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Three Neurotoxins from the Venom of a Sea Snake *Astrotia stokesii*, Including Two Long-Chain Neurotoxic Proteins with Amidated *C*-Termini

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From the venom of a sea snake *Astrotia stokesii* three neurotoxic components, toxins Astrotia stokesii a, b and c were isolated in about 40, 15 and 5% yield by weight respectively of the whole venom. Their LD_{50} values for 20g mice were 0.13, 0.096 and 0.098 μg/g body wt. respectively and accounted for almost all the lethal activity of the venom. Their amino acid sequences were determined. Astrotia stokesii a was composed of 60 amino acid residues with nine half-cystine residues and was quite homologous to other sea-snake short-chain neurotoxins in its amino acid sequence. Toxins Astrotia stokesii b and c were composed of 70 and 72 amino acid residues respectively with 10 half-cystine residues. They are the first long-chain neurotoxins with high activity isolated from sea-snake venoms. The *C*-terminal carboxy groups of toxins b and c were found to be amidated; the amidation is known for some polypeptides, but is novel for a protein. The amide group may make a hydrogen-bond with glutamic acid-39, which replaces a lysine that has so far been found invariably in long-chain neurotoxins. Astrotia stokesii b and c are also novel in having phenylalanine-25 and isoleucine- or valine-42. The ordinary Tyr-Glu pair, which is observed in X-ray structure [Low, Preston, Sato, Rosen, Searl, Rudko & Richardson (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2991–2994] and n.m.r. study [Inagaki, Tatsumi, Miyazawa, Hori & Tamiya (1977) *Abstr. Int. Congr. Pure Appl. Chem. 26th*, p. 336] on erabutoxins may be replaced by a hydrophobic pair. Detailed evidence for the amino acid sequences of the proteins has been deposited as Supplementary Publication SUP 50090 (30 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5.

During the last decade more than 50 neurotoxins have been isolated from the venoms of *Hydrophid* and *Elapid* snakes and sequenced. These neurotoxins are classified into two groups, namely short-chain and long-chain toxins. The former consist of 60–62 amino acid residues with four disulphide bridges (13 toxins from sea snakes and 17 from cobras). The latter consist of 71–74 amino acid residues with five disulphide bridges. From cobras, 22 long-chain toxins have been reported so far. The only long-chain toxin isolated from a sea snake and sequenced is Laticauda semifasciata III (Maeda & Tamiya, 1974), which consists of 66 amino acid residues with five disulphide bridges, and is not very toxic.

The sea snake *Astrotia stokesii* is a large heavily built creature with a broad and massive head, found widely in the waters of tropical Australia and New Guinea and the Arabian Sea. By column chromatography on CM-cellulose, the venom was fractionated into its components, and three strongly neurotoxic components were purified. A short-chain toxin, composed of 60 amino acid residues with nine half-cystine residues, was named toxin Astrotia stokesii a. The other two components named Astrotia stokesii b and Astrotia stokesii c, consisted of 70 and 72 amino acid residues respectively, belonging to the group of long-chain toxins.

The present paper describes the isolation and properties of the venom components and their complete amino acid sequences.

Abbreviation used: Cbz, benzyloxycarbonyl.

Materials and Methods

Sea snakes

During the sea-snake expedition on the research vessel 'Alpha Helix' of Scripps Institution of Oceanography from December 1972 to January 1973 20 *Astrotia stokesii* snakes were collected mostly at Ashmore Reef, Australia.

The venoms were milked from the live sea snakes (Tamiya & Puffer, 1974), diluted with water, freeze-dried and brought back to the laboratory.

Proteinases

Trypsin (twice crystallized, salt-free), carboxypeptidase A (treated with di-isopropyl phosphoro-

fluoridate; from bovine pancreas) and leucine aminopeptidase (from pig kidney) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Thermolysin (three times crystallized) was purchased from Seikagaku Kogyo Co., Tokyo, Japan. α-Chymotrypsin (three times crystallized) was a product of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pronase E was obtained from Kaken Kagaku Co., Tokyo, Japan, and carboxypeptidase Y (from yeast) was from Oriental Yeast Co., Suita, Osaka, Japan.

Reagents

Phenyl isothiocyanate and trifluoroacetic acid were the sequenal grade of Wako Pure Chemical Industries, Osaka, Japan. Dimethylallylamine was synthesized from allyl chloride and dimethylamine (Ahlroth, 1965). Iodoacetic acid was recrystallized from chloroform/light petroleum (b.p. 30–70°C). α-Benzyloxycarbonyl-N^G-nitro-L-arginine (where G indicates that the nitro group is *N*-substituted on either of the amino groups of the guanidine moiety of arginine) was a product of Protein Research Foundation, Minoh, Osaka, Japan, and benzyloxycarbonylglycine and *N*-hydroxysuccinimide were gifts from Dr. Y. Kikuchi of our laboratory. Tetrahydrofuran, benzene and ethyl acetate were redistilled and other solvents and reagents were used without further purification. Sephadexes, CM-cellulose (Brown Co., Berlin, NH, U.S.A.; capacity 0.70 mequiv./g) and DEAE-cellulose (Brown; capacity 0.84 mequiv./g) were purchased from Seikagaku Kogyo Co., Tokyo, Japan. Aminex A-4 was the product of Bio-Rad (Richmond, CA, U.S.A.). Precoated silica-gel aluminium plates (silica gel 60 F_{254}) and cellulose aluminium plates (cellulose F_{254}) were the products of Merck (Darmstadt, Germany).

Carboxypeptidase Y digestion

Peptides or proteins were digested with carboxypeptidase Y in 0.1 M-phosphate buffer, pH 6.5 (made up of KH_2PO_4 and $Na_2HPO_4,12H_2O$) at 37°C usually for 2, 6 and 24 h. The enzyme/substrate ratio was 1:20–50 (w/w). The substrate concentration was about 1 mg/ml. The digests were directly subjected to amino acid analyses.

Mass spectrometry

The peptide (approx. 0.2 μmol) was acetylated as described by Morris & Williams (1971) followed by permethylation by the method described by Gray & del Valle (1970). The derivative was dissolved in 20 μl of chloroform and a part of it was transferred to a direct insertion probe. The magnetic scan spectra were recorded on a Hitachi M-52 instrument (Hitachi, Tokyo, Japan), at 120°C, an ionization potential of 70 eV being used. The resolution setting was 500. The accelerating voltage was maintained at 1.5 kV.

