SYNTHESIS AND BIOLOGIC PROPERTIES OF AN ENKEPHALIN ANALOGUE, A MODEL SUBSTRATE FOR ENZYMIC CONVERSION

Jaroslav VIČAR[a], Martin FLEGEL[b], Linda HAUZEROVÁ[c], Tzezengijn DASH[c]*, Karel HAUZER[c] and Tomislav BARTH[c]

[a] *Chemical Institute of Medical Faculty, Palacký University, 775 15 Olomouc,*
[b] *Léčiva, 140 00 Prague 4 and*
[c] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*

Received December 14th, 1984

An octapeptide analogue of Leu[5]-enkephalin, prolonged in the carboxyterminal part by the tripeptide Lys-Lys-Leu was prepared. Its action on guinea-pig ileum and splitting by trypsin and tissue cathepsin B was investigated.

The existence of a number of small, biologically active peptides raises the question of their origin. Similarly to other peptides, enkephalin sequences are also found in adrenal proenkephalin separated from non-active sequences by a pair of basic amino acids[1,2]. It is becoming evident that particularly enzymes with trypsin-like specificity take part in generating biologically active peptides[3,4]. For the envisaged isolation of tissue enzymes having the above specificity, we prepared a synthetic Leu[5]-enkephalin analogue – Tyr-Gly-Gly-Phe-Leu-Lys-Lys-Leu.** We compared its biologic properties with those of Leu[5]-enkephalin which is another potential product of the expected trypsin-like splitting of the prohormone structure[5] (Tyr-Gly-Gly-Phe-Leu-Lys).

Synthesis of the octapeptide was carried out as shown in Diagram 1. All coupling reactions were carried out with the aid of N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. Benzyl-type protecting groups were split off by catalytic hydrogenolysis, the tert-butyloxycarbonyl groups were removed from the final octapeptide by trifluoroacetic acid and the product was purified by HPLC on a column containing Separon C_{18} in reversed phase.

The octapeptide thus prepared was incubated with trypsin at pH 7·6 and with cathepsins at pH 5·1 – 5·5. Trypsin liberated the terminal dipeptide Lys-Leu from the octapeptide, while adenohypophysial cathepsin B first released the C-terminal leucine

* Predoctoral Fellow of Chemical Institute, Mongolian Academy of Sciences, Ulan Batar.

** All amino acids mentioned in this study are L-enantiomers with the exceptions of glycine.

(position 8) and finally lysine. Even on prolonged incubation, only the carboxyterminal leucine was liberated by the effect of adenohypophysial cathepsin B. While the physiological role of trypsin in the generation of enkephalin is only theoretical, the role played by cathepsins in the organism should be considered.

Determination of Biological Activity

The opioid activity of peptides was determined from their ability to inhibit electrically-evoked contractions of guinea-pig ileum. Activity was expressed in terms of IC_{50} values (refs[9,10]).

EXPERIMENTAL

Samples for analysis were dried at 80°C *in vacuo* (60 Pa) over P_2O_5. Melting points were determined using a Kofler block and are not corrected. The purity of protected intermediates was checked by chromatography on silicagel layers (Silufol, Kavalier, Czechoslovakia), using the

