82 (2)	2.02 (2)		2
Serine					1.15 (1)	0.98 (1)	1.17 (1)	2.85 (3)	4
Glutamic acid		1.29 (1)	1.00† (1)	1.0† (1)	2.01 (2)	2.95 (3)	3.80 (4)		4
Proline		1.19 (1)			1.32 (1)	2.97 (2)	1.99 (2)		2
Glycine					1.00† (1)	0.97 (1)	0.97 (1)		1
Alanine								1.00 (1)	1
Valine	0.88 (1)						1.10 (1)		1
Isoleucine					0.82 (1)	0.87 (1)	0.65 (1)		1
Leucine		0.86 (1)				0.87 (1)	0.91 (1)		1
Tyrosine			0.79 (1)	0.9 (1)	0.77 (1)	0.89 (1)	1.74 (2)		2
Tryptophan							(−)‡	(+)‡ (2)	2
Total	2	7	4	3	10	17	23	10	33
Yield (%)	62	14.5	15	7	16.5	36	71	68	

* *S*-Carboxymethylcysteine tends to give low values.
† The amounts of these amino acids were taken as standards.
‡ Tryptophan was detected by spot test with Ehrlich reagent.

Table 5. *Amino acid sequences of venom*

1–29, Neurotoxins; 30–38, cardiotoxins and other toxins; 1–7 and 29, Hydrophiidae; 8-28, 30-38, Elapidae. The amino acids surrounded by solid lines are acids see the text. p.c., Personal communication.

	5							10					15					20					25				30			35				
1. *Laticauda semifasciata* erabutoxin c	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	H	K	Q	W	S	D	F			R
2. *Laticauda semifasciata* erabutoxin b	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	H	K	Q	W	S	D	F			R
3. *Laticauda semifasciata* erabutoxin a	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	N	K	Q	W	S	D	F			R
4. *Laticauda 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
5. *Laticauda 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
6. *Enhydrina schistosa* toxin 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. *Enhydrina schistosa* toxin 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. *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
9. *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
10. *Naja nivea* toxin δ, *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
11. *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
12. *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
13. *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
14. *Hemachatus haemachatus* toxin 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
15. *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
16. *Bungarus multicinctus* α bungarotoxin	I	V	C	H		T	T	A	T	I	P	S	S	A	V	T	C	P	P	G	E	N	L	C	Y	R	K	M	W	C	D	A	F	C	S	S	R
17. *Dendroaspis polylepis* toxin γ	R	T	C		N			K	T	F	S	D	Q	S	K	I	C	P	P	G	E	N	I	C	Y	T	K	T	W	C	D	A	W	C	S	Q	R
18. *Dendroaspis polylepis* toxin δ	R	T	C		N			K	T	F	S	D	Q	S	K	I	C	P	P	G	E	N	I	C	Y	T	K	T	W	C	D	A	W	C	S	Q	R
19. *Ophiophagus hannah* toxin a	T	K	C	Y				V	T	P	D	V	K	S	Q	T	C	P	A	G	E	N	I	C	Y	T	E	T	W	C	D	A	W	C	S	T	R
20. *Ophiophagus hannah* toxin b	T	K	C	Y				V	T	P	D	A	T	S	Q	T	C	P	D	G	E	N	I	C	Y	T	K	T	W	C	D	G	F	C	S	S	R
21. *Naja nivea* toxin α	I	R	C	F				I	T	P	D	V	T	S	Q	A	C	P	D	G		H	V	C	Y	T	K	M	W	C	D	N	F	C	G	M	R
22. *Naja melanoleuca* toxin b	I	R	C	F				I	T	P	D	V	T	S	Q	I	C	A	D	G		H	V	C	Y	T	K	T	W	C	D	N	F	C	A	S	R
23. *Naja naja siamensis* 3	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	A	F	C	S	I	R
24. *Naja naja naja* toxin 4	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	S	R
25. *Naja naja naja* toxin 3	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	I	R
26. *Naja naja* toxin A	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	I	R
27. *Naja naja* toxin B	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	S	R
28. *Naja naja* toxin C	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	A	F	C	S	I	R
29. *Laticauda semifasciata* component LsIII	R	E	C	Y					L	N	P	H	D	T	Q	T	C	P	S	G	Q	E	I	C	Y	V	K	S	W	C	N	A	W	C	S	S	R
30. *Naja naja atra* cardiotoxin	L	K	C		N			K	L	V	P	L	F	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	F	M	V	A			T	P
31. *Naja naja* cytotoxin II	L	K	C		N			K	L	V	P	L	F	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	Y	M	V	A			T	P
32. *Naja naja* cytotoxin I	L	K	C		N			K	L	I	P	L	A	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	Y	M	V	S			N	K
33. *Naja nigricollis* cardiotoxin 14	L	K	C		N			Q	L	I	P	P	F	W	K	T	C	P	K	G	K	N	L	C	Y		K	M	T	M	R	A			A	P
34. *Naja naja* (Cambodia) cardiotoxin	L	K	C		N			K	L	I	P	I	A	S	K	T	C	P	A	G	K	N	L	C	Y		K	M	F	M	M	S			D	L
35. *Hemachatus haemachatus* DLF (12B)	L	K	C	H	N			K	L	V	P	F	L	S	K	T	C	P	E	G	K	N	L	C	Y		K	M	T	M	L	K			M	P
36. *Naja haje* cardiotoxin	L	K	C	H				K	L	V	P	P	V	W	K	T	C	P	E	G	K	N	L	C	Y		K	M	F	M	V	S			T	S
37. *Naja melanoleuca* cardiotoxin	L	E	C		N			K	L	V	P	I	A	H	K	T	C	P	A	G	K	N	L	C	Y		Q	M	Y	M	V	S			K	S
38. *Dendroaspis angusticeps* toxin F_{VII}	T	M	C	Y	S	H	T	T	T	S	R	A	I	L	T	N	C	G	E			N	S	C	Y	R	K	S	R	R	H	P			P	

