Int. J. Peptide Protein Res. 18, 1981, 113–120

AMINO ACID COMPOSITION AND SEQUENCE ANALYSIS OF SAUVAGINE, A NEW ACTIVE PEPTIDE FROM THE SKIN OF *PHYLLOMEDUSA SAUVAGEI*

PIER CARLO MONTECUCCHI[1] and AGNES HENSCHEN[2]

[1] *Farmitalia-Carlo Erba, S.p.A., Chemical Research Department, Milan, Italy and*
[2] *Max-Planck-Institut für Biochemie, Martinsried, Munich, West Germany*

Received 18 June, accepted for publication 14 October 1980

The complete amino acid sequence of sauvagine, a new active polypeptide from the skin of Phyllomedusa sauvagei, *a frog of Central and South America, has been determined by automated liquid-phase procedure after specific removal of the N-terminal pyrrolidonecarboxylic acid, and specific cleavages at the single methionine and at the two arginine residues. The proposed sequence is:*
Pyr-Gly-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Ser-Leu-Glu-Leu-Leu-Arg-Lys-Met-Ile-Glu-Ile-Glu-Lys-Gln-Glu-Lys-Glu-Lys-Gln-Gln-Ala-Ala-Asn-Asn-Arg-Leu-Leu-Leu-Asp-Thr-Ile-NH_2.

Sauvagine possesses a number of pharmacological actions on diuresis, the cardiovascular system and endocrine glands; it can be considered the prototype of a new family of amphibian peptides, in addition to the tachykinins, bradykinins, dermorphins, caerulein-like and bombesin-like peptides.

Key words: antidiuresis; endocrine effects; hypotension; *Phyllomedusa sauvagei* skin; sauvagine; sequence analysis.

In a preceding paper (Montecucchi *et al.*, 1980) we have reported the purification of a new polypeptide, called sauvagine, present in the skin of *Phyllomedusa sauvagei*, an amphibian of Central America. Sauvagine displayed a long-lasting hypotensive action in all animal species so far examined: rat, dog, monkey and man. Hypotension was particularly striking in the rat, both in intensity and in duration (threshold dose: 0.5 μg/kg subcutaneously, and 5–10 ng/kg intravenously). The pronounced lowering of systemic blood pressure was probably the main cause of the intense antidiuresis observed in hydrated rats, following sauvagine administration. In addition to its properties in the cardiovascular system, this polypeptide displayed conspicuous effects on the rat anterior pituitary, provoking the inhibition of release of PRL, TSH and GH, both *in vitro* and *in vivo*. In contrast, the release of ACTH and β-endorphin was intensively stimulated (Erspamer & Melchiorri, 1980). The ACTH

Abbreviations: DNS, dansyl (dansyl, 5-dimethyaminonaphthalene-1-sulphonyl); DPCC, diphenyl carbamoyl chloride; DFP, diisopropylfluorophosphate; PTH, phenylthiohydantoin derivative of amino acids; ACTH, adrenocorticotropic hormone; PRL, prolactin; GH, growth hormone; TSH, thyroid stimulating hormone; HVPE, high voltage paper electrophoresis; $E_{1,2}$ or $E_{6,5}$, HVPE mobility at pH 1.2 or 6.5 respectively; t.l.c., thin-layer chromatography.

Enzymes: trypsin (EC 3.4.21.4), carboxypeptidase A (EC 3.4.17.1), carboxypeptidase Y (EC 3.4.16.1), L-pyroglutamyl peptide hydrolase (EC 3.4.11.8).

0367–8377/81/070113–08 $02.00/0

release and the inhibition of the TSH release was ascertained also in the dog. For all these pharmacological actions, sauvagine could be considered the prototype of a new peptide family, in addition to the tachykinins, bradykinins, dermorphins, caerulein-like and bombesin-like peptides discovered in the amphibian skin (Erspamer & Melchiorri, 1973, 1980).

The method of isolation of sauvagine yielded a sufficiently pure peptide with a molecular weight of about 4000–5000, determined by gel filtration technique and by sodium dodecyl sulphate gel electrophoresis.

