Enkephalin. Synthesis of Two Pentapeptides isolated from Porcine Brain with Receptor-mediated Opiate Agonist Activity

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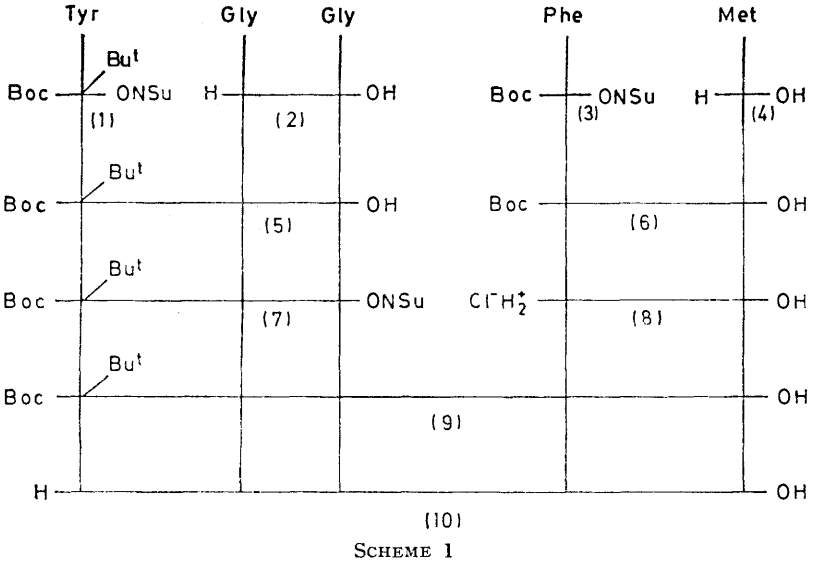
The synthesis and purification of L-tyrosylglycylglycyl-L-phenylalanyl-L-methione (methionine^5-enkephalin) and L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (leucine^5-enkephalin) are described. These synthetic peptides were identical in both physical and biological characteristics with the constituents of enkephalin, a material isolated from porcine brain with potent receptor-mediated opiate agonist activity.

THE discovery of analgesics with the potency but without the addictive potential of opiate-related drugs has long been a major goal of medical research.[1] The possibility of the existence of an endogenous analgesic material has been discussed for some time [2] but only recently has the occurrence of substances in the brain which act as agonists at opiate receptor sites been described [3-5] and materials with agonist activity isolated.[6,7] A subsequent communication [8] described the identification of this material, termed enkephalin, which was found to be a mixture of two related pentapeptides, the major constituent being termed methionine-enkephalin (Met^5-E) (10) and the minor one leucine-enkephalin (Leu^5-E) (18).

Synthesis of both pentapeptides was essential to confirm the structural assignments. The syntheses were achieved by conventional solution methods employing a (3 + 2) fragment coupling approach. The t-butoxycarbonyl and benzyloxycarbonyl groups were used for N-protection and the tyrosine phenolic hydroxy-group was protected by formation of its t-butyl ether.