components from Hydrophiidae and Elapidae

found in common at the same positions and those surrounded by the dotted lines have similar groups at the same positions. For key to IUPAC notations for amino

40	45	50	55	60	65	70	75	Total	Reference
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	62	Tamiya & Abe (1972)
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	62	Sato & Tamiya (1971)
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	62	Sato & Tamiya (1971)
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	62	A. Sato, p.c.
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	62	A. Sato, p.c.
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	60	Fryklund *et al.* (1972)
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	60	Fryklund *et al.* (1972)
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	60	Strydom (1972)
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	61	Botes (1971)
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	61	Botes *et al.* (1971); Botes & Strydom (1969)
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	61	Botes (1972)
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	61	Eaker & Porath (1967)
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	61	Strydom & Botes (1971)
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	61	Strydom & Botes (1971)
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	62	Yang *et al.* (1969)
G K V V E L	G C	A A T C P	S K K P Y E E V T	C C	S T	D K C	N H P P K R Q P G	74	Mebs *et al.* (1972)
G K R V E L	G C	A A T C P	K V K A G V E I K	C C	S T	D D C	D K F Q F G K P R	72	Strydom (1972)
G K I V E L	G C	A A T C P	K V K A G V E I K	C C	S T	D N C	N K F K F G K P R	72	D. J. Strydom, p.c.
G K R V D L	G C	A A T C P	I V K P G V E I K	C C	S T	D N C	N P F P T W R K R P	73	Joubert (1973)
G K R I N L	G C	A A T C P	K V K P G V D I K	C C	S T	D N C	N P F P T W K R R H	73	Joubert (1973)
G K R V D L	G C	A A T C P	K V K P G V N I K	C C	S R	D N C	N P F P T R K R S	71	Botes (1971)
G K R V D L	G C	A A T C P	T V K P G V N I K	C C	S T	D N C	N P F P T R N R P	71	Botes (1972)
G K R V D L	G C	A A T C P	T V K T G V D I Q	C C	S T	D N C	N P F P T R K R P	71	E. Karlsson, p.c.
G K R V D L	G C	A A T C P	T V R T G V D I Q	C C	S T	D N C	N P F P T R K R P	71	E. Karlsson, p.c.
G K R V D L	G C	A A T C P	T V R T G V D I Q	C C	S T	D N C	N P F P T R K R P	71	E. Karlsson, p.c.
G K R V D L	G C	A A T C P	T V R T G V D I Q	C C	S T	D D C	D T F P T R K R P	71	Nakai *et al.* (1971)
G K R V D L	G C	A A T C P	T V R T G V D I Q	C C	S T	D D C	D T F P T R K R P	71	K. Hayashi, p.c.
G K R V D L	G C	A A T C P	T V K T G V D I Q	C C	S T	D D C	D T F P T R K R P	71	K. Hayashi, p.c.
G K V L E F	G C	A A T C P	S V N T G T E I K	C C	S A	D K C	N T Y P	66	Present paper
K V P V K R	G C	I D V C P	K S S L V L K Y V	C C	N T	D R C	N	60	Narita & Lee (1970)
K V P V K R	G C	I D V C P	K S S L V L K Y V	C C	N T	D R C	N	60	Takechi *et al.* (1972)
T V P V K R	G C	I D V C P	K N S L V L K Y E	C C	N T	D R C	N	60	Hayashi *et al.* (1971)
M V P V K R	G C	I D V C P	K S S L L I K Y M	C C	N T	D K C	N	60	O. P. Botes, p.c.
T I P V K R	G C	I D V C P	K S N L L V K Y V	C C	N T	D R C	N	60	O. P. Botes, p.c.
K I P I K R	G C	T D A C P	K S S L L V K V V	C C	N K	D K C	N	61	Fryklund & Eaker (1973)
T V P V K R	G C	I D V C P	K N S A L V K Y V	C C	S T	D K C	N	60	O. P. Botes, p.c.
T I P V K R	G C	I D V C P	K S S L L V K Y V	C C	N T	D R C	N	60	O. P. Botes, p.c.
K M V L G R	G C	G C P	P G D D N L E V K	C C	T S P	D K C	N Y	61	Viljoen & Botes (1973)