Synthesis of glycylserine amide and arginine amide

Benzyloxycarbonylglycine (5.23 g, 25 mmol) was coupled with *N*-hydroxysuccinimide (2.88 g, 25 mmol) in dry tetrahydrofuran (30 ml) by the dropwise addition of dicyclohexylcarbodi-imide (5.15 g, 25 mmol/20 ml of dry tetrahydrofuran) at −13°C. After stirring overnight at room temperature, the mixture was filtered and the filtrate evaporated to dryness. The product, benzyloxycarbonylglycine *N*-hydroxysuccinimide ester (6.6 g, yield 87%), was crystallized from ethyl acetate/light petroleum (b.p. 30–70°C). Part of the product (4.08 g, 13.3 mmol) was mixed with L-serine (2.8 g, 26.8 mmol) in dioxan/water (3:2, v/v; 50 ml) containing $NaHCO_3$ (3.36 g), and incubated overnight while stirring at 37°C. The reaction mixture was evaporated to dryness and the residue redissolved in 40 ml of 1 M-HCl. The product, benzyloxycarbonylglycylserine was extracted with ethyl acetate (four times, total volume 70 ml) and crystallized from ethyl acetate/light petroleum to give 3.1 g (yield 78.7%, m.p. 100–101°C; Found: C, 52.22; H, 5.38; N, 9.44. $C_{13}H_{16}N_2O_6$ requires C, 52.70; H, 5.44; N, 9.46%). Cbz-Gly-Ser (1.5 g, 5 mmol) was esterified with diazomethane in tetrahydrofuran (25 ml) at room temperature. The mixture was evaporated and the product was crystallized from ethyl acetate/light petroleum (1.45 g, yield 93.5%). The methyl ester (500 mg, 1.6 mmol) was treated with aq. NH_3 (28%, w/w; 3 ml) at room temperature for 30 min, and the mixture evaporated to dryness. Cbz-Gly-Ser-NH_2 was crystallized from ethyl acetate/light petroleum. Crystallization was repeated from the same solvent (170 mg, yield 36%, m.p. 169°C. Found: C, 52.85; H, 5.82; N, 14.00. $C_{13}H_{17}N_3O_5$ requires C, 52.87; H, 5.80; N, 14.23%). The Cbz-Gly-Ser-NH_2 (100 mg) was deblocked by hydrogenolysis (10^5 Pa, 1.5 h) with Pd/C (5% w/w, 10 mg) as catalyst in 4.5 ml of 0.01 M-HCl in methanol/acetic acid/water (5:4:1, by vol.). The filtered solution was evaporated and freeze-dried.

Cbz-N^G-nitro-Arg was amidated by the method described by Otsuka *et al.* (1966), and the product recrystallized from 80% (v/v) ethanol (yield 85.7%) (Found: C, 47.67; H, 5.74; N, 23.61. $C_{14}H_{20}N_6O_5$ requires C, 47.72; H, 5.72; N, 23.85%). The compound (92 mg) was deblocked by hydrogenolysis (10^5 Pa) with Pd/C (5%, w/w, 20 mg) as catalyst overnight in methanol/acetic acid/water (7 ml; 3:8:3 by vol.). The filtered solution was added to 1 M-HCl (1.0 ml), evaporated and freeze-dried.

Disc-gel electrophoresis, amino acid analysis, toxicity measurement, enzymic digestions and separation

of the digestion fragments, paper electrophoresis of peptides and Edman degradation

These were carried out as described previously (Maeda & Tamiya, 1974, 1976).

Effects on isolated muscle preparation

The biventer cervicis muscles were isolated from 3–10-day-old chicks. Each muscle was placed in glucose/Ringer solution (9.2g of NaCl, 0.42g of KCl, 0.23g of $CaCl_2$, 0.15g of $NaHCO_3$ and 1.0g of glucose in 1000ml of water; 5ml) through which O_2 was constantly bubbled. The temperature of the solution was maintained at 37°C by use of a water jacket. The action of the neurotoxins on the muscles was studied as described previously (Tamiya & Arai, 1966) with a SB-1T force displacement transducer and multi-purpose recorder RH-20 (Nihon Koden Co., Tokyo, Japan).

Results

Isolation of neurotoxins

In a typical run, the freeze-dried venom (101mg from 0.54ml of wet venom) was dissolved in 0.01M-phosphate buffer (10ml) made up of KH_2PO_4 and $Na_2HPO_4,12H_2O$, pH6.4. A small amount of insoluble precipitate was removed by centrifugation at 8000rev./min for 10min, and the solution was applied to a CM-cellulose column (1.6cm×25cm) equilibrated with the same buffer. The elution pattern is shown in Fig. 1. Toxicity was found in fractions II (0.6% of the whole venom), III (58%), IV (30%) and V (11%). Fraction III was collected, desalted and rechromatographed on the same column with the 0.01M-phosphate buffer, pH6.4, containing 0.08M-NaCl. The protein was eluted in a single peak, and was named toxin Astrotia stokesii a.

Desalted and freeze-dried material of fraction IV was applied on a CM-cellulose CM-52 column (1.6cm×32cm) equilibrated with 0.02M-ammonium acetate buffer, pH7.2, and eluted with the same buffer, gradually increasing the NaCl concentration in the buffer (Fig. 2). The second-peak fraction, which was strongly toxic to mice, was freeze-dried and gel-filtered on a Sephadex G-50 (fine grade) column (1.4cm×95cm) in 0.1M-acetic acid. The component eluted in a single peak was pure on polyacrylamide-gel disc electrophoresis at pH4.0, and named toxin Astrotia stokesii b.

