Amino acid sequences of two novel long-chain neurotoxins from the venom of the sea snake *Laticauda colubrina*

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From the venom of a population of the sea snake *Laticauda colubrina* from the Solomon Islands, a neurotoxic component, Laticauda colubrina a (toxin Lc a), was isolated in 16.6% (A_{280}) yield. Similarly, from the venom of a population of *L. colubrina* from the Philippines, a neurotoxic component, Laticauda colubrina b (toxin Lc b), was obtained in 10.0% (A_{280}) yield. The LD_{50} values of these toxins were $0.12 \mu\text{g/g}$ body wt. on intramuscular injection in mice. Toxins Lc a and Lc b were each composed of molecules containing 69 amino acid residues with eight half-cystine residues. The complete amino acid sequences of these two toxins were elucidated. Toxins Lc a and Lc b are different from each other at five positions of their sequences, namely at positions 31 (Phe/Ser), 32 (Leu/Ile), 33 (Lys/Arg), 50 (Pro/Arg) and 53 (Asp/His) (residues in parentheses give the residues in toxins Lc a and Lc b respectively). Toxins Lc a and Lc b have a novel structure in that they have only four disulphide bridges, although the whole amino acid sequences are homologous to those of other known long-chain neurotoxins. It is remarkable that toxins Lc a and Lc b are not coexistent at the detection error of 6% of the other toxin. Populations of *Laticauda colubrina* from the Solomon Islands and from the Philippines have either toxin Lc a or toxin Lc b and not both of them.

The sea snake *Laticauda colubrina* is widely distributed over a region including the Bay of Bengal, southern Japan, Australia and the islands of Oceania (Smith, 1926). Although the amino acid sequences of some short-chain neurotoxins from the venom of snakes of the Subfamily Laticaudinae have been elucidated (Sato & Tamiya, 1971; Tamiya & Abe, 1972; Maeda & Tamiya, 1977; cf. Kim & Tamiya, 1981b), no true long-chain neurotoxin has ever been found in Laticaudinae venoms. A medium-chain neurotoxin, Laticauda semifasciata III, was found by Maeda & Tamiya (1974) in the venom of *Laticauda semifasciata*.

The present paper describes the isolation, properties and complete amino acid sequences of two long-chain neurotoxins, L. colubrina a (Lc a) and L. colubrina b (Lc b) from *L. colubrina* venom.

Materials and methods

Snake venoms

Laticauda colubrina sea snakes were captured in the waters of the Philippines in August–September

Abbreviations used: toxin Lc a, neurotoxin Laticauda colubrina a; toxin Lc b, neurotoxin Laticauda colubrina b; CmCys (in sequences), carboxymethylcysteine.

1975 and in Lake Te-Nggano of Rennell Island, Solomon Islands, in November–December 1980. The venoms were ‘milked’ from the live snakes (Tamiya & Puffer, 1974), diluted with an equal volume of 0.20 M-acetic acid, and brought back to the laboratory.

Determination of molecular weight

Molecular weight was determined by gel filtration on a Sephadex G-50 (fine grade) column (1.0 cm $\times$ 128 cm) in 0.05 M-phosphate ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffer, pH 7.0, containing 6 M-guanidinium chloride by a slightly modified method of Mann & Fish (1972). Myoglobin (mol.wt. 17000), α -chymotrypsin (mol.wt. 23000), B-chain 14000, C-chain 11000, cytochrome c (mol.wt. 13000), toxin Acanthophis antarcticus b (mol.wt. 8100; Kim & Tamiya, 1981a), toxin Laticauda semifasciata III (mol.wt. 7200; Maeda & Tamiya, 1974) and erabutoxin b (mol.wt. 6900; Sato & Tamiya, 1971) were used as molecular-weight standards.

The other methods of amino acid analysis, disc-gel or paper electrophoresis, measurement of lethal activity, reduction and S-carboxymethylation of the toxins, enzymic digestion and Edman degradation have been described previously (Kim & Tamiya, 1981a).