In this paper the complete primary structure of sauvagine is given and its secondary structure as predicted from the amino acid sequence is presented.

EXPERIMENTAL PROCEDURES

Materials

Sauvagine was isolated from methanol extracts of the skin of *Phyllomedusa sauvagei* as described previously (Montecucchi *et al.*, 1980). Trypsin (DPCC-treated, 8575 BAEE U/mg prot.), and aminopeptidase M (25 U/mg prot.) were purchased from Sigma Chemical Co., St. Louis, USA; carboxypeptidase Y (20 U/mg lyophilisate) and pyroglutamate aminopeptidase (L-pyroglutamyl peptide hydrolase 4 mU/mg lyophilisate) from Boehringer, Mannheim, Germany; fluorescamine (Fluram®) was obtained from Roche, Milan, Italy; sequential grade reagents for Edman degradation were purified as described by Edman & Henschen (1975); 4 N methanesulphonic acid [containing 0.2% 3-(2-aminoethyl) indole] from Pierce, IL, USA; phenylthiohydantoin standard from Fluka A.G. Buchs, Switzerland; polybrene from Aldrich Chemical Co., Beerse, Belgium; Sephadex G-10, G-25 sf and G-50 sf from Pharmacia, Uppsala, Sweden; t.l.c. plates silica gel $60F_{254}$ (layer thickness 0.25 mm) from Merck, Darmstadt, Germany. All other chemicals were of analytical grade purity.

Amino acid composition

The amino acid analyses were performed on a Biotronik apparatus by the method of Spackman *et al.* (1958). The hydrolysis was carried out under vaccum at 110° for 48 h with 6 N HCl or with 4 N methanesulphonic acid (Simpson *et al.*, 1976). No corrections were made for decompositional losses or for incomplete liberation of residues. The hydrolysates of the peptides from cyanogen bromide cleavage were treated with pyridine acetate (pH 6.5 at 105° for 1 h) as indicated by Ambler (1965); the samples were then dried under N_2 and dissolved in pH 2.2 buffer immediately before amino acid analysis.

Hydrolysis with trypsin

Enzymic digestion was performed in 0.5 M ammonium acetate buffer (pH 8) for 15 h at 37°. After lyophilisation the fragments were separated by fingerprint technique (see below).

Arginine-directed trypsin cleavage

Large tryptic peptides were obtained by first blocking the ϵ-amino groups of the lysines with citraconic anhydride, as described by Henschen & Lottspeich (1975). The fragments were separated by gel filtration on Sephadex G-10 and G-25 superfine (column dimension 1 cm x 145 cm, eluent 0.05% trifluoroacetic acid; flow rate 1 ml · h^{-1}). The absorption at 220 nm of the effluent was monitored.

Hydrolysis with pyroglutamate aminopeptidase

About 2 mg sauvagine were incubated with 10 mU of the enzyme at 37° for 16 h in 1 ml buffer containing 0.1 M H_3PO_4, 0.01 M EDTA and $5 \cdot 10^{-3}$ M dithioerythritol (pH 8). The enzymatic digest was desalted on Sephadex G-10 in 0.05% trifluoroacetic acid (column dimension 1 cm x 80 cm, flow rate 1 ml · h^{-1}); the absorption at 220 nm was monitored.

COOH-Terminal analysis

The analysis was performed by digestion with carboxypeptidase A (pH 8.3 in 0.1 M Tris buffer at 40°) as described by Edman & Henschen (1975). For *C*-terminal determination with carboxypeptidase Y (pH 5.5 in 0.1 M sodium citrate at 30°) the method described by Henschen *et al.* (1979) was employed.

Cyanogen bromide cleavage

About 2–3 mg sauvagine were dissolved in 1 ml of 70% formic acid and treated with CNBr as

described by Lottspeich & Henschen (1977). The mixture was then diluted with water, reduced in volume and applied to a column of Sephadex G-50 sf in 0.05% trifluoroacetic acid (column dimension 1 cm x 145 cm, flow rate 1 ml · h^{-1}). The absorption at 220 nm of the effluent was monitored. Peptide-containing peaks were lyophilised.