Desalted and freeze-dried material of fraction V in Fig. 1 was gel-filtered on a Sephadex G-50 (fine grade) column (1.6cm×100cm) in 0.1M-acetic acid. The absorbance of the eluate (Fig. 3) increased gradually and decreased sharply. The material in the former part of the peak was non-toxic to mice, whereas that in the latter half was strongly toxic. The toxic fraction was collected, freeze-dried and further applied to a CM-cellulose CM-52 column (1.8cm×28cm) equilibrated with 0.01M-Tris/HCl buffer, pH8.0, and eluted with the same buffer containing an increasing NaCl concentration (Fig. 4). The component thus obtained was pure on disc electrophoresis and named toxin Astrotia stokesii c.

The protein contents of toxins Astrotia stokesii a, b and c in the venom were about 40, 15 and 5% by weight respectively.

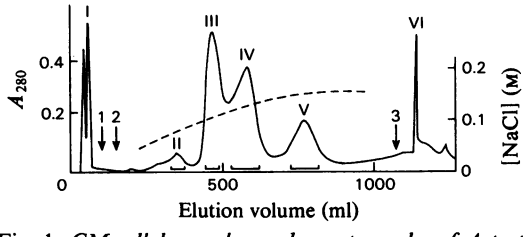

Fig. 1. *CM-cellulose column chromatography of Astrotia stokesii venom*

The crude venom (101mg) was dissolved in 0.01M-phosphate buffer, pH6.4, and chromatographed on a CM-cellulose column (1.6cm×25cm) equilibrated with the same buffer. At arrow 1, the eluting buffer was changed to 0.01M-phosphate buffer, pH6.4, containing 0.05M-NaCl. At arrow 2, a gradient elution with the buffer containing 0.05M-NaCl in the open mixing chamber (diameter 9.0cm, 450ml) and the buffer containing 0.2M-NaCl in the reservoir (diameter 10cm, 550ml) was applied. At arrow 3, the NaCl concentration in the eluting buffer was changed to 0.5M. The flow rate was 170ml/h. Fractions underlined were pooled separately. ——, A_{280}; ----, [NaCl].

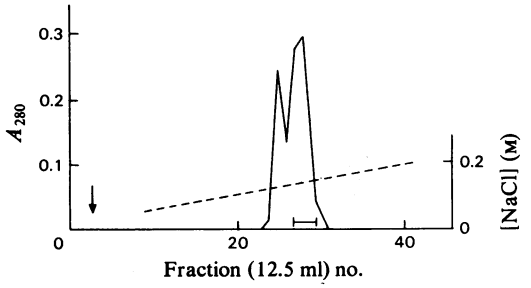

Fig. 2. *Purification of Astrotia stokesii b on CM-cellulose CM-52 column*

At the arrow, linear gradient elution with NaCl concentration was applied with 0.02M-ammonium acetate buffer, pH7.2, containing 0.05M-NaCl in the mixing chamber (300ml) and the same buffer containing 0.3M-NaCl in the reservoir (300ml). Each 12.5ml fraction was collected at a flow rate of 84ml/h. Fractions underlined were collected. ——, A_{280}; ----, [NaCl].

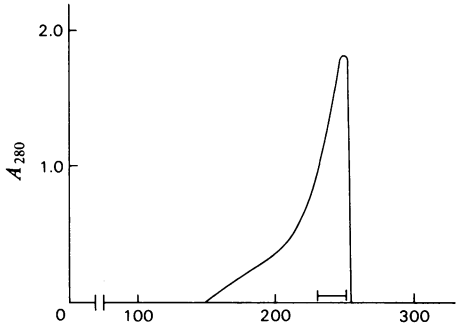

Fig. 3. *Gel filtration of toxic fraction V on Sephadex G-50 column*

The fraction V from CM-cellulose column chromatography (Fig. 1, approx. 44mg) was gel-filtered on a Sephadex G-50 (fine grade) column (1.6cm×100cm) in 0.1M-acetic acid. The flow rate was 37ml/h. Fractions underlined were collected.

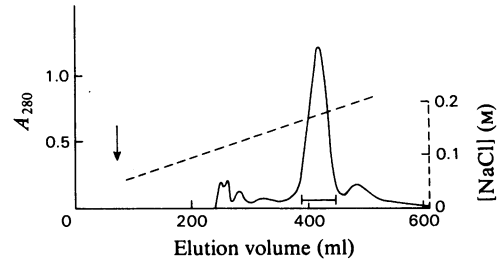

Fig. 4. *Purification of toxin c by CM-cellulose CM-52 column chromatography*

The freeze-dried fractions from Sephadex G-50 gel filtration (Fig. 4, approx. 35mg) was dissolved in 0.01M-Tris/HCl buffer, pH8.0, and applied to a CM-cellulose CM-52 column (1.8cm×28cm) and chromatographed with the same buffer. At the arrow, a linear gradient of NaCl concentration was applied with 300ml of the buffer containing 0.05M-NaCl in the mixing chamber and 300ml of buffer containing 0.3M-NaCl in the reservoir. Each 14ml fraction was collected at a flow rate of 60ml/h. ——, A_{280}; ----, [NaCl].

Disc gel electrophoresis of the components

The electrophoresis was carried out at constant current of 3mA per gel (15% acrylamide) for 3h at pH4.0. Toxins a, b and c from *Astrotia stokesii* all moved toward the cathode as single bands, with migration distances of 2.4, 2.0 and 2.2cm respectively. Although toxins b and c were adsorbed more strongly to CM-cellulose than was toxin a (Fig. 1), the mobilities of the former two were smaller.

Molecular-weight estimation

On gel filtration in 0.1M-acetic acid, Astrotia stokesii a was eluted from a Sephadex G-50 (fine grade) column (1.2cm×100cm) at the same position as erabutoxin b (mol.wt. 6800), whereas Astrotia stokesii b and c were eluted a little faster, giving mol.wts. of 8000, when 1mg of protein for each was applied. However, when more than 10mg of toxins b and c were applied on the same column, the protein peaks were retarded and perfectly coincided with the position of toxin a. This adsorption effect may be caused by their richness in hydrophobic amino acid residues.

An ultracentrifugation study of Astrotia stokesii c by the method of Schachman (1959) revealed its mol.wt. to be about 9000.