Results

Isolation of neurotoxins

Freeze-dried *L. colubrina* venom preparation (each 50mg) from the Solomon Islands or the Philippines populations was dissolved in 0.01M-phosphate ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffer (10ml), pH 6.4, and applied to a column (1.5cm $\times$ 20cm) of CM-cellulose (Whatman CM-52) that had been equilibrated with the same buffer. The elution patterns are shown in Figs. 1(a) and 1(b).

From the venom from the Solomon Islands population, three main fractions with neurotoxic activity (A, B and C) were obtained in 16.6%, 23.0% and 25.7% yields respectively, as measured by A_{280} (Fig. 1a). The total recovery of the protein was 90.7%, as measured by the absorbance. From the venom from the Philippines population, three main fractions (I, II and III) were isolated in 10.0%, 18.7% and 52.7% (A_{280}) yields respectively (Fig. 1b). The total recovery was 91.9%, as measured by the absorbance.

Two components (8.1 and 4.7 A_{280} units respectively), in fraction A from the Solomon Islands population and in fraction I from the Philippines population, were desalted in 0.1M-acetic acid on a Sephadex G-25 (fine grade) column and freeze-dried (yields: component A, 6.0mg; component I, 3.8mg). Each component was pure on disc-gel electrophoresis at pH 4.0. Component A is named toxin *Laticauda colubrina* a (Lc a), and component I toxin *Laticauda colubrina* b (Lc b). Toxins Lc a and Lc b were obtained in larger amounts by repeating the experiments and pooling the products.

Fig. 1. CM-cellulose column chromatography of venoms of *Laticauda colubrina*

Each dried venom sample (50mg) from snakes from the Solomon Islands (a) and from the Philippines (b) was dissolved in 0.01M-phosphate buffer, pH 6.4 (10ml), and applied to a column (1.5cm $\times$ 20cm) of CM-cellulose equilibrated with the same buffer. The elution was in each case performed with a linear 0–0.5M-NaCl gradient in the buffer (1 litre in all) starting at arrow 1, and the flow rate was 45 ml/h. —, A_{280} ; ----, [NaCl].

The components contained in fractions B and C (Fig. 1a) and fractions II and III (Fig. 1b) were desalted and freeze-dried similarly. Fractions C and II contained short-chain neurotoxins, and fractions B and III contained components similar to phospholipases A of *Laticauda semifasciata* (Nishida *et al.*, 1982), judged from the molecular weight and the amino acid composition. These other components are not considered further in the present paper.

Fraction B contained, in addition to the phospholipase A-like component, a different type of neurotoxin. No long-chain neurotoxins were found in fractions other than the Lc a and Lc b fractions.

Fraction D, which was eluted at the position corresponding to that of toxin Lc b, with 3.9% yield by absorbance, on chromatography of the pooled venom from 13 snakes of the Solomon Islands population, showed a lethal activity of 0.5 $\mu\text{g/g}$ in mice. The observation implies that toxin Lc b is present, if at all, at less than 6% of the amount of toxin Lc a in the venom of the Solomon Islands population. The lethal activity of fraction D may well be due to contamination by the neurotoxin in fraction B.

Fraction IV, which was eluted at the position corresponding to that of toxin Lc a, with 0.4% yield by absorbance, on chromatography of the pooled venom from six snakes of the Philippines population, was not lethal when injected at the level of 1.5 $\mu\text{g/g}$ to mice. Toxin Lc a was therefore not detected in the venom from the Philippines population.

Lethal activity

The LD_{50} values of toxins Lc a and Lc b, determined by injection into the hind-leg muscles of mice, were both 0.12 $\mu\text{g/g}$ body wt.

Molecular weights and amino acid compositions

Molecular weights of toxins Lc a and Lc b were determined to be 7600 ($\pm 5\%$) and 7900 ($\pm 5\%$) respectively by gel filtration.

The results of amino acid analysis on 24h, 48h and 72h hydrolysates of toxins Lc a and Lc b are given in Table 1.

