research. Furthermore, ZDERIC et al.[6], in a recent preliminary report, claimed 17α-methyl-androstane-2'-methyl-[3,2-b]-thiazole-17β-ol and some N-substituted 2-aminomethylene derivatives of 17α-methyl-dihydrotestosterone to be highly anabolic active.

17α-Methyl-5α-androstane-17β-ol-3-one (Ia) (dihydromethyltestosterone) was converted[7] to the 3-morpholyl-enamine (IIa) m.p. 209–216°; $[\alpha]_D$ + 6.6° ($c$ = 2, $CHCl_3$) found: C 76.91; H 10.36; N 3.58. IIa when reacted with benzyl azidoformate[8] gave 17α-methyl-3-(N-morpholyl-5α-androstane-17β-ol-[2,3-d]-N¹-carbobenzyloxy-triazole (IIIa) m.p. 141° (dec.); $[\alpha]_D$ + 166° ($c$ = 1, $CHCl_3$) found: C 70.00; H 8.46; N 10.48. Hydrogenation of IIIa with palladium on charcoal or lithium aluminum hydride gave *17α-methyl-5α-androstane-17β-ol-[2,3-d]-triazole* (IVa) m.p. 270–271° (Kofler); $[\alpha]_D$ + 26° ($c$ = 0.63, $CH_3OH$) $\lambda_{max}^{CH_3OH}$ 225 mμ; ε 7.000, found: C 73.18; H 9.61; N 12.87; *IVa hydrochloride* m.p. 225–229°; $[\alpha]_D$ + 40.2° ($c$ = 1, $CH_3OH$) found: C 65.41; H 8.74; N 11.72; Cl 9.45.

By reaction of IIa with phenylazide *17α-methyl-5α-androst-2-ene-17β-ol-[2,3-d]-N¹-phenyltriazole* (Va) was obtained; m.p. 288–291°; $[\alpha]_D$ + 53° ($c$ = 1, $CHCl_3$), $\lambda_{max}^{CH_3OH}$ 230 mμ; ε 9800, found C 76.95; H 8.57; N 10.18.

Similarly, from the 3-morpholyl-enamine of 5α-androstane-17β-ol-3-one (IIb) (dihydrotestosterone) m.p. 180–190°; $[\alpha]_D$ + 31° ($c$ = 2, $CHCl_3$); found C 76.57; H 10.40; N 4.06, 3-(N-morpholyl)-5α-androstane-17β-ol-[2,3-d]-N¹-carbobenzyloxy-triazole (IIIb) was obtained; m.p. 153–154°; $[\alpha]_D$ + 177.5° ($c$ = 1, $CHCl_3$); found: C 69.67; H 8.44; N 10.51; reduction of IIIb gave *5α-androstane-17β-ol-[2,3-d]-triazole* (*IVb*) melting at 237–242° $[\alpha]_D$ + 75° ($c$ = 0.751 $CH_3OH$), $\lambda_{max}^{CH_3OH}$ 225 mμ; ε 7.000; found: C 72.50; H 9.21; N 13.18, *IVb hydrochloride*, m.p. 258–266°, $[\alpha]_D$ + 94° ($c$ = 1.12, $CH_3OH$); found: C 65.00; H 8.70; N 12.11; Cl 10.35.

IIb was reacted with phenylazide to yield *5α-androst-2-ene-17β-ol-[2,3-d]-N¹-phenyltriazole* (*Vb*); m.p. 306–311° (Kofler); $[\alpha]_D$ + 74° ($c$ = 1, $CHCl_3$); $\lambda_{max}^{CH_3OH}$ 230 mμ; ε 9800; found: C 76.49; H 8.21; N 10.41.

A complete chemical and biological study on these compounds is being carried out and the results thereof will be published later.

*Zusammenfassung.* Es werden einige neue am A-Ring mit einem Triazolring kondensierte Steroide beschrieben. Diese werden durch Umsetzung der Enamine der 3-Ketosteroide mit Arylaziden oder Azidoformiaten und nachfolgende Abspaltung des basischen Restes hergestellt.

G. NATHANSOHN, E. TESTA, and N. DI MOLA

*Research Laboratories of Lepetit S.p.A., Milano (Italy), August 7, 1961.*

[6] J. A. ZDERIC, O. HALPERN, H. CARPIO, A. RUIZ, D. C. LIMON, L. MAGANA, H. JIMÉNEZ, A. BOWERS, and H. J. RINGOLD, Chem. and Ind. *1960*, 1625.

[7] F. W. HEYL and M. E. HERR, J. Amer. chem. Soc. *75*, 1918 (1953).

[8] The benzyl azidocarbonate, hitherto not reported in the literature, was obtained by reacting benzyl chlorocarbonate with sodium azide. It is a sufficiently stable oil, which can be purified by distillation *in vacuo*; b.p. 58°C/0.3 mm.

