Biochem. J. (1972) **130**, 547–555
Printed in Great Britain

The Isolation, Properties and Amino Acid Sequence of Erabutoxin c, a Minor Neurotoxic Component of the Venom of a Sea Snake *Laticauda semifasciata*

By NOBUO TAMIYA and HISAKU ABE
Department of Chemistry, Tohoku University, Sendai, Japan

(*Received* 4 *July* 1972)

Erabutoxin c, a minor neurotoxic component of the venom of a sea snake *Laticauda semifasciata*, was isolated in pure form by repeated column chromatography on CM-cellulose columns. The toxin was crystallizable and monodisperse in rechromatography, disc electrophoresis and isoelectric focusing (isoelectric point, pH9.23–9.25). The molecular weight of the toxin, as estimated by gel filtration, was 7000. The toxin showed the same lethal activity to mice (0.13 μg/g body wt., intramuscular injection) and the same effect on isolated frog muscle as erabutoxins a and b, the main toxic components of the venom. The toxin inhibited the acetylcholine contracture but not the potassium chloride contracture of muscle. Erabutoxin c consisted of 62 amino acid residues, containing one fewer lysine and one more histidine than erabutoxin a and one fewer lysine and one more aspartic acid (or asparagine) than erabutoxin b. Erabutoxin c was reduced, *S*-carboxymethylated and hydrolysed with trypsin. The only fragment different from the corresponding fragments from erabutoxin b was hydrolysed further with pepsin. One of the peptic fragments, which was assumed to have the aspartic acid (or asparagine) residue in question at the *C*-terminal end, was treated with carboxypeptidase A. The *C*-terminal residue was found to be an asparagine. It was therefore concluded that erabutoxin c was [51-asparagine]-erabutoxin b.

Erabutoxins a and b are the principal neurotoxic components of the venom of a sea snake *Laticauda semifasciata*, more than 90% of the lethal activity of the venom being ascribed to these toxins (Tamiya & Arai, 1966). The amino acid sequences (Sato & Tamiya, 1971) and the positions of disulphide bridges (Endo *et al.*, 1971) of the toxins have been elucidated. The toxins are typical examples of a group of neurotoxic proteins found in sea snake and cobra venoms (Eaker & Porath, 1967; Yang *et al.*, 1969; Botes & Strydom, 1969; Strydom & Botes, 1971; Botes *et al.*, 1971; Mebs *et al.*, 1971; Nakai *et al.*, 1971; Botes, 1971).

There are some minor toxic components in the venom. One of them, recognized by its elution point from the CM-cellulose column, was named erabutoxin c (Uwatoko-Setoguchi, 1970). The present paper describes the isolation, some properties and the amino acid sequence of erabutoxin c.

Materials and Methods

Materials

Sea snakes. The sea snakes *Laticauda semifasciata* were collected at Amami and Miyako Islands, Japan. The snake heads were kept frozen until use.

Erabutoxins a and b. Erabutoxins a and b were prepared as described by Tamiya & Arai (1966), desalted by ultrafiltration in a Diaflo apparatus (Amicon Corp., Lexington, Mass., U.S.A.) with a UM-2 filter in 0.1M-acetic acid and freeze-dried.

Proteases. Trypsin (twice-crystallized and salt-free) from Worthington Biochemical Corp., Freehold, N.J., U.S.A., was treated with diphenylcarbamoyl chloride (Erlanger *et al.*, 1966). Pepsin (twice-crystallized, Worthington) and di-isopropyl phosphorofluoridate-treated carboxypeptidase A (Carboxypeptidase-A DFP, dialysed and recrystallized, toluene–water suspension; Sigma Chemical Co., St. Louis, Mo., U.S.A.) were commercial preparations.

Methods

Column chromatography. The elution of proteins or peptides from the columns was followed by measuring extinction at 280 or 230nm, either continuously by using a Toyo Uvicon 540M (Toyo Scientific Instruments Co., Tokyo, Japan) or on fractions with a Hitachi–Perkin–Elmer 139 spectrophotometer (Hitachi Co., Tokyo, Japan).