Amino acid compositions

Table 1 shows the amino acid compositions of toxins a, b and c. The values were obtained from triplicate analyses. Values for threonine and serine were corrected to zero time values by using the data of analyses of 24, 48 and 72h hydrolysates. The values of 72h hydrolysates were taken for isoleucine and valine. Astrotia stokesii a is composed of 60 amino acid residues with nine half-cystine residues. The molecular weight calculated from its composition was 6600. Toxins b and c are composed of 70 and 72 amino acid residues respectively with ten half-cystine residues. Compared with toxin b, toxin c has one more valine, one more arginine, one more threonine and one more serine residue and one less aspartic acid and one less isoleucine residue. The molecular weights of toxins b and c calculated from amino acid compositions are 7580 and 7787 respectively.

The $A^{1\%}_{1cm,\ 280nm}$ values for Astrotia stokesii b and c were 18.4 and 20.5 respectively, and that of Astrotia stokesii a was about 11.0.

Toxicity and mode of action

The LD_{50} values of toxins a, b and c obtained by injection into the hind-leg muscles of mice (body wt. approx. 20g) were 0.13, 0.096 and 0.098μg/g body wt. respectively. The injection produced flaccid paralysis in animals.

Astrotia stokesii a (0.4μg) and c (0.2μg) blocked the acetylcholine-induced contracture (12.5μg) of the chick biventer cervicis muscles completely, in 4min after the addition to the medium (5ml). The acetylcholine-induced contracture of the muscles did not recover by washing three times (5ml each time) with glucose/Ringer solution. The toxins did not affect the contracture induced by KCl. This fact suggests the toxins from *Astrotia stokesii* act in the same manner as the snake neurotoxins that specifically

Table 1. *Amino acid compositions of Astrotia stokesii a, b and c*
Values in parentheses are those confirmed by the sequence study.

	Amino acid composition (mol/mol of toxin)		
	Toxin a	Toxin b	Toxin c
Lysine	4.73 (5)	3.83 (4)	4.23 (4)
Histidine	1.84 (2)	1.69 (2)	1.65 (2)
Arginine	1.88 (2)	1.77 (2)	2.71 (3)
Aspartic acid	7.09 (7)	5.94 (6)	5.18 (5)
Threonine‡	6.71 (7)	5.91 (6)	6.96 (7)
Serine‡	5.11 (5)	4.58 (5)	5.83 (6)
Glutamic acid	6.96 (7)	3.00 (3)	3.03 (3)
Proline	2.13 (2)	3.12 (3)	3.00 (3)
Glycine	5.11 (5)	6.89 (7)	6.73 (7)
Alanine	1.18 (1)	5.06 (5)	5.16 (5)
½CyS*	7.84 (9)	9.11 (10)	8.98 (10)
Valine§	1.13 (1)	2.80 (3)	3.77 (4)
Methionine	0.89 (1)	1.06 (1)	1.01 (1)
Isoleucine§	2.74 (3)	3.46 (4)	2.74 (3)
Leucine	1.02 (1)	2.10 (2)	1.93 (2)
Tyrosine	0.87 (1)	2.88 (3)	2.78 (3)
Phenylalanine	— (0)	2.02 (2)	1.88 (2)
Tryptophan	(1)†	(2)†	(2)†
Total	60	70	72
Mol.wt. (formula)	6600	7580	7787

* Cystine residues tend to give smaller values.
† Measured spectrophotometrically (Goodwin & Morton, 1946).
‡ Values are corrected to zero time.
§ Values of 72h hydrolysates are given.

block the acetylcholine receptors on the post-synaptic membrane.

Amino acid sequence of toxin Astrotia stokesii a

Terminal amino acid sequence. Toxin a was reduced and *S*-carboxymethylated as described by Crestfield *et al.* (1963) and desalted by gel filtration on a column (1.3cm×65cm) of Sephadex G-25 (fine grade) in 0.1M-acetic acid. The first 13 *N*-terminal amino acids of reduced and *S*-carboxymethylated toxin a were determined by manual Edman degradation to be Met-Thr-CmCys-CmCys-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Lys-Thr-.

Fractionation of tryptic peptides. Reduced and *S*-carboxymethylated toxin a (47mg) was digested with trypsin in 0.05M-Tris/HCl buffer (2ml), pH8.0, at 37°C for 16h. The enzyme/substrate ratio was 1:100 (w/w). The digestion was stopped by the addition of 0.5ml of 1M-acetic acid, and the digest applied to an Aminex A-4 column and chromatographed as described previously (Maeda & Tamiya, 1976). The elution pattern is given in Supplementary Publication SUP 50090. Out of seven peptide fractions Ta–Tg, separated, only fraction Tc was a mixture of three peptides, Tc-1, Tc-2 and Tc-3, which were separated from each other by DEAE-cellulose column chromatography. The column (1.1cm×15cm) was equilibrated with 0.01M-sodium acetate buffer, pH4.9, and the elution was performed with the same buffer, gradually increasing the NaCl concentration in the buffer solution. Peptides Tc-1, Tc-2 and Tc-3 (=Tb) were eluted at NaCl concentrations of 0.03, 0.04 and 0.5M respectively.

The peptides thus isolated were pure on paper electrophoresis at pH4.8. The isolation, amino acid compositions and some properties of the tryptic peptides are given in the supplement. The amino acid compositions of the peptides agree with those shown in Fig. 5. Peptide Td is an incomplete digestion product, consisting of peptide Tc-1 and lysine. Although the lysine residue in the tryptic peptide mixture of toxin a could be identified clearly on paper electrophoresis and on paper chromatography, the residue released could not be detected as a peak on Aminex column chromatography. After electrophoresis at pH4.8 for 75min, the paper was dried, and the lysine spot cut with the aid of guide strips and extracted with 1M-acetic acid. The yield of lysine residue was 0.03μmol/tryptic digests of 0.5μmol of toxin a by direct amino acid analysis.

Amino acid sequences of tryptic peptides

Each peptide (0.5–2.0μmol) was subjected to Edman degradation. The results are shown in Fig. 5.