Both of them are composed of 69 amino acid residues, with eight half-cystine residues, and are devoid of methionine residues. Compared with toxin Lc b, the toxin Lc a molecule has one more residue each of lysine, aspartic acid, proline, leucine and phenylalanine, one fewer residue each of histidine, serine and isoleucine, and two fewer arginine residues. The molecular weights calculated from amino acid compositions of toxins Lc a and Lc b were 7522 and 7601 respectively.

Amino acid sequence of *Laticauda colubrina* a

Terminal amino acid sequences. Fifteen amino acids from the N-terminus of reduced and S-

carboxymethylated toxin Lc a were determined by Edman degradation to be:

The elution was performed with 0.11 M and 0.19 M buffers for peptides T-Ia and T-Ib respectively. Two

Arg-Ile-CmCys-Tyr-Leu-Ala-Pro-Arg-Asp-Thr-Gln-Ile-CmCys-Ala-Pro-

The amino acids released from reduced and *S*-carboxymethylated toxin Lc a by carboxypeptidase Y digestion are given in Table 4. These results gave the C-terminal sequence:

-Lys-Leu-Ala

Isolation and amino acid sequences of tryptic peptides. Reduced and *S*-carboxymethylated toxin lc a (22mg) was digested with trypsin in 0.05 M-Tris/HCl buffer, pH8.2 (3 ml), as described previously (Kim & Tamiya, 1981a). The digest was resolved into fractions T-I, T-II, T-III and T-IV by gel filtration on a Sephadex G-25 (fine grade) column (1.2 cm × 250 cm) in 0.1 M-acetic acid at elution volumes 143–163 ml, 168–199 ml, 204–235 ml and 240–260 ml respectively.

Fraction T-I gave two peptides T-Ia and T-Ib on chromatography through a DEAE-cellulose (Whatman DE-52) column (1.5 cm × 20 cm) equilibrated with 0.01 M-ammonium bicarbonate buffer, pH8.2.

Table 1. Amino acid compositions of toxins Lc a and Lc b
The composition of each toxin is given, with the nearest integer in parentheses.

Amino acid	Amino acid composition (mol/mol of toxin)	
	Toxin Lc a	Toxin Lc b
Trp*	0.95 (1)	0.92 (1)
Lys	6.01 (6)	5.02 (5)
His	1.09 (1)	1.74 (2)
Arg	3.05 (3)	4.84 (5)
Asp	7.00 (7)	6.00 (6)
Thr	5.10 (5)	4.64 (5)
Ser	1.74 (2)	2.58 (3)
Glu	4.03 (4)	4.04 (4)
Pro	6.45 (6)	5.37 (5)
Gly	5.97 (6)	6.08 (6)
Ala	4.99 (5)	4.89 (5)
CyS†	7.19 (8)	7.37 (8)
Val	1.29 (1)	1.18 (1)
Met	— (0)	— (0)
Ile	4.70 (5)	5.45 (6)
Leu	4.85 (5)	3.94 (4)
Tyr	2.04 (2)	1.54 (2)
Phe	1.97 (2)	1.00 (1)
Total residues	69	69

* Measured by hydrolysis with 4 M-methanesulphonic acid.
† Cystine residues tend to give smaller values. Eight half-cystine residues were found by sequence study.

peptides T-IIa and T-IIb were obtained from fraction T-II by the chromatography on the same column with 0.21 M and 0.27 M buffers respectively. The chromatography of fraction T-III on the same column gave three peptides T-IIIa, T-IIIb and T-IIIc with starting, 0.01 M and 0.08 M buffers respectively. Similarly, from fraction T-IV, two peptides T-IVa and T-IVb were eluted with 0.01 M and 0.28 M buffers respectively.

Peptides T-IIIc1 and T-IIIc2 were separated from the peptide T-IIIc fraction by paper electrophoresis in 0.1 M-pyridine/formic acid buffer, pH2.5, at 40 V/cm for 70 min with no. 50 filter paper (Toyo Roshi Co., Tokyo, Japan). After electrophoresis, the paper was dried, and the spots containing the peptides were cut out with the aid of guide strips and extracted with 10% (v/v) acetic acid at room temperature for 16 h.

The peptides thus isolated were pure on the basis of paper electrophoresis at pH4.8. The amino acid compositions of all the tryptic peptides are given in Table 2.