SCHEME 1

[1] 'Analgetics,' ed. G. de Stevens, Academic Press, New York, 1965.
[2] W. R. Martin, *Pharmacol. Rev.*, 1967, **19**, 463.
[3] L. Terenius and A. Wahlstrom, *Acta Pharm. Tox.*, 1974, **35**, Suppl. 1, 55.
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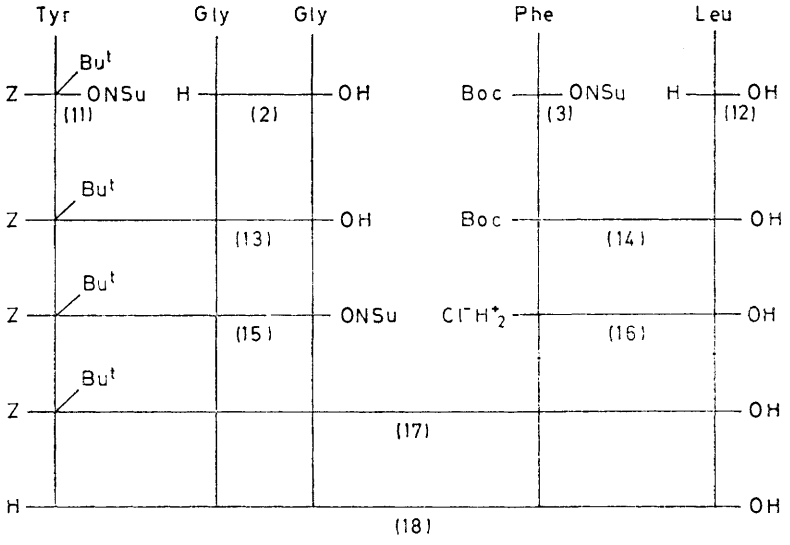
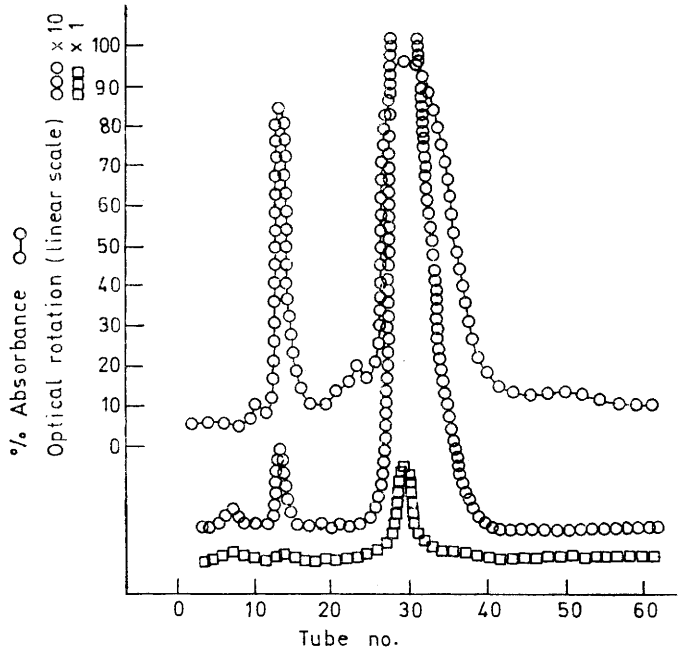
[7] B. M. Cox, K. E. Opheim, H. Terschemacher, and A. Goldstein, *Life Sciences*, 1975, **16**, 1777.
[8] J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, and H. R. Morris, *Nature*, 1975, **258**, 577.



SCHEME 2

Carboxy-group protection was facilitated by the use of ' salt coupling ' techniques throughout the synthesis.

The synthesis of Met[5]-E is shown in Scheme 1. Boc-Phe-ONSu (3) and the sodium salt of methionine were coupled to give Boc-Phe-Met-OH. This was smoothly deprotected with a solution of hydrogen chloride in peroxide-free dioxan to give H-Phe-Met-OH,HCl (8).



Chromatography of synthetic methionine[5]-enkephalin (see Experimental section) on a DEAE-Sephadex column (15 × 800 mm) with aqueous 1% pyridine–0.04% acetic acid buffer. The column was monitored by u.v. absorption at 280 nm (LKB 8300 Uvicord II u.v. absorptiometer) and optical rotation at 546 nm (Thorn Automation 243 automatic polarimeter)

Low yields of products were obtained if dioxan which gave a positive result in a peroxide test [9] was used in deprotections of methionine-containing peptides. The reaction of Boc-Tyr(Bu^t)-ONSu (1) with the sodium salt of glycylglycine gave crystalline Boc-Tyr(Bu^t)-Gly-Gly-OH (5). This was converted into its *N*-hydroxysuccinimido ester (7), which was coupled with the *N*-methylmorpholinium salt of H-Phe-Met-OH to give, after chromatography on silica gel, the pure protected pentapeptide (9). This was deprotected with a solution of hydrogen chloride in peroxide-free dioxan or in 95% trifluoroacetic acid. Purification of the deprotected material on SP-Sephadex, or more conveniently, DEAE-Sephadex (see Figure) gave the pure pentapeptide (10).

