PREPARATION AND PROPERTIES OF THREE ANALOGUES OF [5-LEUCINE]ENKEPHALIN, SUBSTRATES FOR ENZYME CONVERSION STUDIES

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Analogues of [5-Leu]enkephalin, prolonged by methionine on the N-terminus or, by lysine or methionine on the C-terminus were prepared by fragment condensation, purified by ion exchange chromatography or high-pressure liquid chromatography. The substances were characterised by their opioid activity in a test on guinea-pig ileum in comparison with the activity of [5-Leu]-enkephalin.

The genesis of relatively small, biologically active peptides under physiological conditions is becoming a subject of intensive study. Enkephalins, pentapeptides[1] the sequences of which are found in some of the larger proteins of prohormonal or preprohormonal character are enzyme-generated by specific enzymes[2-6]. For the purpose of studying enzymes participating in enzyme-generation of enkephalins in different tissues we have now prepared several model peptides, analogues of [5-Leu]-enkephalin, reporting their synthesis and basic opioid activity in the present communication.

All the three analogues, tyrosyl-glycyl-glycyl-phenylalanyl-leucyl-lysine(*I*)*, tyrosyl-glycyl-glycyl-phenylalanyl-leucyl-methionine(*II*), methionyl-tyrosyl-glycyl-glycyl-phenylalanyl-leucine (*III*) were prepared with the use of the crystalline intermediate tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucine (*IV*) which was synthesized by a 3 + 2 scheme. To prepare the analogues *I* and *II*, ε-benzyloxycarbonyllysine benzyl ester or methionine methyl ester, resp., was condensed (N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole) to the intermediate *IV*. Protecting groups of the benzyl type were removed from the lysine residue by catalytic transfer hydrogenolysis in the ammonium formate–palladium system[7]. The methyl ester group on the methionine residue was saponified in alkaline medium. The N-ter-

* For all amino acids except glycine, the L-configuration is meant.

minal protecting group was removed from the tert-butyloxycarbonyl derivative with trifluoroacetic acid. To prepare the analogue *III*, the tert-butyloxycarbonyl group was first split off from the intermediate *IV* and the [5-leucine]enkephalin obtained was allowed to react in the form of its N-methylmorpholinium salt with tert-butyloxycarbonylmethionine N-hydroxysuccinimide ester. From the protected hexapeptide, the tert-butyloxycarbonyl group was removed with trifluoroacetic acid. In the case of the analogue *I*, final purification was carried out on DEAE-Sephadex, the other two analogues were purified by means of high-pressure liquid chromatography (HPLC).

The opioid activity of the analogues is shown in Table I. With the exception of a single analogue, the carboxyterminal part of which is prolonged by methionine, prolongation of the peptide chain by one amino acid (methionine, lysine, arginine) has a positive effect on the opioid activity of the substance. Comparable activity of the hexapeptide with C-terminal basic amino acid shows, that the intermediates of potential proenkephalin enzyme conversions will likewise be effective agonists of the biologic effect investigated.

EXPERIMENTAL

Samples for analysis were dried *in vacuo* (60 Pa) over phosphorus pentoxide at room temperature. Melting points were determined with a Kofler block and are not corrected. Thin-layer chromatography was performed on silica gel layers (Silufol, Kavalier, Czechoslovakia) using the systems 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4); the solvent system chloroform–methanol (100 : x, where x = = 2, 4, 6, 8) was used to check the purity of the protected intermediates. For column chromato-

TABLE I

Opioid activity of [5-Leu]enkephalin and its analogues, determined on guinea pig ileum

Substance	IC_{50}[a] (nmol l^{-1})	Relative activity
[5-Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu)	307 (32)	1
Met-Tyr-Gly-Gly-Phe-Leu	223 (4)	1·38
Tyr-Gly-Gly-Phe-Leu-Met	990 (6)	0·31
Tyr-Gly-Gly-Phe-Leu-Lys	243 (5)	1·26
Tyr-Gly-Gly-Phe-Leu-Arg[b]	275 (3)	1·12

[a] The number of experiments is given in brackets. [b] Kindly provided by Dr N. M. Titov, Cardiology Center, Moscow, USSR.