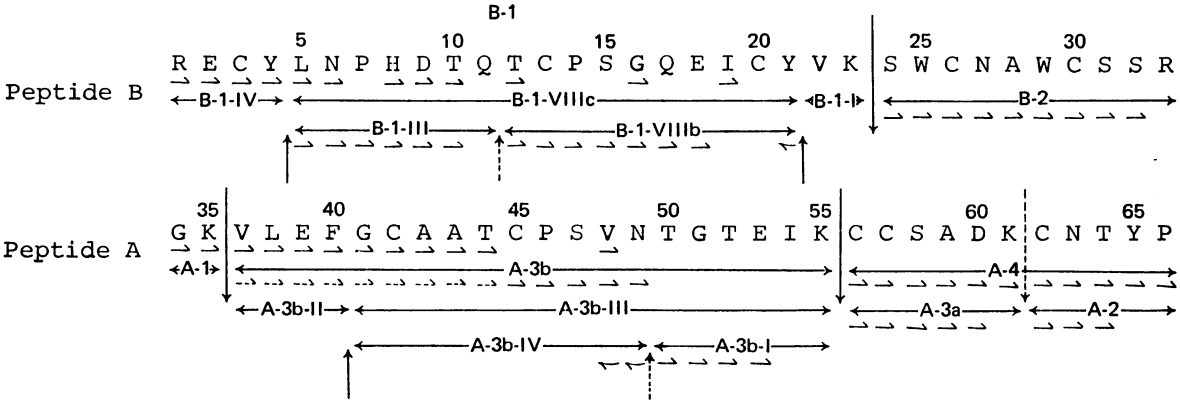


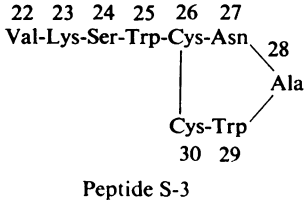
Fig. 2. *Amino acid sequence of component LsIII*

Peptides are shown by bars with names (for notation see the text). →, Amino acids detected by Edman degradation; ⇢, amino acids cleaved off by the sequence analyser for the purpose of obtaining a residual peptide. ↻, Amino acids detected by carboxypeptidase A digestion; ↓ and ↑, indicate the bonds cleaved by trypsin and α-chymotrypsin respectively; ⇣, ⇡ show the partial cleavage by the enzymes.

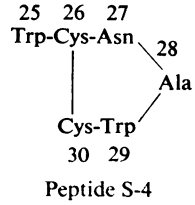
Location of disulphide bridge between Cys-26 and Cys-30

Component LsIII (2.4mg) was treated with iodoacetate in 8M-urea in the same manner as for the preparation of reduced and *S*-carboxymethylated component LsIII, except that the prior reduction with 2-mercaptoethanol was not done, and gel-filtrated on a column (1.2cm×23cm) of Sephadex G-25 (fine grade) in 0.1M-acetic acid. No carboxymethylcysteine was detected in the acid hydrolysate of the freeze-dried preparation, indicating that all the half-cystine residues are in disulphide state.

Component LsIII (7mg) was digested with pepsin in 0.01M-HCl (1.0ml) at an enzyme substrate ratio of 1:25 (w/w) at 37°C for 40h. Two peptides, S-3 and S-4, were eluted later than the salts with 0.1M-acetic acid on application of the digest to a column (1.4cm×58cm) of Sephadex G-25 (fine grade). Both of them were Ehrlich positive, containing 2 tryptophan residues/molecule judged from the u.v.-absorption values. Peptides S-3 and S-4 gave 2 (1.92 and 1.81 respectively) cysteic acid residues/molecule of valine, lysine, serine, aspartic acid and alanine and aspartic acid and alanine respectively, in the amino acid analyses after the performic acid oxidation of the peptides. Their structures are therefore concluded to be as follows:



Peptide S-3



Peptide S-4

The isolation of these peptides suggested the disulphide linkage between half-cystine residues at positions 26 and 30, making an extra loop.