Peptides T-Ia, T-Ib, T-IIIa, T-IIIb, T-IIIc2 and T-IVa were obtained in relatively small yields because of incomplete digestion.

Each peptide (0.2–0.6 μmol), except peptides T-IVa, T-IIIc2 and T-IIIa, was subjected to Edman degradation. The results are shown in Fig. 2, together with the results of direct Edman degradation of reduced and *S*-carboxymethylated toxin Lc a.

The carboxypeptidase Y digestion of peptide T-IIb (Table 4) gave the C-terminal sequence:

-Tyr-Leu-Lys

The carboxypeptidase Y digestion of peptide T-IVb (Table 4) gave the sequence:

-Phe-Leu-Lys

In the carboxypeptidase Y digest of peptide T-Ia, lysine and isoleucine were found in the molar ratios 1:0.11 in 40 min and 1:0.59 in 1 h 40 min, lysine, isoleucine, and aspartic acid were found in the molar proportions 1:0.97:0.05 in 3 h, and lysine, isoleucine, aspartic acid and glycine were found in the molar proportions 1:1.18:0.15:0.02 in 8 h. These results suggest the C-terminal sequence of peptide T-Ia to be either:

-Gly-Ile-Asp-Ile-Lys

or

-Gly-Asp-Ile-Ile-Lys

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

The composition of each peptide is given, with the nearest integer in parentheses. Serine and glycine were considered to be negligible when they gave values less than 0.3. Other amino acids were neglected when they gave values less than 0.2.

Amino acid	Amino acid composition (mol/mol of peptide)									
	T-Ia	T-Ib	T-IIa	T-IIb	T-IIIa	T-IIIb	T-IIIc1	T-IIIc2	T-IVa	T-IVb
Lys	1.98 (2)	1.87 (2)	1.90 (2)	0.97 (1)						1.05 (1)
His			0.82 (1)							
Arg	0.97 (1)				1.00 (1)	1.77 (2)		0.90 (1)	1.0 (1)	
CmCys*	1.61 (2)	1.72 (2)	2.40 (3)	1.54 (2)		0.65 (1)		0.84 (1)		
Asp	2.00 (2)	1.31 (1)	2.00 (2)	1.00 (1)	0.95 (1)					2.00 (2)
Thr	1.86 (2)	1.88 (2)	1.13 (1)	0.88 (1)						0.99 (1)
Ser			0.86 (1)							0.91 (1)
Glu	1.00 (1)	1.14 (1)		2.62 (3)						
Pro	1.98 (2)	1.89 (2)	2.03 (2)	1.01 (1)		0.96 (1)		0.99 (1)		
Gly	2.96 (3)	2.20 (2)		0.88 (1)	0.91 (1)					2.02 (2)
Ala	1.99 (2)	1.84 (2)		0.85 (1)		1.00 (1)	1.00 (1)	1.09 (1)		
Val	1.49 (1)	1.45 (1)								
Ile	1.95 (2)	1.83 (2)		1.62 (2)		0.81 (1)		0.83 (1)		
Leu	0.94 (1)	0.96 (1)		0.87 (1)		0.92 (1)	0.95 (1)	1.05 (1)		1.02 (1)
Tyr				0.80 (1)		0.78 (1)		0.78 (1)		
Phe	0.92 (1)	1.03 (1)								0.99 (1)
Trp†										(1)
Total	22	19	12	15	3	8	2	7	1	10
Yield (%)	40.5	20.0	65.5	86.8	7.5	19.2	52.5	18.5	6.0	76.3

* Carboxymethylcysteine residues tend to give smaller values.

† Tryptophan was detected by the spot test with Ehrlich reagent.

The former sequence was adopted, on the basis of the sequence homology with toxin Lc b (cf. Fig. 3).