Disc electrophoresis. The disc electrophoresis of the toxins was carried out at pH4.0 according to Reisfeld

et al. (1962), with slight modifications described previously (Tamiya & Arai, 1966).

Gel filtration. The molecular weight of erabutoxin c was estimated by gel filtration with Sephadex G-100 in 0.05M-tris hydrochloride buffer, pH7.5, containing 0.1M-potassium chloride. Blue Dextran, lysozyme, erabutoxin b and sucrose were used as standards (Andrews, 1965).

Electrofocusing. The electrofocusing experiments were carried out by LKB 8100 Ampholine electrofocusing equipment according to the instruction manual (LKB Produkter AB, Bromma, Sweden) at pH8–10 with the cathode at the top and the anode at the bottom. Fractions (2g and 3g) were collected in two runs of experiments and measured for the extinction at 280nm and for pH values with the Hitachi 124 spectrophotometer (Hitachi Co., Tokyo, Japan) and the TOA Dempa HM6A pH-meter (TOA Dempa, Tokyo, Japan) respectively.

Paper electrophoresis. The paper electrophoresis of tryptic and peptic fragments was carried out at pH4.8 as described by Sato & Tamiya (1971).

Amino acid analysis. This was done as described by Sato & Tamiya (1971). The carboxypeptidase A digest was analysed also by an automatic analyser. Asparagine appeared at the same position as serine in the automatic analysis. Asparagine added to the control enzyme reaction mixture without the substrate was taken as colour standard.

Measurement of toxicity. The toxicity of erabutoxin c was determined in mice by intramuscular injection of 0.1–0.2ml of a solution in 0.85% sodium chloride as described previously for erabutoxins a and b (Tamiya & Arai, 1966).

Action of erabutoxin c on isolated muscle. The mode of action of erabutoxin c on an isolated rectus abdominis muscle of a frog *Rana nigromaculata* was studied as described previously (Tamiya & Arai,