Partial acid hydrolysis

Partial acid hydrolysis was carried out as described by Partridge & Davis (1950). After treatment with 0.25 M acetic acid (100° for 10 h) the sample was examined by HVPE at pH 6.5.

Hydrazinolysis

Hydrazinolysis was carried out according to the method of Akabori *et al.* (1952).

Liquid-phase Edman degradation

The thiazolinone degradation method was employed. The sequenator procedure was performed as described in detail by Edman & Begg (1967) and Edman & Henschen (1975). The phenylthiohydantoins were identified by thin-layer chromatography (Edman & Henschen, 1975) and by high performance liquid chromatography (Zimmerman *et al.*, 1977).

TABLE 1

Amino acid composition of sauvagine

Amino acid	Amino acid analysis	Sequence analysis
Asp	3.7	2
Asn	–	2
Thr	0.9	1
Ser	2.1	2
Glu	8.8	5
⌐Glu	–	3
Glu	–	1
Pro	1.8	2
Gly	1.3	1
Ala	2.0	2
Met	0.6	1
Ile	4.7	5
Leu	7.0	7
Lys	3.7	4
Arg	2.0	2
Total	–	40

⌐Glu, pyrrolidonecarboxylic acid.

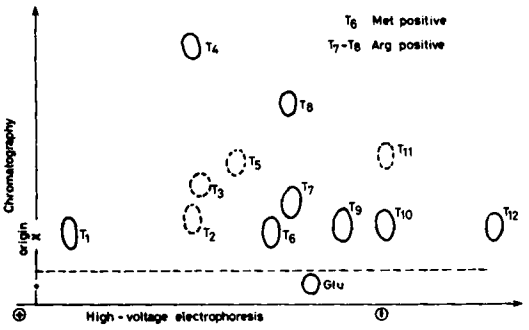

FIGURE 1

Fingerprint of the tryptic peptides (T) of sauvagine. The dotted circles refer to poorly visible spots. The *N*-terminal amino acid residue in each tryptic fragment was identified by the dansyl method, after elution of the spot. The fragment T_1, stained only with chlorine, gave negative results and it was identified as the *N*-terminal nonapeptide by amino acid analysis after total acid hydrolysis.

Dansyl technique

Dansylation was carried out by the method of Bruton & Hartley (1970) and Woods & Wang (1967).

Fingerprint technique

High voltage paper electrophoresis was carried out as previously described (Montecucchi *et al.*, 1980, 1981*a*, *b*). The electrophoresis was run at pH 1.2 (99% formic acid/glacial acetic acid/water, 170:100:730, by vol.) in the first direction at 1500 V for 90 min (about 35 V · cm^{-1}; 100–110 mA). The electrophoretically separated zones were further purified by ascending chromatography in the second dimension using the solvent system *n*-butanol/pyridine/glacial acetic acid/water (4:1:1:1, by vol.). The spots were visualized for elution generally by staining with 0.0002% fluorescamine in acetone by the method of Vandekerckhove & Van Montagu (1974). The fragments were identified with 0.1% ninhydrin (in 95% ethanol containing 2% sym-collidin), chlorine (Rydon & Smith, 1952), platinic iodide (Toennies & Kolb, 1951), and Sakaguchi reagent (Jepson & Smith, 1953). The electrophoretic behaviour of the homogeneous