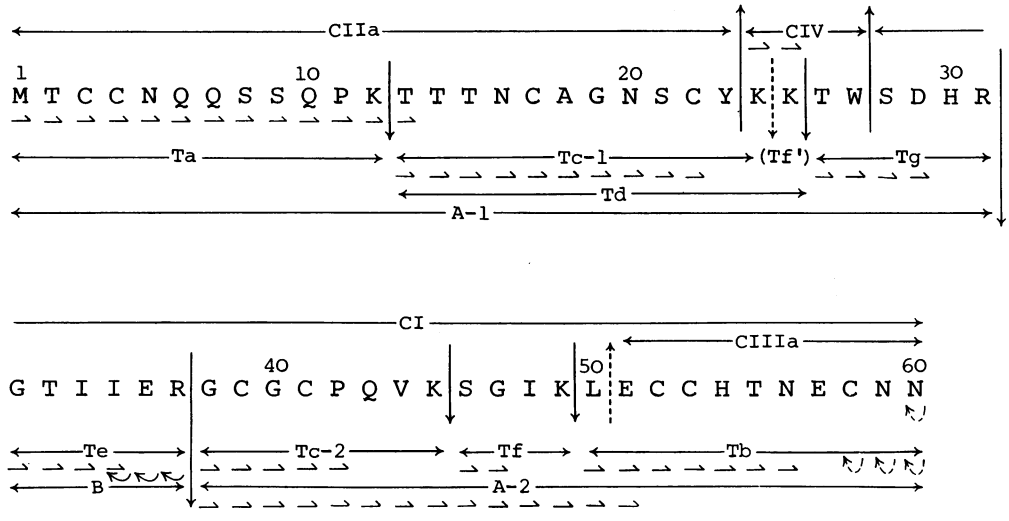

Fig. 5. *Amino acid sequence of Astrotia stokesii a*

Peptides are shown by bars with names. →, Amino acids detected by Edman degradation; ↓ and ↑ indicate the bonds cleaved by trypsin and α-chymotrypsin respectively. The longer arrows indicate the bonds cleaved in citraconylated derivative. ↓ and ↑ show the partial cleavage by the enzymes; the broken and solid curly arrows indicate amino acids detected by carboxypeptidase A and Y digestion respectively. 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.

Asparagine and glutamine residues were confirmed by subjecting the digests of each peptide with leucine aminopeptidase and Pronase E mixture directly to amino acid analysis. Peptide Tb, which contains neither lysine nor arginine, is the *C*-terminal segment of toxin a. Carboxypeptidase A digestion of peptide Tb for 2h yielded 1.08mol of asparagine/mol of peptide, whereas digestion for 24h liberated 1.90mol of aspargine and 0.11mol of *S*-carboxymethylcysteine/mol of peptide. *C*-Terminal sequences of Tyr-Lys for peptide Tc-1 and His-Arg for peptide Tg were deduced according to the specificity of trypsin.

Alignment of tryptic peptides

Tryptic peptide sequence was deduced as shown in Fig. 5 with the aid of the analysis of tryptic peptides A-1, A-2 and B, from citraconylated, reduced and *S*-carboxymethylated toxin Astrotia stokesii a (22.5mg), and of α-chymotryptic peptides CI, CIIa, CIIIa and CIV, of reduced and *S*-carboxymethylated derivative (3.9mg). The isolation and some properties and amino acid compositions of the resulting peptides shown in Fig. 5 are given in the deposited data (Supplementary Publication SUP 50090).

Amino acid sequence of toxin Astrotia stokesii b

Fractionation of tryptic peptides. Because of their richness in hydrophobic amino acids, the reduced and *S*-carboxymethylated derivatives of toxins b and c were hardly soluble in the buffer of acidic pH. Urea and the excess of the reagents for reduction and *S*-carboxymethylation of toxins b and c were removed on a Sephadex G-25 column (fine grade, 1.4cm × 60cm) with 0.05M-ammonium acetate buffer, pH8.0, and the salts were removed by freeze-drying.

Reduced and *S*-carboxymethylated Astrotia stokesii b (25mg) was digested with trypsin in 1.2ml of 0.1M-ammonium acetate buffer, pH7.8, at enzyme/substrate ratio of 1:50 (w/w) for 16h at 37°C. The digests were applied on a Sephadex G-25 (fine grade) column (1.3cm × 58cm), eluted with 0.05M-ammonium acetate buffer, pH7.8, and fractionated into four parts, TI–TIV. Fraction TIV, which was retarded on the column, was pure on paper electrophoresis at pH4.8. Fractions TI and TII were further fractionated by DEAE-cellulose DE-52 column (1.2cm × 12cm) chromatography, into peptides TIa, TIb and TIc, and TIIa, TIIb and TIIc respectively, by increasing the concentration of the NH_4HCO_3 buffer, pH7.8, in the eluent. The chromatograms and the amino acid compositions of the resulting seven