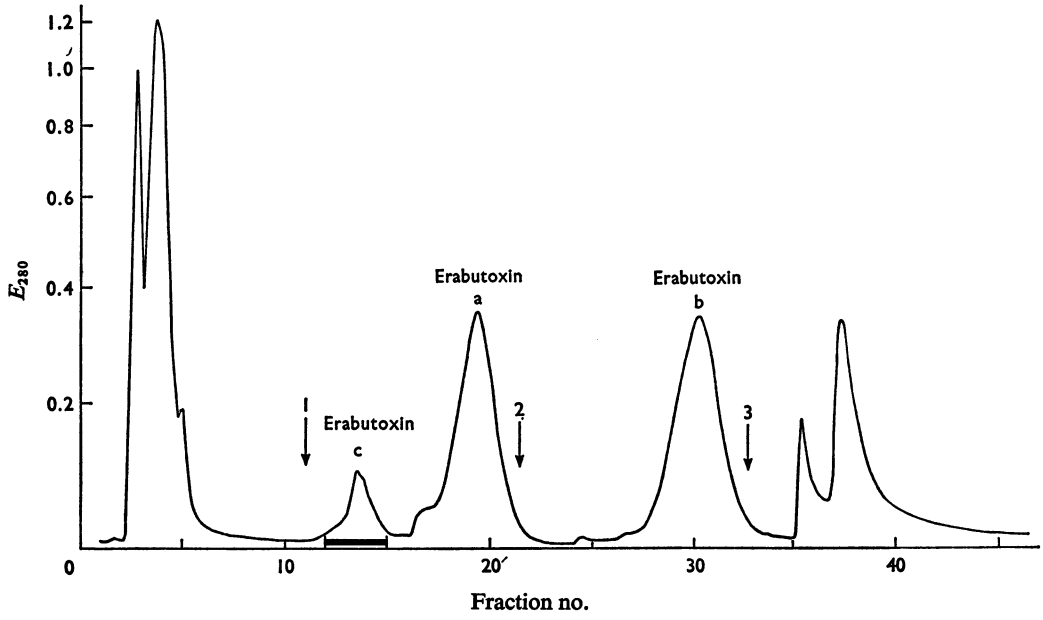

Fig. 1. *CM-cellulose column chromatography of Laticauda semifasciata venom*

Minced glands (2.62g) of ten snakes were extracted as described in the text. The concentrated and desalted extract (total $E^{280}_{1\,cm}$ 125) was chromatographed on a CM-cellulose column (1.7cm × 23cm) with 0.01M-phosphate buffer, pH6.4, at a flow rate of 110ml/h and the elution of proteins was followed by a Toyo Uvicon 540M apparatus at 280nm. Fractions of 18.9g were collected. At arrows 1, 2 and 3, 0.01M-, 0.02M- and 0.1M-sodium chloride respectively were added to the elution buffer. The fractions indicated by the horizontal bar were pooled and subjected to further purification as shown in Fig. 2. The erabutoxin c content, according to the extinction, was 2.2% of the proteins in the extract, whereas content of erabutoxins a and b was 15.4 and 19.3% respectively (Tamiya, 1972).

1966), with SB-1T force-displacement transducers and a multipurpose recorder RM-20 (Nihon Koden Co., Tokyo, Japan).

Results

Isolation of erabutoxin c

Venom glands were taken out from the snake heads, minced with scissors and extracted with an equivalent (w/v) volume of 0.1M-acetic acid. The gland debris was collected by filtration on two layers of gauze and the extraction was repeated six times. The combined extract was centrifuged (9000rev./min for 10min), concentrated and desalted in the Diaflo apparatus with a UM-2 filter and the solvent was replaced with 0.01M-phosphate buffer, pH6.4, on the filter. The final volume was adjusted to about one-third of the original extract and the precipitate removed by centrifugation (4000rev./min for 10min).

The concentrated extract was applied to a CM-cellulose column, which had first been washed thoroughly with 0.025M-sodium hydroxide containing 0.5M-sodium chloride, with water and with 0.01M-phosphate buffer, pH6.4. The elution was carried out with 0.01M-phosphate buffer, pH6.4, with stepwise addition of 0.01M-, 0.02M- and 0.1M-sodium chloride to the buffer. An example of the chromatographic pattern is shown in Fig. 1. The fractions indicated by a bar in the figure were collected from repeated chromatography and kept frozen at −20°C until an adequate amount was accumulated. The later fractions from the chromatography were used for the preparation of erabutoxins a and b.

The pooled erabutoxin c fractions were concentrated in the Diaflo apparatus with a UM-2 filter and chromatographed again on another CM-cellulose column by stepwise change of pH of the elution buffer (Fig. 2). Chromatographically homogeneous erabutoxin c was thus isolated. The final pooled erabutoxin c fractions were concentrated, desalted with 0.1M-acetic acid in the Diaflo apparatus with a UM-2 filter and freeze-dried. The toxin was obtained as a white powder.

In some cases, when the first or second chromatographic separation of erabutoxin c was not very clear, another chromatography step under the same conditions as those of Fig. 1 was added.

Crystallization of erabutoxin c

Erabutoxin c (1.75mg) was dissolved in 0.01M-phosphate buffer, pH6.4 (0.5ml), and saturated ammonium sulphate solution (0.42ml) was added to the solution. The mixture was kept at 37°C for a month. The crystals obtained are shown in Fig. 3.

Properties of erabutoxin c

Disc electrophoresis. Erabutoxin c migrated toward the cathode at pH4.0 as a single band at the same speed as erabutoxin a.

Isoelectric focusing. Erabutoxin c gave a single peak at pH9.23–9.25 in two runs of electrofocusing with carrier Ampholyte 8-10 (Fig. 4).

Gel filtration. Erabutoxin c (1.68mg) was eluted as a single peak at 2.53 times the void volume (84.0ml) from a Sephadex G-100 column (1.65cm×107cm). Co-filtration of erabutoxin c (2.3mg) with erabutoxin b (4.8mg, mol.wt. 6861) gave a single elution peak at the same volume. Lysozyme (2.0mg) and sucrose (5.4mg) were eluted at 2.40 and 2.84 times the void volume respectively from the same column. The flow rate was 7.9ml/h and 1.64ml fractions were collected. Sucrose was detected by the anthrone method.