The positions of other disulphide bridges are assumed to be the same as other venom components from the sequence homology (short-chain toxins, Endo *et al.*, 1971; Yang *et al.*, 1970; Takechi & Hayashi, 1972; long-chain toxins, Botes, 1971; Ohta & Hayashi, 1973).

Discussion

The amino acid sequence of component LsIII is given in Fig. 2. The sequence is clearly homologous to all the neurotoxins and cardiotoxins from *Hydrophiidae* and *Elapidae* snakes for which amino acid sequence analysis is available. Ten half-cystine residues are at the same positions as in long-chain toxins, making an additional loop between residues 26–30 (Fig. 2) or 30–34 (Table 5). Component LsIII is the first sea-snake venom component with five disulphide bridges.

It is of interest that component LsIII is a weak and reversible neurotoxin and not a cardiotoxin and that its structure lies between the two types of toxins. Component LsIII shares tryptophan-29, arginine-37 and glycine-38 with neurotoxins in addition to eight half-cystine residues, tyrosine-25, glycine-44 and proline-50, which are common to all neuro- and cardio-toxins.

Tryptophan-29 is considered to be an indispensable residue from chemical modification studies (Chang & Hayashi, 1969; Seto *et al.*, 1970; Chicheportiche *et al.*, 1972; Karlsson & Eaker, 1972; Chang & Yang, 1973).

Component LsIII is different, however, from other neurotoxins, especially in five features.

(1) In component LsIII, the tail is missing compared with a long-chain toxin. Thus component LsIII has a total length of 66 residues, which lies between the length of short-chain and long-chain toxins. Karlsson *et al.* (1972) reported that the removal of the *C*-terminal four residues from *Naja naja siamensis* toxin 3 (no. 23, Table 5) decreased its toxicity to one-half the original value. Strydom (1973) discussed the possibility that the seven extra residues of 'nonsense' sequence at the *C*-terminus could easily be added by a single base change in the original terminating codon.

(2) Component LsIII is a neutral protein with an isoelectric point of pH7.2, whereas other neurotoxins are basic proteins. Component LsIII has seven carboxyl groups and seven amino and guanidyl groups; the only extra charge may be due to histidine-8. Giving the molecule a net positive charge by modifying the carboxyl groups with a carbodi-imide and glycine methyl ester, however, did not affect the toxicity (H. Hori & N. Tamiya, unpublished work).

(3) Aspartic acid-31, which is common to neurotoxins, is missing. Chang *et al.* (1971) reported that the modification of this residue in cobrotoxin (no. 15, Table 5) did not destroy the toxicity.

(4) The usual positive charge at position 53 is missing in component LsIII. Chang *et al.* (1971) reported that the modification of the lysine at this position of cobrotoxin (no. 15, Table 5) with trinitrobenzenesulphonate destroyed the toxicity, and Karlsson *et al.* (1972) reported that the acetylation of lysine at this position of *Naja naja* neurotoxins (nos. 23 and 25, Table 5) did not affect the toxicity.

(5) The usual hydroxyl group at position 9 is missing in component LsIII.

It may be possible to explain the weak and reversible neurotoxicity of component LsIII by one or some of these features. It is noteworthy that this fairly ineffective component accounts for about 10% by weight of the venom protein. Strydom (1973) suggested from computer analysis of the sequences of venom components (nos. 10, 9, 14, 13, 12, 15, 3, 2, 21, 22, 11, 8, 17, 30, 16 and 26, Table 5) that *Naja naja atra* cardiotoxin (no. 30, Table 5) is most closely related to the prototype of venom components. The poorly effective component LsIII, with a structure between those of neuro- and cardio-toxins, may be a fossil component preserved in the venom.

We are grateful to Dr. D. P. Botes, Dr. K. Hayashi, Dr. E. Karlsson, Dr. A. Sato and Dr. D. J. Strydom for providing the amino acid sequences of the venom components in advance of the publication. The research was supported by grants from the Ministry of Education, Japanese Government.