graphy, silicagel L 40/100 (Lachema, Czechoslovakia) was used. Electrophoresis was carried out on Whatman paper No 4 in a moist chamber (20 V/cm) for 1 h in 1M acetic acid (pH 2·4) and in pyridine–acetate buffer (pH 5·7). Compounds were detected with ninhydrine or chlorination method. Samples for amino acid analysis were hydrolysed with 6M-HCl at 105°C for 20 h and analysed using a Type 6020 automatic analyser (Development Workshops, Czechoslovak Academy of Sciences). Optical rotation values were measured using the photoelectric polarimeters Polamat 17 (Zeiss, Jena, GDR: measurements were carried out at 546 and 578 nm, results were converted to wavelength 589 nm) and PE 141 (Perkin–Elmer, USA). High-pressure liquid chromatography was carried out with and apparatus consisting of a Type HPP 6001 pump (Laboratorní přístroje, Czechoslovakia), Type SP 8440 detector and Type SP 4100 integrator (Spectra-Physics, USA). Samples (10 µl) were injected onto a Type CGC column (Laboratorní přístroje) filled with Separon C_{18} or Lichrosorb RP-18 using a loop-type sampling device (Rheodyne. USA). Preparative liquid chromatography was performed with a column of our own design (2·5 × × 25 cm) filled with Separon C_{18} with a flow-rate of 6 ml/min. Substances were detected in the UV-region by a Type UVM 4 detector (Development Workshops, Czechoslovak Academy of Sciences) at a wavelength of 280 nm. In the following text, the term standard treatment is understood to mean: washing with $KHSO_4$–K_2SO_4 buffer (pH 2), 0·5M-$NaHCO_3$, water, drying with Na_2SO_4 and evaporation using a rotating evaporator at bath temperatures lower than 40°C.

Tert-butyloxycarbonyltyrosyl-glycyl-glycine Benzyl Ester (*V*)

Tert-butyloxycarbonyltyrosine (3·1 g) and glycyl-glycine benzyl ester (liberated from glycyl-glycine benzyl ester hydrochloride[8] with the aid of saturated solution of ammonia in chloroform) (3·8 g) were dissolved in dimethylformamide (60 ml), 1-hydroxybenzotriazole (1·84 g) was added and after cooling to −20°C, dicyclohexylcarbodiimide (2·5 g) was added under constant agitation. The mixture was allowed to reach 0°C over a period of 1 h and kept overnight at this temperature. After removal of dicyclohexylurea by filtration, dimethylformamide was evaporated, the residue dissolved in ethyl acetate and another portion of dicyclohexylurea was filtered off after 1 h at 0°C. The residue obtained by standard treatment was chromatographed on a silicagel column (250 g), methanol–chloroform 4 : 100 being used for elution. Fractions found homogeneous by TLC were collected and evaporated, yielding 4·1 g (72%) of the product, m.p. 104–106°C, cit.[9] states m.p. 97–102°C.

Tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucine Benzyl Ester (*VI*)

The tripeptide *V* (3·3 g) was hydrogenolysed in tert-butanol (30 ml) with palladium black (0·2 g) for 8 h. The catalyst was removed by filtration, washed with methanol and the filtrate evaporated. The residue was dissolved in chloroform (30 ml), phenylalanyl-leucine benzyl ester (liberated from phenylalanyl-leucine benzyl ester hydrochloride[10] (2·8 g) with saturated solution of ammonia in chloroform) was added to the solution, the mixture was cooled to −20°C and dicyclohexylcarbodiimide (1·6 g) was added under agitation. The mixture was brought to 0°C over a period of 1 h, agitated at this temperature for 3 h and allowed to stand overnight at room temperature. Dicyclohexylurea was removed by filtration, the filtrate was evaporated, the residue dissolved in ethyl acetate and after 1 h at 0°C another portion of dicyclohexylurea was filtered off. The residue obtained by standard treatment was chromatographed on a silicagel column (250 g), methanol–chloroform 4 : 100 being used for elution. Fractions found homogeneous by TLC were collected and evaporated, yielding 4·1 g (81%) of a foam, $[\alpha]_D^{25}$ −10·6° (*c* 0·8, methanol). For $C_{40}H_{51}N_5O_9$ (745·9) calculated: 64·41% C, 6·89% H, 9·39% N; found: 64·17% C, 6·87% H, 9·09% N.

Tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucine (*IV*)

The pentapeptide (*VI*) (1·00 g) was hydrogenolysed in tert-butanol (20 ml) with palladium black (0·07 g). After 8 h, a second portion of the catalyst (0·07 g) was added and hydrogenolysis was continued for another 8 h. After filtration to remove the catalyst, the filtrate was evaporated, the residue dissolved in 0·2M-NaOH (10 ml). After extraction (twice) with chloroform, the aqueous layer was acidified with 0·5M-H_2SO_4 (2·2 ml) and extracted three times with ethyl acetate. The ethyl acetate extract was dried with Na_2SO_4, evaporated and the residue was triturated with ether. The crystals obtained were filtered and washed with ether, yielding 0·76 g (86%) of the product with m.p. 138–141°C. For analysis, a sample was recrystallised from a mixture of ethyl acetate (containing several drops of methanol)–ether, m.p. 138–142°C, $[\alpha]_D^{25}$ −5·3° (*c* 0·5, methanol). The compound *IV* is described in the literature[11,12] as an oil. For $C_{33}H_{45}N_5O_9$ (655·7) calculated: 60·44% C, 6·92% H, 10·68% N; found: 59·98% C, 6·82% H, 10·57% N.

Tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucyl-ε-benzyloxycarbonyllysine Benzyl Ester (*VII*)

ε-Benzyloxycarbonyllysine benzyl ester (143 mg) and 1-hydroxybenzotriazole (59 mg) was added to a solution of substance *IV* (252 mg) in dimethylformamide (4 ml) and after cooling to −20°C, dicyclohexylcarbodiimide (87 mg) was added under agitation. The mixture was allowed to reach 0°C over 1 h and kept at this temperature overnight. Dimethylformamide was evaporated, the residue dissolved in ethyl acetate and after 1 h at 0°C dicyclohexylurea was filtered off. The filtrate was subjected to the standard treatment, the residue was chromatographed on a silica gel column (50 g), the eluent was methanol–chloroform 5 : 100. Fractions homogeneous by TLC were collected and evaporated to yield 220 mg (57%) of the product, m.p. 161–165°C. After recrystallisation from chloroform, the sample for analysis was found to have m.p. 165–167°C, $[\alpha]_D^{25}$ −12·4° (*c* 0·4, methanol). For $C_{54}H_{69}N_7O_{12}$ (1 008) calculated: 64·33% C, 6·90% H, 9·72% found: 64·25% C, 6·86% H, 9·50% N.

Tyrosyl-glycyl-glycyl-phenylalanyl-leucyl-lysine (*I*)

The hexapeptide *VII* (220 mg) was dissolved in methanol (3 ml), ammonium formate (100 mg) and 10% palladium-on-carbon (100 mg) was added, the mixture was agitated at room temperature for 3 h. The catalyst was removed by filtration, the filtrate was evaporated and the residue triturated with ether. Anisol (0·3 ml) and trifluoroacetic acid (3 ml) were added to the product. After 1 h at room temperature the solution was evaporated, the residue triturated three times with ether and applied to a column of DEAE-Sephadex (100 ml, acetate cycle), the buffer for column equilibration and elution was water–pyridine–acetic acid (1 000 : 10 : 0·4), adjusted with acetic acid to pH 6·18. Evaporation of the chromatographically homogeneous fractions, dissolution of the residue in a minimum amount of methanol and precipitation of the product with ether yielded 58 mg of the acetate of product *I*, m.p. 207–209°C, $[\alpha]_D^{25}$ +3·7° (*c* 0·2, water): $E_{5.7}^{His}$ 0·51, $E_{2.4}^{His}$ 0·65, $E_{2.4}^{Gly}$ 1·40; R_F 0·20 (S1), 0·00 (S2), 0·19 (S3), 0·45 (S4). For $C_{34}H_{49}N_7O_8.CH_3COOH$ (743·8) calculated: 58·12% C, 7·18% H, 13·18% N; found: 58·47% C, 7·20% H, 13·47% N. Amino acid analysis: Tyr 1·0, Gly 2·12, Phe 1·04, Leu 0·95, Lys 0·92.

Tert-butyloxycarbonyltyrosyl-glycyl-glycyl-phenylalanyl-leucyl-methionine Methyl Ester (*VIII*)

Methionine methyl ester (75 mg) and 1-hydroxybenzotriazole (70 mg) were added to a solution of substance *IV* (302 mg) in dimethylformamide (4 ml) and after cooling to −20°C, dicyclohexyl-

carbodiimide (93 mg) was added under agitation. The mixture was brought to 0°C over 1 h and kept at this temperature overnight. After removal of dicyclohexylurea by filtration, the filtrate was evaporated, the residue refluxed (10 ml) in ethyl acetate (10 ml) and cooled. The crystals which separated were filtered off and washed with ethyl acetate and ether to yield 320 mg (87%) of the product, m.p. 219–221°C. Crystallisation from methanol afforded 282 mg (76%) of the product, m.p. 229–231°C, $[\alpha]_D^{25}$ −13·1° (*c* 0·5, methanol). For $C_{39}H_{56}N_6O_{10}S$ (801·0) calculated: 58·48% C, 7·05% H, 10·49% N; found: 58·81% C, 7·04% H, 10·49% N.