## Structure and Pharmacological Actions of Eledoisin, the Active Endecapeptide of the Posterior Salivary Glands of Eledone[1]

It has been known for several years that acetone extracts of the posterior salivary glands of *Eledone* (*moschata* and *Aldrovandi*) contain a principle which possesses a powerful hypotensive action and potently stimulates extravascular smooth muscle[2]. This principle, called *eledoisin*[3], has now been isolated in a pure form and identified as the endecapeptide

Pyr-Pro-Ser-Lys-Asp(OH)-
Ala-Phe-Ileu-Gly-Leu-Met-$NH_2$[4]

Synthesis has fully confirmed the above constitution and aminoacid sequence[5].

*Isolation procedure and determination of structure.* 5368 g of posterior salivary glands, corresponding to approximately 10 000 pairs of glands obtained from 1450 kg of *Eledone*, were removed, at S. Margherita Ligure, from living animals and extracted twice with 3 vol of methanol. The combined filtered extracts were kept in the refrigerator and served as a standard crude extract for the isolation of the active principle.

The first step in the purification of eledoisin consisted in the absorption of the crude polypeptide dissolved in 95% ethanol on an alkaline alumina column and subsequent elution with descending concentrations of ethanol. According to experimental conditions, eledoisin was eluted, together with a large amount of aminoacids, by 60–40% ethanol, whilst biogenic amines were eluted by 90–80% ethanol.

Alternatively, eledoisin could be purified from a crude aqueous solution upon addition of serum albumin at pH 5. Salting out with ammonium sulphate resulted in precipitation of the protein complex which was collected by centrifugation, dialysed against water, then dissociated by treatment with trichloroacetic acid. The resulting protein-free eledoisin appeared to be freed from most of the low molecular weight contaminants. Further purification of the partially purified eledoisin obtained by any one of the above steps was carried out by ion-exchange chromatography on a column of Amberlite CG-50 in the $H^+$ form, performing the elution with a M ammonium acetate buffer at pH 8.4, and finally by a 160 transfer counter current distribution between 0.5 N acetic acid and *n*-butanol.

The above procedures gave a 1000–1200 fold increase in the activity/weight ratio and a yield of 20 to 40% of highly purified peptide.

Pure preparations appeared to be homogeneous on paper chromatography and electrophoresis giving a single peptide spot associated with biological activity. On ascending chromatograms run with the *n*-butanol : acetic acid : water mixture (5:1:4), eledoisin had an Rf of 0.6. On electropherograms carried out with aqueous buffers it appeared to be poorly soluble and showed a very low mobility in the range of pH 2–12.

[1] Supported in part by a grant from the Rockefeller Foundation, New York.

[2] V. ERSPAMER, Exper. *5*, 79 (1949).

[3] V. ERSPAMER, Arzneimittelforsch. *2*, 253 (1952).

[4] Pyr- = Pyroglutamyl-.

[5] R. A. BOISSONNAS and ED. SANDRIN, Exper. *18*, 59 (1962).

On acid hydrolysis the purified peptide yielded serine, leucine, isoleucine, methionine, glycine, alanine, phenylalanine, proline, lysine, aspartic and glutamic acids in a 1:1 ratio. The chromatographic and electrophoretic spots gave the same aminoacid pattern.

The aminoacid sequence was elucidated by chemical and enzymatic degradation. No splitting of C-terminal residue was achieved by carboxypeptidase digestion and both the fluorodinitrobenzene (FDNB) and Edman techniques failed to reveal a free N terminal residue. Chymotrypsin split two peptide bonds giving a heptapeptide containing glutamic and aspartic acids, serine, proline, lysine, alanine and phenylalanine, a tripeptide composed of isoleucine, leucine and glycine and a single residue identified as methioninamide. Trypsin digestion produced two fragments one of which contained glutamic acid, serine, proline and lysine and the other the remaining seven residues. When the heptapeptide produced by chymotrypsin digestion was submitted to trypsin attack, the same tetrapeptide as above was obtained together with a tripeptide composed of alanine, phenylalanine and aspartic acid.

The structure of the two tripeptides produced by chymotrypsin and trypsin hydrolysis was made clear by the FDNB and carboxypeptidase methods and resulted respectively in Asp(OH)-Ala-Phe and Ileu-Gly-Leu.

Under the action of carboxypeptidase, lysine was split from the tetrapeptide isolated from tryptic digests; but attempts to break other peptide bonds were unsuccessful, and even at high concentrations of enzyme only lysine and an acidic peptide negative to ninhydrin could be isolated. As for eledoisin, and also for the tetrapeptide, the N-terminal degradation failed with both the Edman and FDNB procedures. The relative positions of the proline, serine and glutamic acid residues were established by partial acid hydrolysis experiments, and therefore the structure of the tetrapeptide resulted in Pyr-Pro-Ser-Lys.

Consequently, the sequence of eledoisin was clearly established as Pyr-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ileu-Gly-Leu-Met-$NH_2$. On the basis of digestion with leucinaminopeptidase all aminoacids appeared to have the *L*-configuration.