The leucine analogue (18) was synthesised by a similar route (Scheme 2). The absence of methionine in this analogue allowed use of the benzyloxycarbonyl group. The pentapeptide derivative (17) was deprotected more efficiently by hydrogenation followed by acidolysis. In purification of the deprotected material on DEAE-Sephadex a trace was obtained similar to that of the methionine analogue (Figure), and the pure pentapeptide (18) was isolated.

Both pentapeptides were shown to be homogeneous by t.l.c. in several solvent systems, by high voltage paper electrophoresis, and by ion-exchange chromatography. Their behaviour in high voltage electrophoresis was identical with that of natural enkephalin, as was their behaviour in high-pressure liquid chromatography.[8] The mass spectrum of a mixture of the acetylated permethylated synthetic peptides was identical with that of acetylpermethylenkephalin isolated from brain tissue.[8,10]

The biological activity of the synthetic peptides as determined by the inhibition of electrically evoked

[9] J. M. Stewart and J. D. Young, ' Solid Phase Peptide Synthesis,' Freeman, San Francisco, 1969, p. 31.
[10] H. R. Morris, in preparation.
[11] G. Henderson, J. Hughes, and H. W. Kosterlitz, *Brit. J. Pharmacol.*, 1972, **46**, 764.
[12] H. W. Kosterlitz and A. J. Watt, *Brit. J. Pharmacol.*, 1968, **33**, 266.

contraction of the mouse vas deferens [11] and the guinea pig ileum [12] was in excellent agreement with the results obtained with the natural material.[8]

EXPERIMENTAL

Thin-layer chromatograms were run on Keiselgel GF_{254} plates in the following systems: 2A, chloroform–methanol–acetic acid (18 : 2 : 1); 3A, chloroform–methanol–acetic acid–water (60 : 18 : 2 : 3); 3B, chloroform–methanol–acetic acid–water (30 : 20 : 4 : 6); 4B, butan-1-ol–ethyl acetate–acetic acid–water (1 : 2 : 2 : 1); 6A, butan-1-ol–pyridine–acetic acid–water (60 : 20 : 6 : 24); 7B, ethyl acetate–pyridine–acetic acid–water (60 : 20 : 6 : 11); 7C, ethyl acetate–pyridine–acetic acid–water (120 : 20 : 6 : 11). Compounds were revealed by irradiation with u.v. light or by treatment with ninhydrin, chlorine–starch–potassium iodide, or Pauli's reagent. Acidic hydrolyses of peptide derivatives were carried out with 6M-HCl (108 °C; 24 h). Hydrolysates were evaporated, diluted with sodium citrate buffer (0.2M; pH 2.2), and analysed with a JEOL 6AH machine. Optical rotations were determined with a Perkin-Elmer 141 automatic polarimeter for solutions in dimethylformamide ($c = 1$) unless otherwise stated. N.m.r. spectra were recorded with a Varian T60 instrument. Organic solutions were dried with anhydrous sodium sulphate and evaporated under reduced pressure in a rotary evaporator at 35—40 °C. M.p.s were determined with a Kofler hot-stage apparatus. Microanalyses were carried out by the Butterworth Microanalytical Consultancy Ltd. Saline refers to a saturated sodium chloride solution. DMF refers to *NN*-dimethylformamide and DCCI to *NN'*-dicyclohexylcarbodiimide.

N-*t-Butoxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycine* (5).—Glycylglycine (4.74 g, 36 mmol) was dissolved in M-sodium hydroxide (36 ml) and solid sodium hydrogen carbonate (6.0 g, 71 mmol) was added to the stirred solution. A solution of *N*-t-butoxycarbonyl-*O*-t-butyl-L-tyrosine *N*-hydroxysuccinimido-ester [13] (13.0 g, 30 mmol) in DMF (100 ml) was slowly added, followed by water (20 ml) to give a homogeneous solution. After stirring for 20 h at 23 °C the solution was evaporated to dryness and the residue distributed between 10% citric acid (200 ml) and ethyl acetate (100 ml). The ethyl acetate layer was separated and the aqueous phase adjusted to pH 2—3 with solid citric acid and further extracted with ethyl acetate (4 × 100 ml). The combined ethyl acetate extracts were washed with saline (100 ml), dried, and evaporated to give a pasty mass (*ca.* 12.5 g). This was dissolved in hot ethanol; the solution was filtered, concentrated to 30 ml and diluted with water to the point of turbidity (*ca.* 110 ml). After 15 h the crystals were collected and dried to give the protected *tripeptide* (5) (10.0 g, 74%), m.p. 128° (softened at 90°), $[\alpha]_{589}^{22}$ −9.2°, $[\alpha]_{578}^{22}$ −10.2°; R_F(7C) 0.32, R_F(3B) 0.65 (Found: C, 58.4; H, 7.2; N, 9.2. $C_{22}H_{33}N_3O_7$ requires C, 58.5; H, 7.35; N, 9.3%).