Tyrosyl-glycyl-glycyl-phenylalanyl-leucyl-methionine (*II*)

The hexapeptide *VIII* (180 mg) was dissolved in a mixture of methanol (3 ml) and 1M-NaOH (0·5 ml). After 5 h at room temperature, water (10 ml) was added to the solution, the solution was filtered, methanol was evaporated from the solution, the aqueous solution was twice extracted with chloroform and acidified with 0·5M-H_2SO_4 (0·53 ml). The precipitated crystals were filtered, washed with water and dried over P_2O_5 *in vacuo*. The product obtained (120 mg) was dissolved in trifluoroacetic acid (3 ml). After 30 min the solution was evaporated and the residue was repeatedly triturated with ether. The product obtained was purified by preparative liquid chromatography using a methanol/0·01M trifluoroacetic acid (4 : 6) mixture as mobile phase. Evaporation of the HPLC-homogeneous fractions and lyophilisation of the residue from 2M acetic acid yielded 65 mg (36%) of the trifluoroacetate of product *II*, $[\alpha]_D^{25}$ +4·3° (*c* 0·3, water): $E_{2.4}^{His}$ 0·36, $E_{2.4}^{Gly}$ 0·62; R_F 0·68 (S1), 0·17 (S2), 0·58 (S3), 0 (S4). For $C_{33}H_{46}N_6O_8S.CF_3COOH$ (800·8) calculated: 52·49% C, 5·86% H, 10·48% N; found: 52·06% C, 5·63% H, 10·32% N. Amino acid analysis: Tyr 0·97, Gly 1·93, Phe 1·00, Leu 1·01, Met 0·97.

Tert-butyloxycarbonylmethionyl-tyrosyl-glycyl-glycyl-phenylalanyl-leucine (*IX*)

Tyrosyl-glycyl-glycyl-phenylalanyl-leucine (189 mg, obtained from *IV* by splitting off the protecting group with trifluoroacetic acid using the same procedure as for compound *I*), tert-butyloxycarbonylmethionine N-hydroxysuccinimide ester (171 mg), N-methylmorpholine (0·075 ml) were dissolved in dimethylformamide (5 ml) and kept for 5 days at room temperature. The solution was evaporated, the residue dissolved in 0·1M-NaOH (15 ml), the solution was extracted four times wih chloroform and acidified with 0·5M-H_2SO_4 to pH 3. The aqueous phase was extracted three times with ethyl acetate, the ethyl acetate solution was dried with Na_2SO_4 and evaporated. The residue was triturated with ether, filtered and washed with ether yielding 200 mg (73%) of product *IX*, homogeneous by TLC, m.p. 129–135°C, $[\alpha]_D^{25}$ −21·3° (*c* 0·5, methanol). For $C_{38}H_{54}N_6O_{10}S.H_2O$ (804·9) calculated: 56·71% C, 7·01% H, 10·44% N; found: 56·55% C, 7·00% H, 10·35% N.

Methionyl-tyrosyl-glycyl-glycyl-phenylalanyl-leucine (*III*)

The hexapeptide *IX* (130 mg) was dissolved in trifluoroacetic acid (3 ml), after 25 min at room temperature the solution was evaporated and triturated with ether. After filtration and washing with ether the product was purified by preparative liquid chromatography using methanol/0·05M ammonium acetate (4 : 6) as mobile phase. The product was applied to the column after being dissolved in a mixture of water (2 ml) and acetic acid (0·5 ml). Fractions found to be homogeneous by HPLC were collected, water (200 ml) was added, the solution was desalted by application to a column of Separon C_{18}, washed with water (300 ml) and eluted from the column with a methanol/water (7 : 3) mixture. After evaporation of the solution the product was lyophilised to yield 59 mg (49%) of the acetate of product *III*, $[\alpha]_D^{25}$ +14·4° (*c* 0·5, acetic acid): $E_{2.4}^{His}$ 0·37, $E_{2.4}^{Gly}$ 0·70;

R_F 0·66 (S1), 0·12 (S2), 0·57 (S3), 0 (S4). For $C_{33}H_{46}N_6O_8S.CH_3COOH$ (746·8) calculated: 56·29% C, 6·75% H, 11·25% N; found: 55·82% C, 6·63% H, 11·41% N. Amino acid analysis: Met 0·95, Tyr 1·00, Gly 2·00, Phe 1·01, Leu 1·04.

Determination of Activity

The opioid activity of the peptides was determined on the basis of their ability to inhibit contractions of guinea pig ileum caused by electrical stimulation[13] and expressed by the IC_{50} value, see Table I.

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