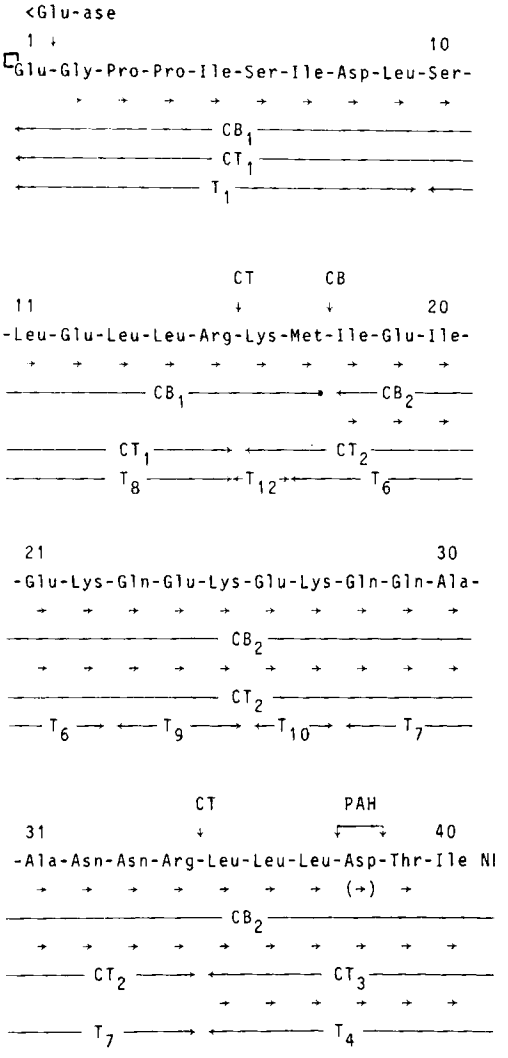

FIGURE 2

Complete amino acid sequence of sauvagine. <Glu-ase, action of pyroglutamate aminopeptidase; $\overset{\downarrow}{CT}\overset{\downarrow}{}$ trypsin cleavage points after citraconylation; $\overset{\downarrow}{CB}$ cyanogen bromide cleavage point; → amino acid residue located by direct thiazolinone degradation; (→) residue located with lower degree of confidence ↔ denotes length of peptides; CB denotes a peptide isolated after cyanogen bromide cleavage, CT after arginine-direct trypsin cleavage; T after trypsin cleavage (see Fig. 1); the vertical arrows in CT_3 between Leu (37), Asp (38) and Thr (39) indicate the sites of partial acid hydrolysis (PAH).

peptides was determined in HVPE at pH 6.5 (pyridine/glacial acetic acid/water, 50:2:948, by vol.) ($35\,V \cdot cm^{-1}$; 50 mA) after elution with 50% ethanol. The electrophoretic mobility of each spot was calculated in relation to glutamic acid (HVPE at pH 1.2) or histidine (HVPE at pH 6.5).

RESULTS

Amino acid composition

After total acid hydrolysis the molecule of sauvagine appeared to comprise 40 amino acid residues (Table 1). The molar ratio values reported in the Table were calculated assuming that sauvagine contained two residues of arginine. In effect, when the tryptic peptides from sauvagine were separated by the finger-print technique, two fragments (T_7 and T_8 in Fig. 1) positive for the Sakaguchi reaction were observed.

Sequence analysis

When sauvagine was analysed for primary structure by the Edman degradation, no phenylthiohydantoin derivatives could be identified. This indicated that the amino end was blocked. However, after treatment with pyroglutamate aminopeptidase, almost the complete sequence could be directly determined in the Edman sequenator (Fig. 2). After the enzymatic treatment, the sample was desalted on a column of Sephadex G-10 (Fig. 3). Sauvagine was eluted in pool A; the released *N*-terminal pyrrolidonecarboxylic acid could be identified as glutamic acid in pool B, after acid hydrolysis and amino acid analysis.

The automated sequencing of the deblocked *N*-terminal fragment was unambiguously extended to leucine 37.

The *C*-terminal part of the sequence was obtained by analysing in the sequenator the fragment CB_2 from cyanogen bromide cleavage at the single methionine residue. The cleavage products were first fractionated by gel filtration on a column of Sephadex G-50 sf (Fig. 4). Four recognizable ultraviolet-absorbing peaks were collected separately. After total acid hydrolysis and amino acid analysis (Table 2) it was possible to establish that pool A contained the intact molecule, probably because the

methionyl residue was in the sulphoxide form. Pool B was recognised as the *C*-terminal part of the molecule by the absence of methionine (or, really, homoserine) in its amino acid composition. Pool C could be identified as the *N*-terminal fragment by the presence of homoserine, which was the only residue released from it by carboxypeptidase A. Pool D did not contain peptidic material. Upon rechromatography on the same column, pool B (fragment CB_2) appeared as a single peak, with the same effluent volume. This fragment was analysed in the sequenator and it was possible to establish the complete sequence (Fig. 2).