Alignment of fragment peptides. The alignment of tryptic peptides was deduced as shown in Fig. 2 mainly from the analysis of fragments of reduced and S-carboxymethylated toxin Lc a (6.0 mg) obtained by digestion with α -chymotrypsin. The digest was applied to a column (1.2 cm $\times$ 140 cm) of Sephadex G-25 (fine grade) in 0.1 M-acetic acid. Fractions C-I, C-II, C-III, C-IV and C-V were eluted at 81–99 ml, 102–117 ml, 120–132 ml, 135–153 ml and 156–180 ml respectively.

Fraction C-I gave two peptides C-Ia (Leu-32–Ala-69) and C-Ib (Gly-40–Ala-69) on chromatography through a DEAE-cellulose column (1.5 cm $\times$ 20 cm) equilibrated with 0.01 M-ammonium bicarbonate buffer, pH 8.2. The elution was performed with 0.19 M and 0.22 M buffers for peptides C-Ia and C-Ib respectively. The chromatography of fraction C-IV on the same column gave two peptides C-IVa (Arg-1–Tyr-4) and C-IVb (Leu-22–Phe-31) with 0.20 M and 0.25 M buffers respectively.

Fractions C-II, C-III and C-V each contained a single component peptide, C-II (Leu-5–Tyr-21), C-III (Leu-32–Phe-39) and C-V (Arg-1–Tyr-4) respectively. The resulting peptides gave single spots on paper electrophoresis at pH 4.8. Fig. 2 shows the whole amino acid sequence of toxin Lc a. Tryptic

peptides T-IIIb and T-IIb were placed at the N-terminus in this order from the results of Edman degradation of reduced and S-carboxymethylated toxin Lc a. Tryptic peptide T-IIIc1 was placed at the C-terminus because it was the only tryptic peptide without lysine or arginine and its sequence agreed with the C-terminal sequence of the whole molecule. Tryptic peptides T-Ia, T-IIa and T-IIIc1 were placed at the C-terminus in this order by the isolation and Edman degradation of chymotryptic peptide C-Ib. Tryptic peptide T-IVb was unavoidably placed between these N- and C-terminal sequences.

Amino acid sequence of Laticauda colubrina b

Terminal amino acid sequences. Fifteen amino acids from the N-terminus of reduced and S-carboxymethylated toxin Lc b were determined by Edman degradation to be identical with those of toxin Lc a.

The amino acids released by carboxypeptidase Y digestion of reduced and S-carboxymethylated toxin Lc b are given in Table 4. These results gave the C-terminal sequence of toxin Lc b:

-Lys-Leu-Ala

Isolation and amino acid sequences of tryptic peptides. Reduced and S-carboxymethylated toxin

Fig. 2. Amino acid sequence of toxin *Laticauda colubrina* a

Peptides are shown by the horizontal bars (↔ and ↔). The prefixes T and C refer to peptides produced by digestion with trypsin and α -chymotrypsin respectively. — and — indicate amino acid residues detected by Edman degradation and carboxypeptidase Y digestion respectively.

Lc b (15 mg) was digested with trypsin in 0.05 M-Tris/HCl buffer, pH 8.2 (2 ml). From the digest, fractions T-1, T-2, T-3 and T-4 were separated by chromatography on a Sephadex G-25 (fine grade) column (1.2 cm $\times$ 250 cm) in 0.1 M-acetic acid.

Four peptides T-1a, T-1b, T-1c and T-1d were obtained from fraction T-1 by chromatography on a DEAE-cellulose column (1.5 cm $\times$ 17 cm) equilibrated with 0.01 M-ammonium bicarbonate buffer, pH 8.2. The elution was performed with 0.10 M, 0.15 M, 0.22 M and 0.30 M buffers for peptides T-1a, T-1b, T-1c and T-1d respectively. Chromatography of fraction T-2 on the same column gave peptides T-2a and T-2b with starting buffer and peptide T-2c with 0.25 M buffer. Similarly, fraction T-3 gave two peptides T-3a and T-3b with 0.01 M and 0.09 M buffers respectively, and fraction T-4 was separated into three peptides T-4a, T-4b and T-4c with starting, 0.11 M and 0.26 M buffers respectively.