Toxicity. The LD_{50} value of erabutoxin c to mice

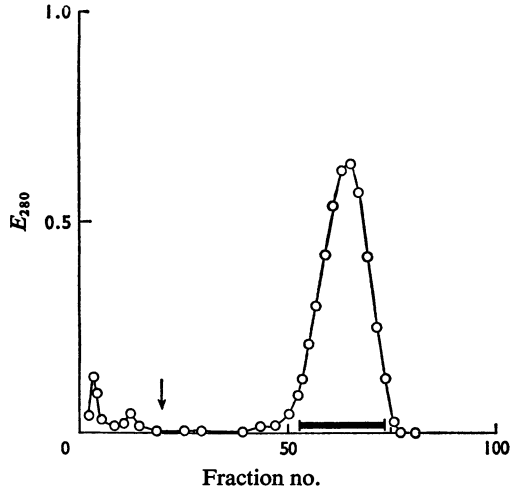

Fig. 2. *Second CM-cellulose column chromatography of erabutoxin c fractions*

The pooled and freeze-dried erabutoxin c (see the text, 40.9mg) from the first chromatographic step (Fig. 1) was dissolved in 0.01M-phosphate buffer, pH5.9 (4.0ml), and chromatographed on a CM-cellulose column (1.3cm×16.5cm), which had been washed and equilibrated with the buffer. The pH of the elution buffer was raised to 6.1 at the point indicated by the arrow. The flow rate was 45ml/h and 3.8ml fractions were collected. The erabutoxin c contained in the fractions indicated by the horizontal bar was collected, desalted and freeze-dried (yield 21.3mg).

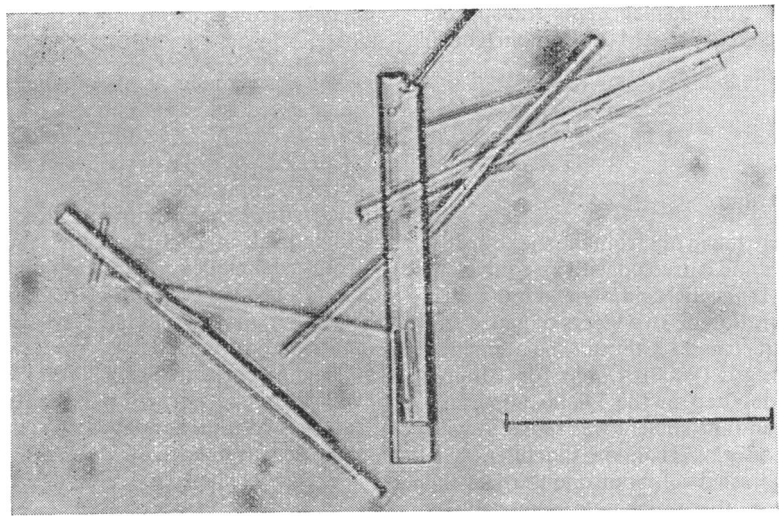

Fig. 3. *Crystals of erabutoxin c*

The scale mark indicates 0.1 mm.

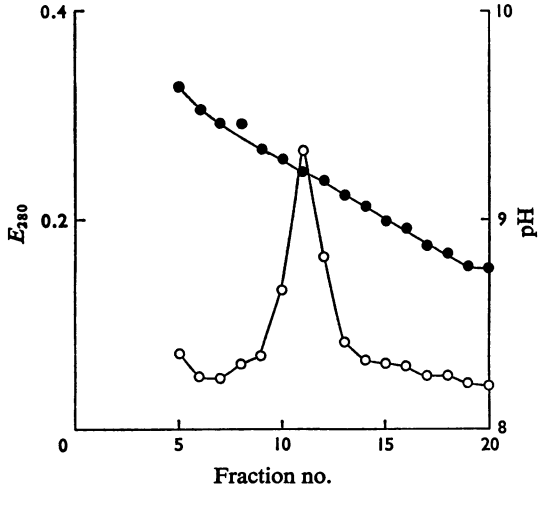

Fig. 4. *Isoelectric focusing of erabutoxin c*

Erabutoxin c (2mg) was subjected to isoelectric focusing with arginine hydrochloride (80mg) and carrier Ampholyte 8–10 (40% soln.; 2ml) in a total volume of 100ml. After 2 days at 200V, 2 days at 400V and 1 day at 500V, 2.0g fractions were collected and the pH (●) and extinction at 280nm (○) were measured.

by intramuscular injection was the same as that for erabutoxins a and b, being 0.13 μg/g body wt.