No amino acid was released from the intact sauvagine molecule by treatment with carboxypeptidase A or by hydrazinolysis. With carboxypeptidase Y, isoleucine was found. This indicated a blocked *C*-terminus. Sauvagine was selectively cleaved at the arginyl bonds by trypsin. After gel filtration on Sephadex G-10

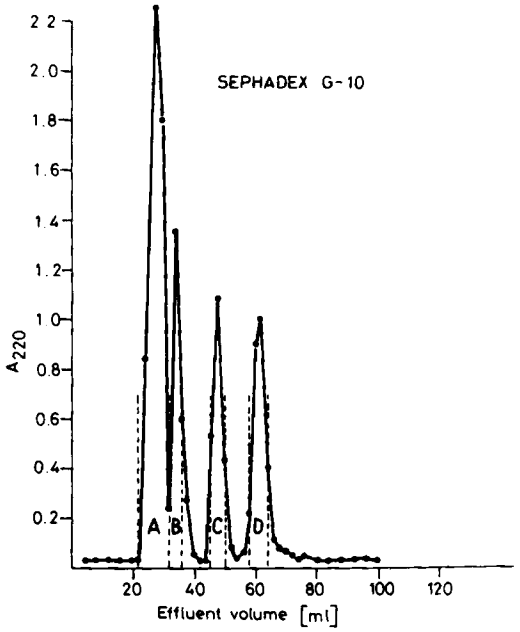

FIGURE 3

Gel filtration on Sephadex G-10 (1 × 80 cm) of sauvagine treated with pyroglutamate aminopeptidase. Pooling is indicated by broken lines. Pool A contained the polypeptide; pool B pyrrolidonecarboxylic acid; pool C and pool D salts.

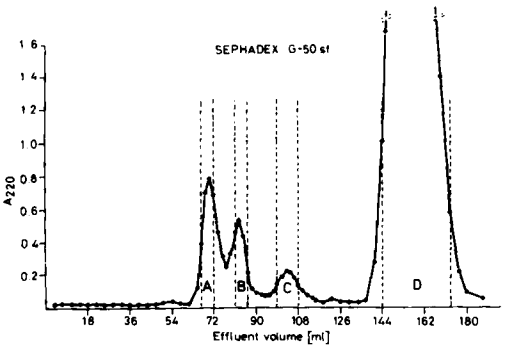

FIGURE 4

Gel filtration on Sephadex G-50 (1 × 145 cm) of cyanogen bromide-cleaved sauvagine. Pooling is indicated by broken lines. Pool A corresponds to the intact molecule; pool B to the *C*-terminal part (fragment CB_2 in Fig. 2); pool C to the *N*-terminal part (fragment CB_1 in Fig. 2) and pool D contained salts.

and G-25, the *C*-terminal hexapeptide (fragment CT_3 in Fig. 2) was obtained in pure form. On HVPE this fragment was located in a single spot ($E_{1.2}$ 0.56 Glu, no migration at pH 6.5) giving positive ninhydrin and chlorine reactions. On silica gel t.l.c. (solvent system *n*-butanol/glacial acetic acid/water, 4:1:1, by vol.) its Rf value was 1.67 Leu. The fragment CT_3 was also submitted to sequence analysis by automated Edman degradation (Fig. 2). After partial acid hydrolysis, it was examined on HVPE at pH 6.5 (Fig. 5).

The cathodic spot Ac_3 ($E_{6.5}$ 1.36 His), yellowish with ninhydrin, was eluted. This fragment, with the amino acid composition (1 Thr, 1 Ile), showed the same electrophoretic mobility ($E_{1.2}$ 0.97 Glu; $E_{6.5}$ 1.36 His) and the same chromatographic behaviour (Table 3) as the synthetic dipeptide H-Thr-Ile-NH_2. As a result of amino acid analysis and sequence studies, the primary structure proposed for sauvagine is indicated in Fig. 2.