All the peptides isolated were pure on the basis of paper electrophoresis at pH 4.8. The amino acid compositions of tryptic peptides are given in Table 3. Peptides T-1c and T-2c were indistinguishable from each other in their amino acid composition, mobility in electrophoresis and elution positions from the DEAE-cellulose column. These results indicate that a single peptide was distributed in both frac-

tions T-1 and T-2 on Sephadex G-25 column chromatography.

Each peptide (0.2–0.4 μ mol), except for peptides T-4a, T-3b, T-1b and T-2a, was subjected to Edman degradation. The results are shown in Fig. 3. Carboxypeptidase Y digestion of peptides T-1d, T-4c, T-1b and T-2a released the amino acids shown in Table 4. These results gave the C-terminal sequences of the peptides as shown in Fig. 3.

The alignment of tryptic peptides as shown in Fig. 3 was deduced on the basis of sequence homology with toxin Lc a at positions 8–9, 23–24, 33–34 and 55–56. The alignment at positions 49–50–51 was established by the isolation of two pairs of tryptic peptides, T-1a and T-2b, and T-1b and T-2a. Peptide T-4b was placed at the C-terminus for the same reasons as for peptide T-IIIc1 from toxin Lc a.

Discussion

Both toxin Lc a and toxin Lc b are composed of molecules containing 69 amino acid residues including eight half-cystine residues, and their amino acid sequences have been elucidated in the present study. The chemical structures of toxins Lc a and Lc b are novel as compared with those of previously known

Table 3. *Tryptic peptides from reduced and S-carboxymethylated toxin Lc b*

The composition of each peptide is given, with the nearest integer in parentheses. Serine and glycine were considered to be negligible when they gave values less than 0.3. Other amino acids were neglected when they gave values less than 0.2.

Amino acid	Amino acid composition (mol/mol of peptide)											
	T-1a	T-1b	T-1c	T-1d	T-2a	T-2b	T-2c	T-3a	T-3b	T-4a	T-4b	T-4c
Lys	1.05 (1)	1.06 (1)	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)	2.00 (2)					
His			0.88 (1)		0.73 (1)	0.77 (1)	0.93 (1)					
Arg	1.79 (2)	1.05 (1)			1.08 (1)			1.85 (2)	1.09 (1)	1.00 (1)		0.94 (1)
CmCys	1.64 (2)	1.75 (2)	2.56 (3)	1.63 (2)			2.67 (3)	0.91 (1)	0.90 (1)			
Asp	1.14 (1)	1.00 (1)	2.14 (2)	1.04 (1)			2.15 (2)					2.01 (2)
Thr	1.95 (2)	1.85 (2)	1.09 (1)	0.97 (1)			1.04 (1)					0.96 (1)
Ser			0.84 (1)				1.03 (1)					1.86 (2)
Glu	0.95 (1)	0.94 (1)		2.70 (3)								
Pro	1.14 (1)	1.17 (1)	1.91 (2)	1.08 (1)			1.88 (2)	1.11 (1)	1.01 (1)			
Gly	1.80 (2)	1.80 (2)		1.08 (1)	0.84 (1)	1.01 (1)						1.93 (2)
Ala	1.76 (2)	1.88 (2)		0.82 (1)				0.95 (1)	0.98 (1)		1.00 (1)	
Val	0.95 (1)	1.03 (1)										
Ile				1.58 (2)	1.68 (2)	1.84 (2)		0.84 (1)	0.87 (1)			1.00 (1)
Leu	0.89 (1)	0.96 (1)		0.83 (1)				1.10 (1)	1.02 (1)		1.01 (1)	
Tyr				0.92 (1)				0.94 (1)	0.93 (1)			
Phe	0.90 (1)	0.93 (1)										
Trp*												(1)
Total	17	16	12	15	6	5	12	8	7	1	2	10
Yield (%)	45.0	33.9	57.2	73.3	23.3	30.0	23.3	42.8	27.8	31.1	67.8	73.9

* Tryptophan was detected by the spot test with Ehrlich reagent.