*Pharmacological actions.* Eledoisin is a powerful vasodilator and hypotensive agent in most animal species. In the dog, the threshold hypotensive dose is 0.5–2 mμg/kg, by i.v. injection. The pressure fall is proportional, both in intensity and duration, to the dose of the polypeptide, and there is no sign of tachyphylaxis. Subcutaneous doses of 10–150 μg/kg eledoisin produce a pressure fall lasting 6 to 10 h, and with doses of 300 μg/kg sometimes hypotension terminates in death due to heart failure. A persistent, controllable lowering of blood pressure may also be obtained with i.v. infusion of eledoisin, the threshold being 5–15 mμg/kg/min.

Eledoisin displays a potent vasodilator action on the musculo-cutaneous vessels of the dog's hind leg (threshold intraarterial dose 0.3–1.5 mμg), as well as on the coronary arteries and probably on the brain vessels. The vessels of the splanchnic area, lung and kidney are less affected by the polypeptide.

10–20 μg eledoisin are capable of abolishing completely the hypertensive action of 1000 μg *L*-noradrenaline; 1–2 μg counteract the hypertensive action of 3–20 μg angiotensin. Weight-for-weight eledoisin is at least 50 times more potent than acetylcholine, histamine and bradykinin in lowering the blood pressure of the dog.

Following subcutaneous doses of 100–300 μg/kg, eledoisin is detectable in the blood stream only for 10–15 min, whereas hypotension lasts for hours.

Like the kinins, eledoisin potently lowers the permeability of the skin vessels in the guinea-pig and man.

Of the many preparations of extravascular smooth muscles examined the following proved to be particularly sensitive to the polypeptide: terminal portion of the rabbit large intestine (threshold concentration 0.3–0.6 mμg/ml), guinea-pig ileum (0.3–0.6 mμg/ml), dog duodenum and large intestine (0.6–3 mμg/ml), and frog stomach (2–4 mμg/ml). On the rat duodenum eledoisin displays a purely stimulant action; the oestrus rat uterus is poorly sensitive to the polypeptide (0.3–1 μg/ml). The powerful action of eledoisin on the gastro-intestinal smooth muscle becomes apparent also *in vivo*, when high doses of the polypeptide are administered subcutaneously to intact unanaesthetized dogs.

High doses of eledoisin stimulate salivary, and probably also gastrointestinal secretion. This effect, like that on vascular and extravascular smooth muscle, is atropine-resistant.

In parallel assays eledoisin may easily be distinguished from the kinins (bradykinin, wasp kinin), from substance P, and from all other known active tissue products. The natural polypeptide which so far is most similar to eledoisin in its biological properties, especially in its action on smooth muscles, is substance P. The behaviour of the kinins is distinctly different.

Rabbit large intestine, guinea-pig ileum and dog blood pressure are particularly suitable for the quantitative estimation of crude and pure preparations of eledoisin, owing to their high sensitivity and the excellent dose/effect relationship.

Full reports of the experiments and results described in this paper will be published elsewhere.

*Riassunto.* Viene descritto il procedimento che ha permesso l'isolamento ed il chiarimento della struttura chimica dell'*eledoisina*, l'endecapeptide attivo delle ghiandole salivari posteriori dell'*Eledone*. L'eledoisina è dotata di potente azione ipotensiva, particolarmente spiccata nel cane, e di intensa azione stimolante su alcuni preparati di muscoli lisci extravasali. L'eledoisina è facilmente distinguibile, mediante saggi paralleli, da tutti gli altri polipeptidi biogeni finora descritti.

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## Synthesis of Eledoisin

The structure proposed for Eledoisin[1] (H-Pyr-Pro-Ser-Lys-Asp-(OH)-Ala-Phe-Ileu-Gly-Leu-Met-$NH_2$) was confirmed in our laboratories by a total synthesis, which was performed according to the scheme given in the Figure.

Condensation of *p*-nitrophenyl N-carbobenzoxy-*L*-pyroglutamate with *L*-proline gave N-carbobenzoxy-*L*-pyroglutamyl-*L*-proline (54% yield. M.p. 198°. $[\alpha]_D^{21} = -109°$ in 95% acetic acid. Analysis calculated for $C_{18}H_{20}O_6N_2$: C 59.8; H 5.6; O 26.7; N 7.8. Found: C 60.2; H 5.5; O 26.1; N 8.0), while condensation of N-trityl-*L*-serine with methyl ε-N-carbobenzoxy-*L*-lysinate by dicyclohexylcarbodiimide afforded methyl N-trityl-*L*-seryl-ε-N-carbobenzoxy-*L*-lysinate (66% yield. M.p. 102°. $[\alpha]_D^{22} = -45°$ in dimethylformamide. Analysis calculated for $C_{37}H_{41}O_6N_3$: C 71.3; H 6.6; O 15.4; N 6.7. Found C 71.0; H 6.9 O; 15.5;