DISCUSSION

The skin of the South American Phyllomedusinae frogs contains bradykinin-like (Anastasi *et al.*, 1966), caerulein-like (Anastasi *et al.*, 1969), physalaemin-like (Anastasi & Falconieri-Erspamer, 1970, bombesin-like (Erspamer,

TABLE 2

Amino acid composition of isolated pools from cyanogen bromide cleavage of sauvagine

Amino acid	pool A		pool B		pool C	
	Amino acid analysis	Sequence analysis	Amino acid analysis	Sequence analysis	Amino acid analysis	Sequence analysis
Asp	4.20	2	3.00	1	1.00	1
Asn	–	2	–	2	–	–
Thr	1.10	1	0.90	1	0.20	–
Ser	1.70	2	0.08	–	1.20	2
Hse	–	–	–	–	present N.D.	
Glu	9.30	5	7.30	4	2.50	1
Gln	–	3	–	3	–	–
pGlu	–	1	–	–	–	1
Pro	2.30	2	–	–	1.50	2
Gly	1.40	1	0.10	–	1.10	1
Ala	2.00	2	2.20	2	0.60	–
Met	present N.D.	1	–	–	–	1
Ile	4.68	5	2.80	3	1.70	2
Leu	7.23	7	3.12	3	3.50	4
Lys	3.60	4	3.20	3	1.20	1
Arg	2.00	2	1.00	1	0.80	1
Total	–	40	–	23	–	17

N.D. = not determined.

personal communication) and opiate-like peptides (Montecucchi *et al.*, 1981*a*, *b*). In addition, the methanol extracts of the skin of *Phyllomedusa sauvagei* and other *Phyllomedusa* species contain variable amounts of a new active constituent, called sauvagine, which possesses a number of pharmacological actions on diuresis, the cardiovascular system and endocrine glands (Erspamer & Melchiorri, 1980). In the effects on blood pressure and diuresis sauvagine showed a close biological resemblance to urotensin I, a peptide isolated from teleost urophysis, which is considered a caudal neurosecretory system (Lederis & Medakovic, 1974; Medakovic *et al.*, 1975).

It has been repeatedly pointed out that peptides found in mammalian gut and brain have very frequently counterparts in amphibian skin and, vice versa, that amphibian skin peptides may offer a key to the discovery of new, analogous peptides in mammalian tissues (Erspamer & Melchiorri, 1979). There are good reasons to believe that sauvagine (Mr 4599.9) is the prototype of a new peptide family, having representatives not only in the amphibian skin and in the bony fish urophysis, but also in the mammalian gut and brain. This assumption is based on preliminary results obtained in the rat gastrointestinal tract and brain with an anti-sauvagine serum raised in rabbits with a semipurified *Ph. sauvagei* extract (Erspamer & Melchiorri, personal communication). Should the sauvagine structure be confirmed by synthesis, this polypeptide would represent one of the few examples of natural active polypeptides lacking aromatic amino acid residues in their molecule. Up to now, no active peptide from amphibian skin has shown this peculiarity.

Studies are now in progress to determine the minimum chain length required for the pharmacological effects of sauvagine. The possibility that the peptide is an active precursor polypeptide which, through enzymic fragmentation, gives rise to shorter chains with yet unknown hormonal activities, is suggested by the occurrence of two adjoining basic residues (positions 15–16). Similar sequences

are known to be the cleavage site for generation of active peptides (Steiner & Oyer, 1967; Kimura *et al.*, 1979).

The sauvagine sequence has a clearly hydrophobic character in the regions 1–20 and 30–40, while polar amino acids are concentrated in the middle of the structure. Repetitive sequences are abundant. Repeats of the type Pro-Pro are found seven times. The combinations Ile-Glu and Glu-Lys occur two and three times, respectively. The sequence Ile-Ser-Ile-Asp is followed by the highly analogous sequence Leu-Ser-Leu-Glu.

The secondary structure has been predicted according to the methods of Chou & Fasman (1975, 1977, 1978) as follows: α-helix in positions 9–31, β-sheet in positions 36–40 and β-turns (probability $> 0.5 \times 10^{-4}$) in positions 2–5 and 32–35.