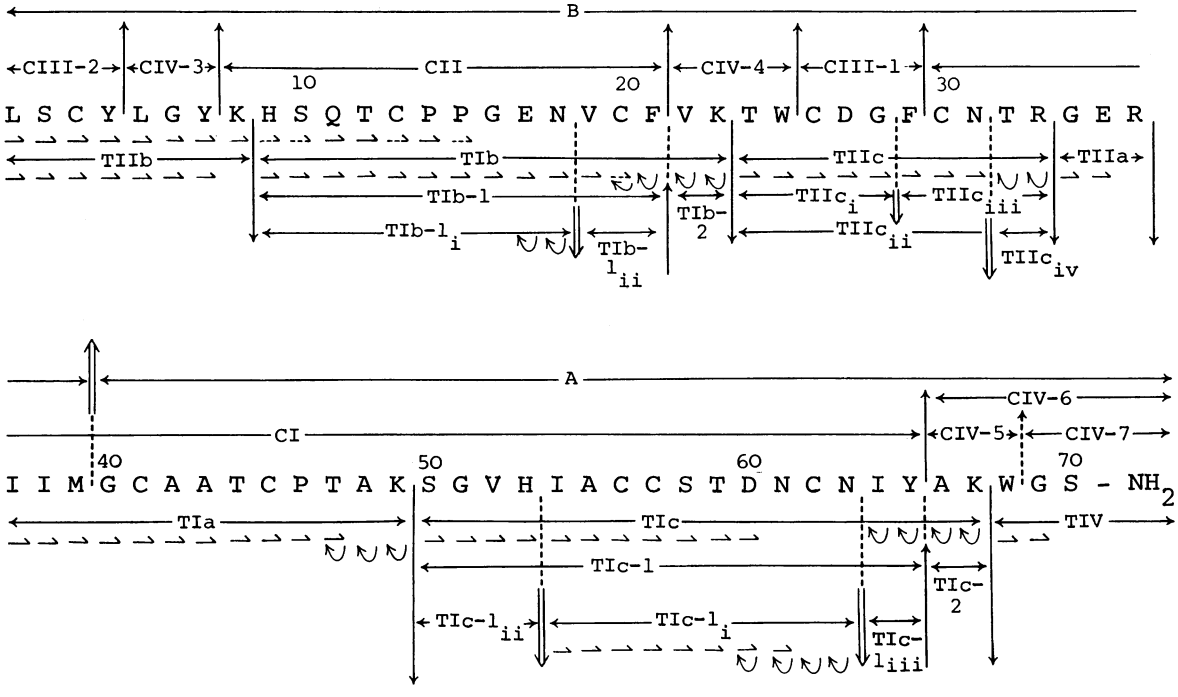

Fig. 6. *Amino acid sequence of Astrotia stokesii b*

Peptides are shown by bars with names. →, Amino acids detected by Edman degradation; ↻, amino acids detected by carboxypeptidase Y digestion; ↓, ↑ and ⇓ indicate the bonds cleaved by trypsin, α-chymotrypsin and thermolysin respectively; ↑ shows the partial cleavage by the enzyme; ⇑ indicates the bonds cleaved by CNBr. The key to one-letter notation for amino acids is given in Fig. 5.

peptides that account for the composition of Astrotia stokesii b, are given in the deposited data (Supplementary Publication SUP 50090).

Cyanogen bromide cleavage of reduced and S-carboxymethylated toxin b. Reduced and *S*-carboxymethylated toxin b (approx. 33mg) was dissolved in 70% (w/v) formic acid (1.5ml) and cleaved with CNBr (65mg) in the dark at room temperature for 22h. The reaction mixture was diluted with water (10ml) and freeze-dried. The freeze-drying was repeated twice after the addition of water (10ml). The material was chromatographed on a Sephadex G-50 column (1.6cm×290cm) in 0.05M-ammonium acetate buffer, pH8.1. The peptide fraction eluted at 340–370ml was collected, freeze-dried and applied to a DEAE-cellulose DE-52 column (1.2cm×12cm) and eluted with 0.01M-ammonium acetate buffer, pH8.0, increasing the NaCl concentration of the eluent. The amino acid compositions of two peptides, A and B, which were eluted at NaCl concentrations of 0.1 and 0.15M respectively, could account for the composition of toxin b (Supplementary Publication SUP 50090). Peptide B, which contained homoserine and its lactone, is the *N*-terminal half of the original toxin.

Separation of α-chymotryptic peptides. Reduced and *S*-carboxymethylated toxin b (5mg) was digested with α-chymotrypsin (1:80, w/w) in 0.1M-ammonium acetate buffer (1.0ml), pH8.0, at 37°C for 40min. The digests were separated into four fractions, CI–CIV, by Sephadex G-25 column (1.3cm×58cm) chromatography in 0.05M-ammonium acetate buffer, pH7.9. Fractions CI and CII were further purified on a DEAE-cellulose DE-52 column (1.2cm×12cm). Fractions CIII and CIV were separated into their components preparatively by paper electrophoresis at pH4.8. Peptides CIII-1, CIII-2 (=CIV-1), CIV-3 (=CIII-3), CIV-4, CIV-5, CIV-6 and CIV-7 (=CIII-4) were obtained (Fig. 6). The amino acid compositions of α-chymotryptic peptides of toxin b agreed with those shown in Fig. 6 and are given in the deposited data (Supplementary Publication SUP 50090).

Amino acid sequences of tryptic peptides. Each peptide (0.5–1.0μmol) was subjected to Edman degradation (Fig. 6). Peptide TIc was further digested with α-chymotrypsin (enzyme/substrate ratio, 1:60,

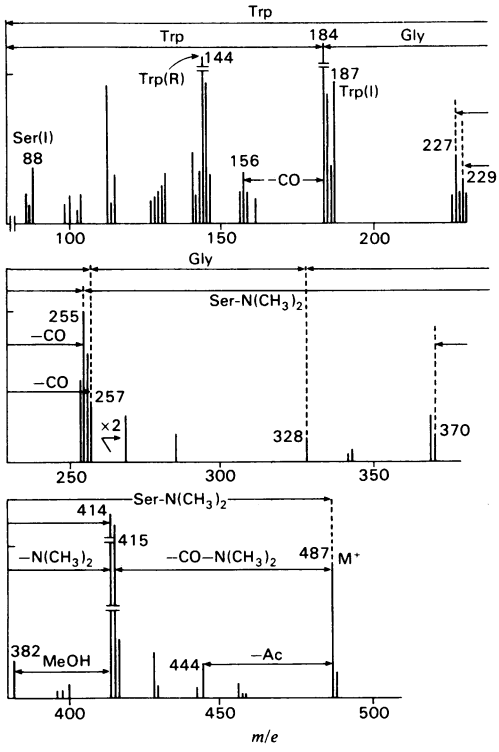

Fig. 7. *Mass spectrum of the modified peptide TIV*
Sequence ions at *m/e* 257, 328 and 487 (M^+) and those at *m/e* 184, 255 and 414 give the sequence of Trp-Gly-Ser-NH_2. The peaks at *m/e* 144 and 187 and *m/e* 88 confirm the presence of tryptophan and serine respectively. In the Figure the normal amino acid abbreviations are used to represent the modified amino acid residues.

w/w) at 37°C for 30min and separated into peptides TIc-1 and TIc-2 by gel filtration on a column (1.3cm × 58cm) of Sephadex G-25 (fine grade). Hydrolysis of peptide TIc-1 (approx. 1.5μmol), which is the *N*-terminal segment of peptide TIc, with thermolysin (enzyme/substrate ratio 1:50, w/w) at 37°C for 2.5h in 0.1M-ammonium acetate buffer (1ml), pH7.8, yielded three peptides, TIc-1-i, TIc-1-ii and TIc-1-iii. They were separated from each other on a Sephadex G-25 column (1.3cm × 58cm) in 0.02M-ammonium acetate buffer. The amino acid compositions of these peptides and that of TIc-2 (given in Supplementary Publication SUP 50090) could account for the composition of peptide TIc (Fig. 6).