Action on isolated muscle. The erabutoxin c (1.3 μg/ml of Ringer–glucose solution) completely inhibited the contracture of an isolated rectus abdominis muscle of a frog induced by the addition of acetylcholine (0.6 μg/ml of Ringer–glucose solution). The toxin at the same concentration, however, did not affect contracture of the muscle induced by the addition of potassium chloride (final concn. 0.05M).

Amino acid composition. The molar proportions of the amino acids in the hydrolysates of erabutoxin c and reduced and *S*-carboxymethylated erabutoxin c (see below) are shown in Table 1. The amino acid contents of erabutoxins a and b are also shown for comparison. The molecular weight calculated assuming an amino acid content of 62 agrees well with the observed value of 7000. The erabutoxin c contains one fewer lysine and one more histidine than erabutoxin a and one fewer lysine and one more aspartic acid (or asparagine) than erabutoxin b.

Amino acid sequence. The reduction and S-carboxymethylation of erabutoxin c (62mg) was performed as described previously (Sato & Tamiya, 1971) by the method of Crestfield *et al.* (1963). The *S*-carboxymethylated erabutoxin c (5.00 μmol) was digested with diphenyl carbamoyl chloride-treated trypsin (0.8mg) in 0.1M-phosphate buffer, pH7.8, for 20h at room temperature. The tryptic peptides were

Table 1. *Amino acid analysis of erabutoxin c and of reduced and S-carboxymethylated erabutoxin c*

Values in parentheses give the nearest whole numbers.

	Molar proportions of amino acids				
	Erabutoxin c		Reduced and *S*-carboxymethylated erabutoxin c	Erabutoxin a¶	Erabutoxin b¶
Time of hydrolysis (h)	28	53	24		
Lysine	2.99	2.91	2.98 (3)	4	4
Histidine	1.64	1.66	1.89 (2)	1	2
Arginine	2.6	2.45	3.35 (3)	3	3
S-Carboxymethylcysteine			6.96*		
Aspartic acid	5.38	5.06	5.11 (5)	5	4
Threonine	5.22	4.90	4.61 (5)	5	5
Serine	6.92	6.27	7.44 (8)	8	8
Glutamic acid	6.87	6.82	8.21 (8)	8	8
Proline	4.20	4.27	4.24 (4)	4	4
Glycine	4.83	4.77	4.78 (5)	5	5
Alanine	0	0	0 0	0	0
Cystine	7.62	7.86	(8)	8	8
Valine	2.10	2.16	1.92 (2)	2	2
Methionine	0	0	0 0	0	0
Isoleucine	3.48	3.77	3.18 (4)	4	4
Leucine	1.35	1.28	0.98 (1)	1	1
Tyrosine	1.45	1.48	0.90 (1)	1	1
Phenylalanine	1.92	1.90	1.96 (2)	2	2
Tryptophan			(1)	1	1
Total			62	62	62
Formula wt.			6847	6838	6861
Mol.wt.			7000†	7430‡	7430‡

*S-Carboxymethylcysteine tends to give a lower value.
† Estimated by gel filtration.
‡ Determined by ultracentrifugation.
¶ Sato *et al.* (1969); Sato & Tamiya (1971).

separated from each other by Sephadex G-10 column chromatography and by paper electrophoresis at pH4.8 and analysed for the amino acid contents. The results are summarized in Table 2. The six tryptic fragments from reduced and *S*-carboxymethylated erabutoxin c correspond to the total amino acid composition of erabutoxin c. Five fragments out of six coincided with the corresponding fragments from erabutoxin b (Sato & Tamiya, 1971). The only different peptide (T_4) was the largest and the most acidic of all and had an amino acid composition corresponding to the sum of two *C*-terminal tryptic peptides from erabutoxin b, except that peptide T_4 contained one fewer lysine and one more aspartic acid (or asparagine).