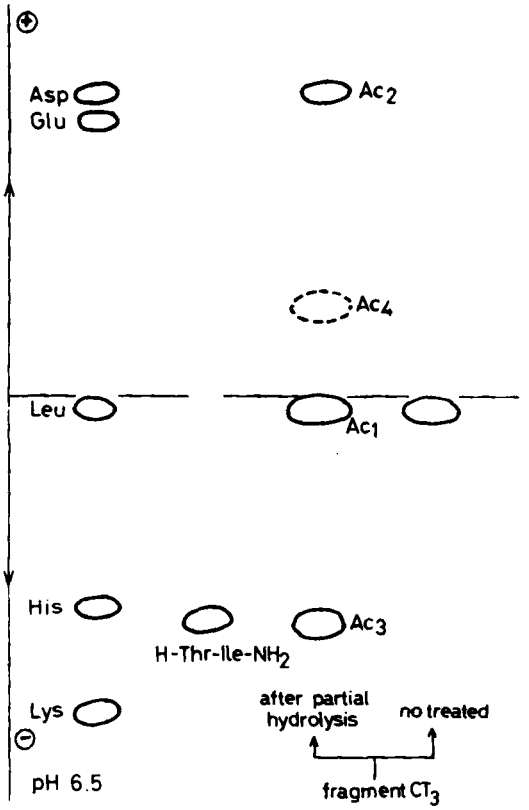

FIGURE 5

Electrophoretic pattern of fragment CT_3 after partial acid hydrolysis. Ac denotes a peptide isolated after PAH. Ac_1 H-Leu-Leu-Leu-OH, Ac_2 H-Asp-OH, Ac_3 H-Thr-Ile-X, Ac_4 H-Leu-Leu-Leu-Asp-OH (this spot was very faint).

TABLE 3

Migration of the fragment Ac_3 on silica gel thin-layer chromatography

	Relative Rf values	
	Fragment Ac_3	Synthetic H-Thr-Ile-NH_2
n-butanol/acetic acid/water (4:1:1, by vol.)	1 Leu	1 Leu
n-butanol/pyridine/acetic acid/water (4:1:1:1, by vol.)	1.26 Leu	1.26 Leu

During purification of the peptide, we isolated a minor active component with the same biological spectrum of sauvagine. The two forms have similar molecular weight and exhibit similar sensitivity to treatment with cyanogen bromide trypsin, chymotrypsin, carboxypeptidase A and Y. The amino acid composition, as determined on the acid hydrolysates, is also identical. They showed, however, different electrophoretic mobility. The more acidic behaviour of sauvagine II at pH 8.5 suggests the presence of at least an additional carboxyl group arising from deamidation of a glutaminyl or asparaginyl residue.

Note added in proof. Sauvagine-like immunoreactivity has been recently observed in Dalgreen's neurons and perivascular neural nets of the *Tinca* urophysis, in the neurons and neural fibres of cerebral cortex, in the molecular layer of the cerebellar cortex, in some diencephalic neurons and in a few endocrine-like cells of the intestinal crypts. The sauvagine-like immunoreactivity in urophysis confirms the proposed structural analogy between urotensin I and sauvagine (Renda *et al.*, 1980).

ACKNOWLEDGEMENTS

We thank Dr. R. de Castiglione for his interest in this work and for the supply of the synthetic dipeptide H-Thr-Ile-NH_2. The authors wish to express their appreciation to Dr. L. Gozzini for prediction of the secondary structure. It is a pleasure to acknowledge the excellent technical assistance of Miss H. Gross and Mrs. C. Lottspeich.

REFERENCES

Akabori, S., Ohno, K. & Narita, K. (1952) *Bull. Chem. Soc. Japan* **25**, 214–218

Ambler, R.P. (1965) *Biochem. J.* **96**, 32p

Anastasi, A. & Falconieri-Erspamer, G. (1970) *Experientia* **26**, 866

Anastasi, A., Erspamer, V., Bertaccini, G. & Cei, J.M. (1966) in *Hypotensive Peptides* (Erdös, E.G., Back, N., Sicuteri, F. & Wilde, A.F., eds), pp. 76–85, Springer, New York