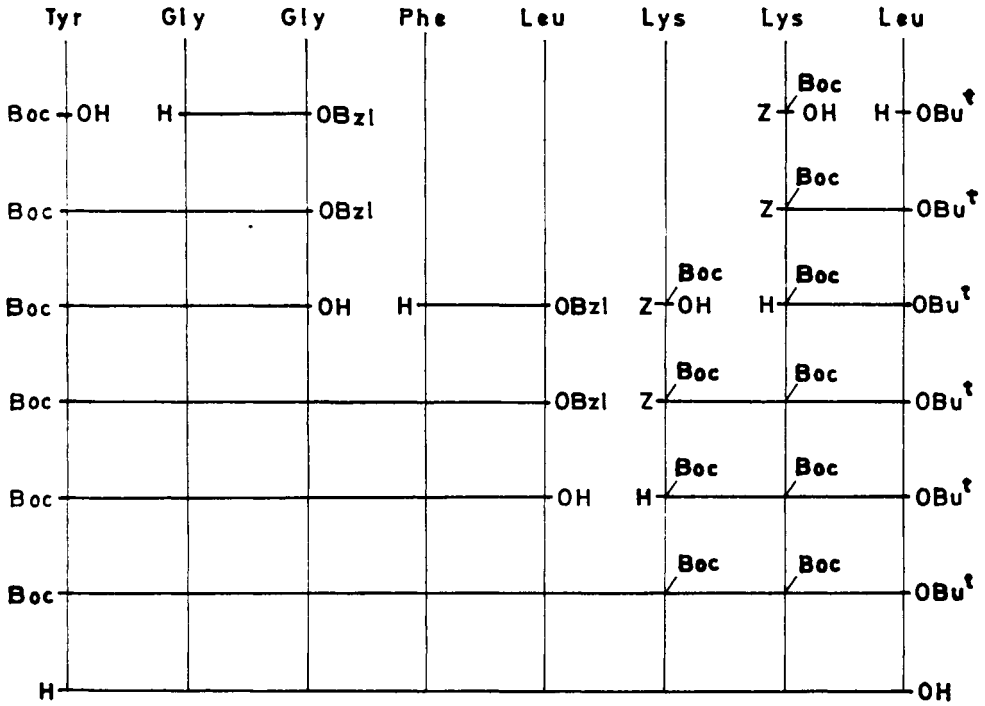

DIAGRAM 1
Synthesis of the octapeptide

system chloroform–methanol (100 : x, where x = 0·5, 2, 4). The purity of the final product was checked by means of high-pressure liquid chromatography. Column chromatography was done on Grade L 40/100 silicagel (Lachema, Czechoslovakia). Electrophoresis was carried out on paper Whatman N° 4 in a moist chamber (20 V/cm) for 1 h in 1M-acetic acid (pH 2·4) and in pyridine–acetate buffer solution (pH 5·7). Samples for amino acid analysis were hydrolysed with 6M-HCl at 105°C for 20 h and analysed on a Type 6020 automatic analyser (Instrumentation Research Workshops of the Czechoslovak Academy of Sciences, Czechoslovakia). Optical rotation data were measured with a photoelectric polarimeter Polamat A (Zeiss, Jena, GDR) at the wavelengths 546 nm and 578 nm and converted to 589 nm. High-pressure liquid chromatography was carried out with an apparatus composed of a Type HPP 5001 linear dosage device (Laboratorní přístroje, Czechoslovakia), CGC column (Laboratorní přístroje) filled with Separon C_{18} in reversed phase, Model SP 8440 detector (Spectra Physics, USA) and Model SP 4100 integrator (Spectra Physics). The apparatus used for preparative liquid chromatography consisted of a Type HPP 4003 pump (Laboratorní přístroje), a column of our own design (25 × 1·5 cm) filled with Separon C_{18} in reversed phase, using methanol–0·1% trifluoroacetic acid (30 : 70) at a flow-rate of 180 ml/h as the mobile phase, a Type UVM 4 detector (Instrumentation Research Workshops, Czechoslovak Academy of Sciences) and TZ 4200 recorder (Laboratorní přístroje).

Bovine trypsin was a product of the Léčiva, Prague, bovine spleen cathepsin B prepared by Dr J. Pohl according ref.[6] of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, cathepsin B from bovine adenohypophysis was prepared by a procedure developed by Dash and coworkers[7]. The hydrolytic products were analysed by thin layer chromatography using the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) and 1-butanol–acetic acid–water (3 : 1 : 1).