The peptide T_4 (1.83μmol) was digested with pepsin (0.12mg) in 0.01M-hydrochloric acid (0.5ml) at 37°C for 22h and freeze-dried. The peptic fragments were separated from each other by paper electrophoresis at pH4.8. The yields and the amino acid compositions of the peptic peptides from the tryptic peptide T_4 are given in Table 3. The amino acid compositions of the five peptides (P_1, P_2, P_3, P_6 and P_7) correspond to the amino acid composition of peptide T_4, whereas the two peptides with small yields (P_4 and P_5) correspond to the sum of peptides P_3 and P_7 and P_2 and P_6.

The peptic peptide P_1 (0.30μmol) was treated with carboxypeptidase A (0.2mg) in 0.05M-tris hydrochloride buffer, pH8.0 (1.0ml). After 0, 20 and 51h at 37°C, each 0.3ml of the reaction mixture was subjected to amino acid analysis. Asparagine (0.3μmol,

Table 2. *Amino acid compositions of tryptic peptides from reduced and S-carboxymethylated erabutoxin c*

Reduced and *S*-carboxymethylated erabutoxin c (5.00 μmol) was digested with diphenylcarbamoyl chloride-treated trypsin and the resulting peptides were separated as described in the text. The results are expressed as molar proportions of the amino acids. Values in parentheses give the nearest whole numbers.

	Fragment filtrate from filter UM-05	Fragment absorbed on Sephadex G-10	Peptides separated by paper electrophoresis				Total	Erabutoxin c
			T_1	T_2	T_3	T_4		
Lysine			1.00 (1)		0.93 (1)	0.94 (1)	3	3
Histidine			0.98 (1)		0.93 (1)		2	2
Arginine	(1)	1.01 (1)		1.12 (1)			3	3
S-Carboxymethylcysteine			0.75 (1)		1.88 (2)	4.84 (5)	8	8
Aspartic acid		1.00* (1)	1.00* (1)			3.00* (3)	5	5
Threonine			1.84 (2)	0.91 (1)	0.90 (1)	0.79 (1)	5	5
Serine		1.04 (1)	1.81 (2)		2.58 (3)	1.81 (2)	8	8
Glutamic acid		1.05 (1)	2.92 (3)	1.00* (1)	1.00* (1)	1.99 (2)	8	8
Proline			0.98 (1)		0.94 (1)	1.92 (2)	4	4
Glycine				0.98 (1)	1.39 (1)	3.46 (3)	5	5
Valine						1.68 (2)	2	2
Isoleucine			0.88 (1)	1.51 (2)		1.01 (1)	4	4
Leucine						1.00 (1)	1	1
Tyrosine					0.91 (1)		1	1
Phenylalanine		0.99 (1)	1.00 (1)				2	2
Tryptophan		1 (1)					1	1
Total	1	6	14	6	12	23	62	62
Yields (%)	100	43	36	36	23	36		

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

100%) and isoleucine (0.15 μmol, 50%) were detected in both 20 and 51 h digests. It is therefore concluded that erabutoxin c is [51-asparagine]-erabutoxin b.

Discussion

Erabutoxin c is monodisperse in rechromatography on a CM-cellulose column, disc electrophoresis, isoelectric focusing and gel filtration. The toxin is crystallizable. The toxicity (LD_{50} = 0.13 μg/g body wt.; intramuscular injection into mice) and the mode of action are the same as those of erabutoxins a and b. Erabutoxin c inhibits the acetylcholine contracture of an isolated frog muscle but does not affect the potassium chloride contracture of the muscle.

The amino acid analysis and the molecular weight estimation of erabutoxin c show that the toxin consists of 62 amino acid residues, as do erabutoxins a and b. A comparison of the tryptic peptides of reduced and *S*-carboxymethylated erabutoxin c with those of reduced and *S*-carboxymethylated erabutoxin b showed that erabutoxin c was [51-aspartic acid (or asparagine)]-erabutoxin b. Further hydrolysis of the peptide in question with pepsin and carboxypeptidase A showed that the residue 51 was an asparagine. The course of the sequence determination is shown schematically in Fig. 5. The structure of erabutoxin c, assuming the same disulphide bridges as erabutoxin a (Endo *et al.*, 1971), cobrotoxin (Yang *et al.*, 1970) or toxin α of *Naja nivea* (Botes, 1971), is shown in Fig. 6. The structures of erabutoxins a and b are also given for comparison.