Peptide TIb was digested into small fragments, TIb-1-i, TIb-1-ii and TIb-2 enzymically in the same method. The amino acid compositions of these three peptides could account for that of TIb.

Digestion of peptide TIIc (approx. 1.0μmol) with thermolysin under the same conditions described earlier yielded four segments, TIIc-i–TIIc-iv, which were separated from each other by paper electrophoresis at pH4.8. Details are given in Supplementary Publication SUP 50090.

Mass spectrometry of peptide TIV. Though peptide TIV is the *C*-terminal segment of toxin b, containing no basic amino acid residue, its mobility on paper electrophoresis at pH4.8 was 81 as a ratio to the mobility of arginine (100). This fact, as well as the fact that reduced and *S*-carboxymethylated toxin b is resistant to carboxypeptidase A digestion, suggests that the *C*-terminal carboxy group is blocked. Fig. 7 shows the mass spectrum of an acetylated and permethylated derivative of peptide TIV. A peak at *m/e* 487 corresponds to the molecular ion (M^+) of *C*-terminal amidated peptide, Trp-Gly-Ser-NH_2. A very intense peak at *m/e* 414 is the ion corresponding to the loss of *N*-methylacetamide from *N*-terminal tryptophan probably by a McLafferty-type rearrangement (Grüzmacher & Heyns, 1966). A sequence Trp-Gly-Ser-NH_2 is deduced for peptide TIV.

C-Terminal amidation was further confirmed by the properties of peptide CIV-7, which is one of the *C*-terminal segments produced on chymotryptic digestion. The mobility on paper electrophoresis at pH4.8 (120), R_F values on cellulose t.l.c. [0.24 in butan-1-ol/acetic acid/water (12:3:5 by vol.), 0.32 in t-butyl alcohol/formic acid/water (70:1:30, by vol.)] and the retention time when applied directly to the amino acid analyser (short column, 27min) of peptide CIV-7 perfectly agreed with those of standard Gly-Ser-NH_2, which was synthesized chemically as described in the Materials and Methods section.

Taking account of the results so far obtained, the amino acid sequence of toxin Astrotia stokesii b was deduced to be as shown in Fig. 6.

Amino acid sequence of toxin Astrotia stokesii c

The amino acid sequence of toxin c was deduced to be as shown in Fig. 8, by Edman degradation of tryptic and α-chymotryptic peptides of reduced and *S*-carboxymethylated toxin c. Details are described in Supplementary Publication SUP 50090. No *C*-terminal blocked peptide was detected in the tryptic digest. The amino acid composition of tryptic peptide T'Ib was the same as that of TIb of toxin b. The sequence of the *C*-terminal five residues of peptide T'Ib was deduced on homology to TIb. Compared with peptide TIc of toxin b, peptide T'Ic of toxin c had one more threonine and one less alanine residue. Carboxypeptidase Y digestion of the peptide TIc for 20h yielded lysine (1.0mol), alanine (0.9mol), tyrosine (0.9mol) and isoleucine (0.1mol)/mol of peptide, and digestion of peptide T'Ic yielded lysine (1.0mol), threonine (1.0mol), tyrosine (1.1mol) and isoleucine (0.2mol)/mol of peptide. The isolation of tripeptides CIV-5 and C'IV-4 (Figs. 6 and 8) from α-chymotryptic

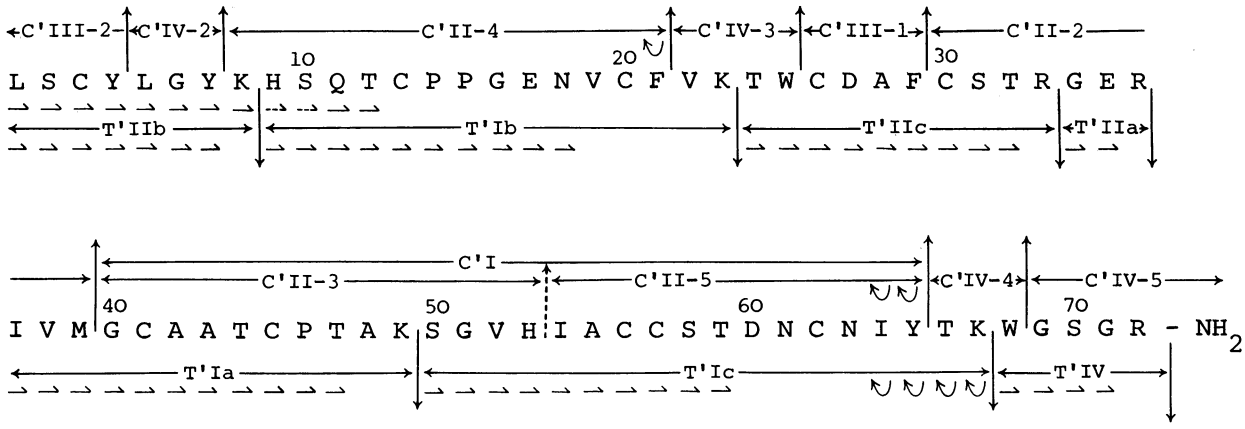

Fig. 8. *Amino acid sequence of Astrotia stokesii c*

Peptides are shown by bars with names. → Amino acids detected by Edman degradation; ↻, amino acids detected by carboxypeptidase Y digestion; ↓ and ↑ indicate the bonds cleaved by trypsin and α-chymotrypsin respectively. ↕ show the partial cleavage by the enzyme. The key to one-letter notation for amino acids is given in Fig. 5.

digests of toxins b and c respectively also accounts for the replacement: peptide CIV-5 consists of one residue each of alanine, lysine and tryptophan and C'IV-4 of one residue each of threonine, lysine and tryptophan.