In the following text, standard processing is understood to mean: removal of N,N′-dicyclohexylurea by filtration, evaporation of the filtrate, dissolution of the residue in ethyl acetate, placing the solution in a refrigerator for 1 h, filtering a second portion of N,N′-dicyclohexylurea, extraction of the filtrate with 0·25M-HCl, 0·5M-$NaHCO_3$, water, drying with Na_2SO_4 and evaporation of ethyl acetate.

N^{α}-Benzyloxycarbonyl-N^{ε}-tert-butyloxycarbonyllysyl-leucine Tert-butyl Ester (*I*)

N,N′-dicyclohexylcarbodiimide (1·25 g) was added under agitation at −15°C to a mixture of N^{α}-benzyloxycarbonyl-N^{ε}-tert-butyloxycarbonyllysine[8] (2·0 g), leucine tert-butyl ester (1·0 g)

TABLE I

Inhibition of electrically-evoked contractions of guinea-pig ileum by Leu^5-enkephalin and its analogues

Compound	IC_{50} nmol l^{-1}	Relative potence[a]
Tyr-Gly-Gly-Phe-Leu	307	1
Tyr-Gly-Gly-Phe-Leu-Lys	243	1·26
Tyr-Gly-Gly-Phe-Leu-Lys-Lys-Leu	245	1·27

[a] Related to the potence of Leu^5-enkephalin.

and 1-hydroxybenzotriazole (0·7 g) in a mixture of chloroform (20 ml) and dimethylformamide (3 ml). Agitation was continued for 1 h at −15°C, then the mixture was allowed to stand overnight in a refrigerator. Standard processing yielded 2·5 g (87%) of an oil, shown by TLC to contain some 5% impurities. The substance was used in this state for the further reaction; a sample for analysis was purified by chromatography on silicagel and elution with 5% methanol in chloroform. $[\alpha]_D^{25}$ −27·9° (*c* 0·5 methanol). For $C_{29}H_{47}N_3O_7$ (549·7) calculated 63·36% C, 8·61% N, 7·64% H; found 62·84% C, 8·44% H, 7·45% N.

N^{α}-Benzyloxycarbonyl-N^{ε}-tert-butyloxycarbonyllysyl-N^{ε}-tert-butyloxycarbonyllysyl-leucine Tert-butyl Ester (*II*)

The protected dipeptide *I* (1·35 g) in methanol (15 ml) was hydrogenolysed with palladium black (0·10 g) for 6 h, the catalyst was removed by filtration, washed with methanol and the filtrate evaporated. The residue (0·80 g) was dissolved in a mixture of chloroform (12 ml) and dimethylformamide (2 ml), N^{α}-benzyloxycarbonyl-N^{ε}-tert-butyloxycarbonyllysine (0·75 g) and 1-hydroxybenzotriazole (0·30 g) were added to the solution and N,N′-dicyclohexylcarbodiimide (0·42 g) was added under agitation after cooling to −15°C. The mixture was agitated for 1 h at −15°C and allowed to stand overnight in a refrigerator. Standard processing yielded 1·0 g of compound *II*, m.p. 85−88°C, according to TLC the substance contained two other contaminants. Chromatography on silicagel (65 g), eluent 5% methanol in chloroform yielded 0·66 g (36%) of compound *II*, m.p. 105−108°C, $[\alpha]_D^{25}$ −32·9° (*c* 0·5 methanol). For $C_{40}H_{67}N_5O_{10}$ (778·0) calculated: 61·75% C, 8·68% H, 9·00% N; found: 62·11% C, 8·80% H, 8·74% N.

Tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucyl-
-N^{ε}-tert-butyloxycarbonyllysyl-N^{ε}-tert-butyloxycarbonyllysyl-leucine
Tert-butyl Ester (*III*)

The protected tripeptide *II* (0·40 g) in methanol (5 ml) was hydrogenolysed with palladium black (0·10 g) for 6 h. The catalyst was removed by filtration, washed with methanol and the filtrate evaporated. The residue was dissolved in dimethylformamide (4 ml), tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucine[5] (0·36 g), 1-hydroxybenzotriazole (0·08 g) were added and N,N′-dicyclo-hexylcarbodiimide (0·12 g) under agitation after cooling to −20°C. Agitation was continued for 1 h at −15°C, 10 h at 4°C, and 10 h at room temperature. After standard processing the evaporated residue was dissolved in ethyl acetate (5 ml), light petroleum was added to the solution, the precipitate formed was filtered and washed with light petroleum: 0·22 g (33%) of compound *III*, m.p. 165−173°C, was obtained. A sample for analysis was recrystallized twice from an ethyl acetate–light petroleum mixture, m.p. 205−208°C, $[\alpha]_D^{25}$ −20·2° (*c* 0·5 methanol). For $C_{65}H_{104}N_{10}O_{16}$ (1 281·6) calculated: 60·92% C, 8·18% H, 10·93% N; found 60·54% C, 7·99% H, 10·72% N.

Tyrosyl-glycyl-glycyl-phenylalanyl-leucyl-lysyl-lysyl-leucine

The protected octapeptide *III* (130 mg) was dissolved in trifluoroacetic acid (2 ml). After 25 min the solution was evaporated, the residue triturated with ether, the precipitate removed by filtration and washed with ether. After 12 h of drying in a dessicator over P_2O_5/KOH the substance was dissolved in 10% methanol (2 ml) and in this form subjected to HPLC-purification. The mobile phase was a mixture of methanol and 0·1% trifluoroacetic acid (30 : 70), detection at 230 nm, flow-rate 3 ml/min. Fractions containing the HPLC-pure product (analytical HPLC was done with the mobile phase methanol–0·1% trifluoroacetic acid (50 : 50)) were collected, the solvents were evaporated and the residue lyophilized. 32 mg (21%) of the HPLC-homogeneous product

were obtained, $[\alpha]_D^{25}$ −22·0° (*c* 0·15, methanol), $E_{5,7}^{His}$ 0·56, $E_{2,4}^{Gly}$ = 1·59, $E_{2,4}^{His}$ = 0·80. Amino-acid analysis: Tyr 1·00, Gly 2·04, Phe 0·97, Leu 2·08, Lys 2·00. For $C_{46}H_{72}N_{10}O_{10}.5\,CF_3COOH$ (1 495·2) calculated: 44·98% C, 5·19% H, 9·37% N; found: 44·56% C, 5·11% H, 9·51% N.

REFERENCES

1. Lewis R. V., Stern A. S., Kimura S., Rossier I., Stein S., Udenfriend S.: Science *208*, 1459 (1980).
2. Lewis R. V., Stern A. S.: Ann. Rev. Pharmacol. Toxicol. *22*, 353 (1983).
3. Stern A. S., Lewis R. V., Kimura S., Rossier I., Stein S., Udenfriend S.: Arch. Biochem. Biophys. *205*, 606 (1980).
4. Jones B. N., Shively I. E., Kilpatrick D. L., Stern A. S., Lewis R. V., Kojima K., Udenfriend S.: Proc. Nat. Acad. Sci. U.S.A. *79*, 2096 (1982).
5. Vičar J., Servítová L., Flegel M., Hauzer K., Barth T.: This Journal *50*, 1329 (1985).
6. Keilová H., Tomášek V.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *29*, 335 (1973).
7. Dash C.: Unpublished results.
8. Scott I. W., Parker D., Parrish D. R.: Syn. Commun. *11*, 303 (1981).
9. Paton W. P. M.: J. Physiol. (London) *127*, 40P (1955).
10. Hutchinson M., Kosterlitz H. V., Leslie F. M., Waterfields A. A.: Brit. J. Pharmacol. Chemother. *55*, 541 (1975).

Translated by the author (T. B.).