It is noteworthy that three very similar toxins are found in the same venom. Erabutoxin a is [26-asparagine]-erabutoxin b and erabutoxin c is [51-asparagine]-erabutoxin b. It was found that a single snake has all three or at least two toxins in the venom, although the proportions of erabutoxins a, b and c vary (Tamiya, 1972). The mechanism by which a snake synthesizes three toxins with single amino acid replacements is of interest.

Table 3. *Amino acid compositions of peptic peptides from peptide T_4 of reduced and S-carboxymethylated erabutoxin c*

Tryptic peptide T_4 (1.83 μmol) was digested with pepsin and the resulting peptides were separated by paper electrophoresis. The results are expressed as molar proportions of the amino acids. Values in parentheses give the nearest whole numbers.

Peptic peptides ...	P_1	P_2	P_3	P_4 (P_3+P_7)	P_5 (P_2+P_6)	P_6	P_7	Total $P_1+P_2+P_3+P_6+P_7$	Peptide T_4
Lysine	0.74 (1)							1	1
S-Carboxymethylcysteine	1.69 (2)	1.00 (1)	0.53 (1)	1.57 (2)	0.80 (1)		1 (1)	5	5
Aspartic acid	1.00* (1)	2.00* (2)			2.00* (2)			3	3
Threonine	0.93 (1)							1	1
Serine			0.77 (1)	1.16 (1)	1.09 (1)	1.09 (1)		2	2
Glutamic acid					1.71 (2)	2.00* (2)		2	2
Proline	1.83 (2)							2	2
Glycine	2.96 (3)							3	3
Valine	0.75 (1)	0.92 (1)			0.87 (1)			2	2
Isoleucine	0.91 (1)							1	1
Leucine			1.00* (1)	1.00* (1)				1	1
Total	12	4	3	4	7	3	1	23	23
Yields (%)	99	40	31	12	12	76	33		

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

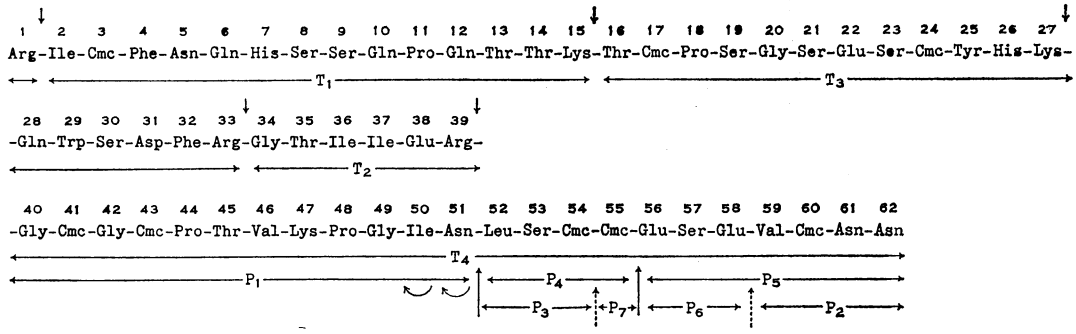

Fig. 5. *Schematic presentation of enzymic digestion of reduced and S-carboxymethylated erabutoxin c*

Fragments T_1 etc. and P_1 etc. are denoted by horizontal arrows. ↓, Bonds cleaved with trypsin; ↑, bonds cleaved with pepsin (bonds partially cleaved with pepsin are marked with broken arrows). Hydrolysis with carboxypeptidase A is denoted by curved arrows. Fragment 1 was separated by ultrafiltration and fragments 28–33 by absorption on a Sephadex G-10 column. Cmc, *S*-carboxymethylcysteine.