Table 4. *Amino acids released from reduced and S-carboxymethylated toxins Lc a and Lc b and from some of their tryptic peptides by carboxypeptidase Y digestion*

Amino acid	Toxin Lc a									Toxin Lc b														
	T-IIb						T-IVb			T-1b						T-1d			T-2a			T-4c		
	2h	4h	1h	2h	4h	1h	2h	4h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h		
Lys	0.28	0.39	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	0.34	0.39	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*					
Arg																				1.0*	1.0*	1.0*		
Thr											0	0.04	0.11											
Ser																				0	0.07	0.18		
Ala	1.0*	1.0*							1.0*	1.0*														
Val											0.89	0.84	0.92											
Ile																			0.15	0.38	0.60	0.46	0.79	0.84
Leu	0.87	0.90	0.99	0.96	0.94	0.99	0.96	0.99	0.88	0.92				0.87	0.87	0.88								
Tyr			0.21	0.35	0.61									0.18	0.36	0.56								
Phe						0.08	0.16	0.34																

* The amount of these amino acids are taken as unity.

neurotoxins, namely short-chain and long-chain toxins. Although these toxins have four disulphide bridges per molecule, similarly to the short-chain neurotoxins and differently from the long-chain neurotoxins, which have five disulphide bridges, the whole amino acid sequences of these two new toxins are more similar to those of long-chain neurotoxins than to those of short-chain neurotoxins. The

sequences of toxins Lc a and Lc b are compared with some other long-chain neurotoxins in Fig. 4.

Two half-cystine residues, which form an 'extra loop' between positions 30 and 34 (Fig. 4), were previously considered to be invariant among long-chain neurotoxins. The two residues are, however, replaced by aspartic acid and glycine residues in the sequences of toxins Lc a and Lc b. The previously

Fig. 3. Amino acid sequence of toxin *Laticauda colubrina* b
Details are the same as in Fig. 2 legend.

adopted definition of long-chain neurotoxins, namely that they consist of 70–74 amino acid residues with five disulphide bridges, must be revised, as follows. The long-chain neurotoxins consist of 66–74 amino acid residues (including the medium-chain neurotoxin *L. semifasciata* III) with four or five disulphide bridges. Although the cysteine/glycine replacement can be introduced by a single base replacement, the cysteine/aspartic acid replacement needs two base replacements in the codon.

It was suggested by Maeda & Tamiya (1978) that the C-terminal carboxy group of long-chain neurotoxin is salt-linked to the amino group of lysine-35, which is nearly invariant among the long-chain neurotoxins. The lysine-35 is replaced by an asparagine residue in the sequences of toxins Lc a and Lc b. This substitution may replace the salt-linkage by a hydrogen bond.

It is noteworthy that, in spite of the replacements mentioned above, toxins Lc a and Lc b are fully active as compared with the other neurotoxins. It is noteworthy that, in connection with these observations, the peptide bonds at Trp-25–Asp-26 and Arg-36–Leu-37 of reduced and *S*-carboxymethylated toxins Lc a and Lc b resisted α -chymotrypsin and trypsin actions respectively.

There are five amino acid replacements between

toxins Lc a and Lc b, namely phenylalanine/serine at position 31, leucine/isoleucine at position 32, lysine/arginine at position 33, proline/arginine at position 50 and aspartic acid/histidine at position 53. Each replacement can be introduced by a single base replacement in the codon. Toxin Lc a has a lysine residue at position 33; the position has been occupied only by an arginine residue in previously known neurotoxins.

It is remarkable that *L. colubrina* snakes from the Solomon Islands or the Philippines have either toxin Lc a or toxin Lc b respectively in their venom. Toxin Lc b is present, if at all, in the venom of the Solomon Islands population at less than 6% of the amount of toxin Lc a. Toxin Lc a was not detected in the venom of the Philippines population. The situation is quite different from what is observed with *Laticauda semifasciata*, a sea snake of the same genus. In *L. semifasciata*, short-chain neurotoxins, namely erabutoxins a, b and c, which are different from one another at their one (a/b and b/c) or two (a/c) residues out of their 62 amino acid residues, are found in the venom of individual snakes (Tamiya, 1973) and the ratio of the contents of the toxins remains constant for a given individual, although the ratio varies from one snake to another (Takeda *et al.*, 1974).

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