Anastasi, A., Bertaccini, G., Cei, J.M., De Caro, G., Erspamer, V. & Impicciatore, M. (1969) *Brit. J. Pharmacol.* **37**, 198–206

Bruton, G.J. & Hartley, B.S. (1970) *J. Mol. Biol.* **52**, 165–178

Chou, P.Y. & Fasman, G.D. (1975) *Biochemistry* **14**, 2536–2541

Chou, P.Y. & Fasman, G.D. (1977) *J. Mol. Biol.* **115**, 135–175

Chou, P.Y. & Fasman, G.D. (1978) *Ann. Rev. Biochem.* **47**, 251–276

Edman, P. & Begg, G. (1967) *European J. Biochem.* **1**, 80–91

Edman, P. & Henschen, A. (1975) in *Protein Sequence Determination* (Needleman, S.B., ed.), 2nd edn., pp. 232–279, Springer, Berlin

Erspamer, V. & Melchiorri, P. (1973) *Pure Appl. Chem.* **35**, 463–494

Erspamer, V. & Melchiorri, P. (1980) in *Growth Hormone and Other Biologically Active Peptides* (Pecile, A. & Müller, E.E., eds.), pp. 185–200, Excerpta Medica, Amsterdam

Henschen, A. & Lottspeich, F. (1975) *Hoppe-Seylers Z. Physiol. Chem.* **356**, 1985–1988

Henschen, A., Lottspeich, F., Brantl, V. & Techemacher, H. (1979) *Hoppe-Seylers Z. Physiol. Chem.* **360**, 1217–1224

Jepson, J.B. & Smith, I. (1953) *Nature* **172**, 1100–1101

Kimura, S., Lewis, R.V., Gerber, L.D., Brink, L., Rubinstein, M., Stein, S. & Udenfriend, S. (1979) *Proc. Natl. Acad. Sci. US* **76**, 1756–1759

Lederis, K. & Medakovic, M. (1974) *Brit. J. Pharmacol.* **51**, 315–324

Lottspeich, F. & Henschen A., (1977) *Hoppe-Seylers Z. Physiol. Chem.* **358**, 1521–1524

Medakovic, M., Chan, D.A.O. & Lederis, K. (1975) *Pharmacology* **13**, 419–426

Montecucchi, P.C., Anastasi, A., de Castiglione, R. & Erspamer, V. (1980), *Int. J. Peptide Protein Res.* **16**, 191–199

Montecucchi, P.C., de Castiglione, R., Piani, S., Gozzini, L. & Erspamer, V. (1981*a*) *Int. J. Peptide Protein Res.* **17**, 275–283

Montecucchi, P.C., de Castiglione, R. & Erspamer, V. (1981*b*) *Int. J. Peptide Protein Res.* **17**, 316–321

Partridge, S.M. & Davis, H.F. (1950) *Nature* **165**, 62–63

Renda, T., Negri, L., D'Este, L., Salvi, E., Lomato, D. & Erspamer, V. (1980) in *Abstracts of the Third International Symposium on Gut Hormones*, Cambridge, September 14–18, 1980 – *Regulatory Peptides*, supplement 1, S92

Rydon, H.N. & Smith, P.W.G. (1952) *Nature* **169**, 922–923

Simpson, R.J., Neuberger, M.R. & Liu, T.Y. (1976) *J. Biol. Chem.* **251**, 1936–1940

Spackman, D.M., Stein, W.H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206

Steiner, D.F. & Oyer, P.E. (1967) *Proc. Natl. Acad. Sci US* **57**, 473–480

Toennies, G. & Kolb, J.J. (1951) *Anal. Chem.* **23**, 823–826

Vandekerckhove, J. & Van Montagu, M. (1974) *European J. Biochem.* **44**, 279–288

Woods, K.R. & Wang, K.T. (1967) *Biochem. Biophys. Acta* **133**, 369–370

Zimmerman, C.L., Appella, E. & Pisano, J. (1977) *Anal. Biochem.* **77**, 569–573

Address:

Pier Carlo Montecucchi
Farmitalia Carlo Erba S.p.A.
Ricerca & Sviluppo Chimico
Via dei Gracchi, 35
20146 Milan
Italy