References

Ahlroth, A. (1965) *Acta Chem. Scand.* **19**, 2420
Botes, D. P. (1971) *J. Biol. Chem.* **246**, 7383–7391
Botes, D. P. (1972) *J. Biol. Chem.* **247**, 2866–2871
Botes, D. P. & Strydom, D. J. (1969) *J. Biol. Chem.* **244**, 4147–4151
Botes, D. P., Strydom, D. J., Anderson, C. G. & Christensen, P. A., (1971) *J. Biol. Chem.* **246**, 3132–3139
Chang, C. C. & Hayashi, K. (1969) *Biochem. Biophys. Res. Commun.* **37**, 841–846
Chang, C. C. & Yang, C. C. (1973) *Biochim. Biophys. Acta* **295**, 595–604
Chang, C. C., Yang, C. C., Nakai, K. & Hayashi, K. (1971) *Biochim. Biophys. Acta* **251**, 334–344
Chicheportiche, R., Rochat, C., Sampieri, F. & Lanzdunski, M. (1972) *Biochemistry* **11**, 1681–1691
Crestfield, A., Moore, S. & Stein, W. (1963) *J. Biol. Chem.* **238**, 622–627
Eaker, D. & Porath, J. (1967) *Jap. J. Microbiol.* **11**, 353–355
Endo, Y., Sato, S., Ishii, S. & Tamiya, N. (1971) *Biochem. J.* **122**, 463–467
Fryklund, L. & Eaker, D. (1973) *Biochemistry* **12**, 661–667
Fryklund, L., Eaker, D. & Karlsson, E. (1972) *Biochemistry* **11**, 4633–4640
Gibbons, I. & Perham, R. N. (1970) *Biochem. J.* **116**, 843–849
Goodwin, T. W. & Morton, R. A. (1946) *Biochem. J.* **40**, 628–632
Hayashi, K., Takechi, M. & Sasaki, T. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1357–1362
Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A. & Blombäck, B. (1969) *Eur. J. Biochem.* **8**, 189–199
Jeppsson, J.-O. & Sjöquist, J. (1967) *Anal. Biochem.* **18**, 264–269
Jepson, J. B. & Smith, I. (1953) *Nature (London)* **172**, 1100–1101
Joubert, F. (1973) *Biochim. Biophys. Acta* **317**, 85–98
Karlsson, E. & Eaker, D. (1972) *J. Formosan Med. Assoc.* **71**, 358–371
Karlsson, E., Eaker, D. & Ponterius, G. (1972) *Biochim. Biophys. Acta* **257**, 235–248
Maeda, N., Takagi, K., Tamiya, N., Chen, Y.-M. & Lee, C.-Y. (1974) *Biochem. J.* **141**, 383–387
Mann, T. & Leone, E. (1953) *Biochem. J.* **53**, 140–148
Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. & Lee, C. Y. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 243–262
Nakai, K., Sasaki, T. & Hayashi, K. (1971) *Biochem. Biophys. Res. Commun.* **44**, 893–897
Narita, K. & Lee, C. Y. (1970) *Biochem. Biophys. Res. Commun.* **41**, 339–343
Ohta, M. & Hayashi, K. (1973) *Biochem. Biophys. Res. Commun.* **55**, 431–438
Porath, J. (1960) *Biochim. Biophys. Acta* **39**, 193–207
Sato, S. & Tamiya, N. (1971) *Biochem. J.* **122**, 453–461
Seto, A., Sato, S. & Tamiya, N. (1970) *Biochim. Biophys. Acta* **214**, 483–489
Smith, I. (1953) *Nature (London)* **171**, 43–44
Strydom, D. J. (1972) *J. Biol. Chem.* **247**, 4029–4042
Strydom, D. J. (1973) *Comp. Biochem. Physiol.* **44B**, 269–281

Strydom, A. J. C. & Botes, D. P. (1971) *J. Biol. Chem.* **246**, 1341–1349
Takechi, M. & Hayashi, K. (1972) *Biochem. Biophys. Res. Commun.* **49**, 584–590
Takechi, M., Hayashi, K. & Sasaki, T. (1972) *Mol. Pharmacol.* **8**, 446–451
Tamiya, N. & Abe, H. (1972) *Biochem. J.* **130**, 547–555
Tamiya, N. & Arai, H. (1966) *Biochem. J.* **99**, 624–630
Viljoen, C. C. & Botes, D. P. (1973) *J. Biol. Chem.* **248**, 4915–4919
Yang, C. C., Yang, H. J. & Huang, J. S. (1969) *Biochim. Biophys. Acta* **188**, 65–77
Yang, C. C., Yang, H. J. & Chiu, R. H. C. (1970) *Biochim. Biophys. Acta* **214**, 355–365