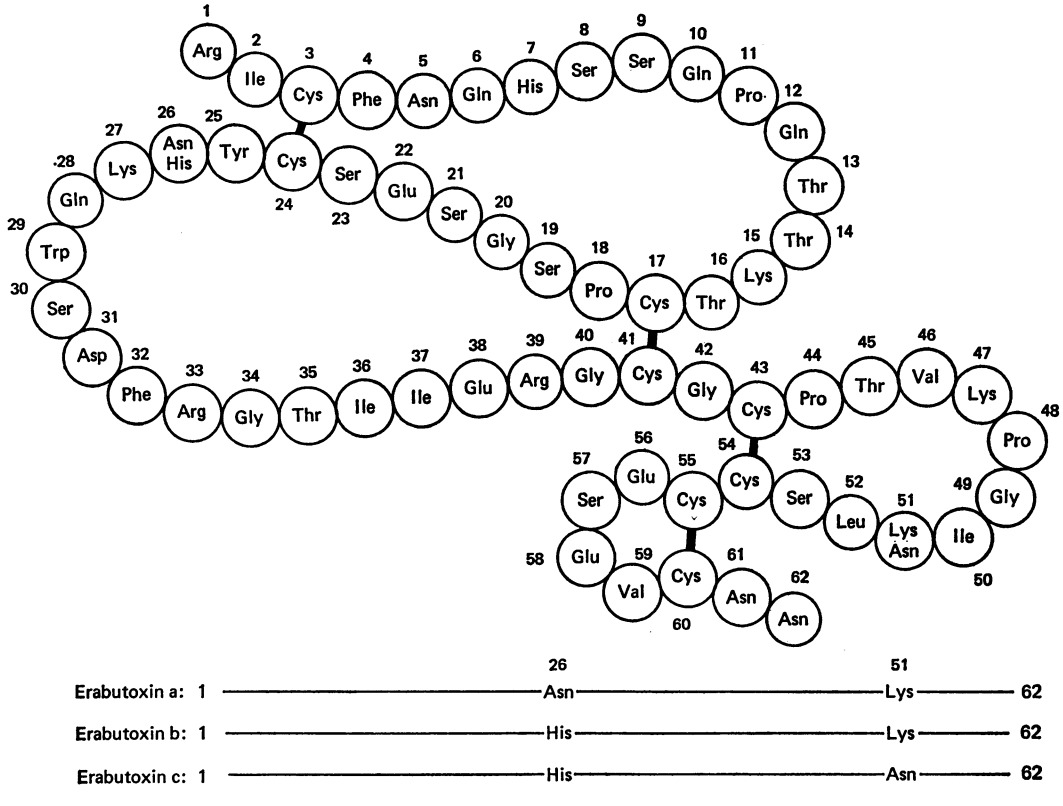

Fig. 6. *Structure of erabutoxins a, b and c*

The study of toxin homologues is also of interest for the deduction of minimal structure requirements for the neurotoxic activity.

The work was supported by grants from Kurata Memorial Funds and from the Ministry of Education, Japanese Government.

References

Andrews, P. (1965) *Biochem. J.* **96**, 595–605
Botes, D. P. (1971) *J. Biol. Chem.* **246**, 7383–7391
Botes, D. P. & Strydom, D. J. (1969) *J. Biol. Chem.* **244**, 4147–4157
Botes, D. P., Strydom, D. J., Andersen, C. G. & Christensen, P. A. (1971) *J. Biol. Chem.* **246**, 3132–3139
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
Erlanger, B. F., Cooper, A. G. & Cohen, W. (1966) *Biochemistry* **5**, 190–196
Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. & Lee, C. Y. (1971) *Biochem. Biophys. Res. Commun.* **44**, 711–723
Nakai, K., Sasaki, T. & Hayashi, K. (1971) *Biochem. Biophys. Res. Commun.* **44**, 893–897
Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
Sato, S. & Tamiya, N. (1971) *Biochem. J.* **122**, 453–461
Sato, S., Yoshida, H., Abe, H. & Tamiya, N. (1969) *Biochem. J.* **115**, 85–90
Strydom, A. J. C. & Botes, D. P. (1971) *J. Biol. Chem.* **246**, 1341–1349
Tamiya, N. (1972) *Toxicon* in the press
Tamiya, N. & Arai, H. (1966) *Biochem. J.* **99**, 624–630
Uwatoko-Setoguchi, Y. (1970) *Acta Med. Univ. Kagoshima* **12**, 73–95
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**, 355–363