C-Terminal peptide C'IV-5, isolated from α-chymotryptic digests of toxin c, was subjected to Edman degradation, and three residues were split off from the *N*-terminus. The remainder gave a spot corresponding to authentic Arg-NH_2 on paper electrophoresis at pH4.8 (mobility 150) and on cellulose t.l.c. [R_F 0.24 in butan-1-ol/acetic acid/water (12:3:5, by vol.) and 0.22 in t-butyl alcohol/formic acid/water (70:1:30, by vol.)]. The *C*-terminal carboxy group of Astrotia stokesii c is concluded to be amidated.

Discussion

From the venom of a sea snake *Astrotia stokesii* three strongly neurotoxic components were obtained and sequenced. Fig. 9 shows the comparison of amino acid sequences of these toxins. Astrotia stokesii a is a short-chain toxin composed of 60 amino acid residues, the sequence of which is very homologous to other sea-snake neurotoxins (Maeda & Tamiya, 1976). The other two toxins Astrotia stokesii b and Astrotia stokesii c are composed of 70 and 72 amino acid residues respectively. Astrotia stokesii b is the smallest long-chain toxin so far found except for Laticauda semifasciata III (66 residues; Maeda *et al.*, 1974). Toxin c is two residues longer at the *C*-terminus compared with toxin b. Besides the above addition, there are four amino acid replacements between them. Both toxins b and c are amidated at their *C*-termini, having serine amide and arginine amide respectively. There are several reports on the amidated *C*-termini for polypeptides, such as oxytocin (du Vigneaud *et al.*, 1953), melittin (Habermann & Jentsch, 1967), secretin (Mutt *et al.*, 1970), but it is quite novel for larger protein molecules synthesized by the usual protein-synthesizing system (Takeda *et al.*, 1974). Mass spectrometry was useful to identify the peptide amide.

The X-ray crystallographic structures of the short-chain toxins erabutoxins a and b have been elucidated by Low *et al.* (1976) and by Tsernoglou & Petsko (1976, 1977). In a model constructed according to their results, the *C*-terminal carboxy group lies near the guanidyl group of arginine-43, which is one of the invariant residues among short-chain neurotoxins, suggesting a salt-link formation between them. For the long-chain neurotoxins, Dufton & Hider (1977), using the method of Chou & Fasman (1974), predicted a tertiary structure and suggested that a similar salt link was found between the *C*-terminal carboxy group and lysine-39, which was also invariant among long-chain neurotoxins, with the single exception of Naja naja toxin E (Ohta *et al.*, 1976). The replacement of the lysine-39 by a glutamic acid residue in Astrotia stokesii b and c is accompanied by the amidation of the *C*-terminal residue. The tertiary structure may be maintained by hydrogen-bonding between glutamate-39 and the *C*-terminal amide, and the toxins may exhibit a strong toxicity, whereas *Naja naja* toxin E is less toxic without the bonding.

Another interesting feature in the sequences of

```
                                5                                  10                                  15
(a) Met-Thr-CmCys-CmCys-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Lys-Thr-Thr-Thr-

(b) Leu-Ser-CmCys-Tyr----------Leu-Gly-Tyr-----Lys-His-Ser-----Gln-

(c) Leu-Ser-CmCys-Tyr----------Leu-Gly-Tyr-----Lys-His-Ser-----Gln-

                           20                                 25                                  30
    Asn-CmCys---------Ala-Gly-Asn-Ser-CmCys-Tyr-Lys-Lys-Thr-Trp-Ser-

    Thr-CmCys-Pro-Pro-Gly-Glu-Asn-Val-CmCys-Phe-Val-Lys-Thr-Trp-CmCys-

    Thr-CmCys-Pro-Pro-Gly-Glu-Asn-Val-CmCys-Phe-Val-Lys-Thr-Trp-CmCys-

                           35                                 40                                  45
    Asp-------------------His-Arg-Gly-Thr-Ile-Ile-Glu-Arg-Gly-CmCys-

    Asp-Gly-Phe-CmCys-Asn-Thr-Arg-Gly-Glu-Arg-Ile-Ile-Met-Gly-CmCys-

    Asp-Ala-Phe-CmCys-Ser-Thr-Arg-Gly-Glu-Arg-Ile-Val-Met-Gly-CmCys-

                           50                                 55                                  60
    --------Gly-CmCys-Pro-Gln-Val-Lys-Ser-Gly-Ile-Lys-Leu-Glu-CmCys-

    Ala-Ala-Thr-CmCys-Pro-Thr-Ala-Lys-Ser-Gly-Val-His-Ile-Ala-CmCys-

    Ala-Ala-Thr-CmCys-Pro-Thr-Ala-Lys-Ser-Gly-Val-His-Ile-Ala-CmCys-

                           65                                 70                                  75
    CmCys-His-Thr-Asn-Glu-CmCys-Asn-Asn-------------------------------

    CmCys-Ser-Thr-Asp-Asn-CmCys-Asn-Ile-Tyr-Ala-Lys-Trp-Gly-Ser-NH2----

    CmCys-Ser-Thr-Asp-Asn-CmCys-Asn-Ile-Tyr-Thr-Lys-Trp-Gly-Ser-Gly-Arg-NH2
```

Fig. 9. *Comparison of the amino acid sequences of three toxins from the venom of Astrotia stokesii*
(*a*) Astrotia stokesii a; (*b*) Astrotia stokesii b; (*c*) Astrotia stokesii c. Deletions are arranged to maximize the homology to other toxins (Maeda & Tamiya, 1974).

Astrotia stokesii b and c, compared with those of other neurotoxins, is the occurrence of phenylalanine-25. All neurotoxins (and most related proteins) so far sequenced have tyrosine at this position. For erabutoxins it is hard to modify this tyrosine residue chemically (Sato & Tamiya, 1970), and X-ray structure (Low *et al.*, 1976) and n.m.r. study (Inagaki *et al.*, 1977) suggest the presence of a hydrogen-bond between it and the conservative carboxy group of glutamic acid at position 42. In addition to the replacement at position 25 by phenylalanine, in Astrotia stokesii b and c, position 42 is replaced by isoleucine or valine respectively. It is striking that the toxicity remains unaffected in spite of these replacements as compared with those of other strongly acting neurotoxins. It may be possible that hydrophobic interaction between phenylalanine-25 and isoleucine-42 (or valine-42) stabilizes the molecules in a similar manner to the tyrosine–carboxylate pair.

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