Concentration of the mother liquors gave a second crop (0.96 g, 7%), m.p. 126—128° (softened at 85°).

N-*t-Butoxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycine* N-*Hydroxysuccinimido-ester* (7).—*N*-t-Butoxycarbonyl-*O*-t-butyl-L-tyrosylglycylglycine (9.02 g, 20 mmol) and *N*-hydroxysuccinimide (2.53 g, 22 mmol) were dissolved in dry 1,2-dimethoxyethane (25 ml) and the stirred solution was cooled in an ice-bath. A solution of DCCI (4.53 g, 22 mmol) in dry 1,2-dimethoxyethane (25 ml) was added and the mixture stirred for 1 h at 0 °C and then at ambient temperature for a further 15 h. After cooling at −16 °C for 1 h the precipitate of *NN'*-dicyclohexylurea was removed (4.46 g, 90%) and the filtrate evaporated to leave the protected tripeptide ester (7) as a cream-coloured foam (12.35 g, 112%), which was used without further purification.

N-*t-Butoxycarbonyl*-L-*phenylalanyl*-L-*methionine* (6).—L-Methionine (597 mg, 4 mmol) was dissolved in 0.5M-sodium hydroxide (7 ml) and solid sodium hydrogen carbonate (336 mg, 4 mmol) was added to give a solution of pH 9. DMF (15 ml) was added, followed by a solution of t-butoxycarbonyl-L-phenylalanine *N*-hydroxysuccinimido-ester [14] (1.45 g, 4 mmol) in DMF (5 ml). The mixture was stirred at 20 °C for 18 h, then evaporated, and the residue was distributed between 10% citric acid (50 ml) and ethyl acetate (50 ml). The ethyl acetate layer was separated, washed with saline (20 ml), dried, and evaporated to give a crystalline white residue (*ca.* 1.5 g). Recrystallisation from hot ethyl acetate (10 ml)–light petroleum (30 ml) gave *t-butoxycarbonyl*-L-*phenylalanyl*-L-*methionine* (6) (1.206 g, 78%) as a white crystalline solid, m.p. 138—140°; $[\alpha]_{589}^{25}$ −9.8°; R_F(7C) 0.64, R_F(2A) 0.74 (Found: C, 57.65; H, 7.0; N, 7.05; S, 8.05. $C_{19}H_{28}N_2O_5S$ requires C, 57.6; H, 7.05; N, 7.05; S, 8.1%).

L-*Phenylalanyl*-L-*methionine Hydrochloride* (8).—t-Butoxycarbonyl-L-phenylalanyl-L-methionine (396 mg, 1 mmol) was added to a solution of hydrogen chloride in dioxan (4.75M; 2 ml). The solution was stirred at laboratory temperature for 1 h then evaporated, and the residue was dissolved in dioxan (2 ml). This solution was then evaporated. Precipitation of the residue from dioxan–dry ether gave L-phenylalanyl-L-methionine hydrochloride (312 mg, 94%) as a chromatographically homogeneous hygroscopic white solid, R_F(3A) 0.29, R_F(7C) 0.07.

N-*t-Butoxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycyl*-L-*phenylalanyl*-L-*methionine* (9).—L-Phenylalanyl-L-methionine hydrochloride (3.66 g, 11 mmol) was dissolved in DMF (22.5 ml) and a solution of *N*-methylmorpholine (2.22 g, 22 mmol) in DMF (10 ml) was added. A solution of *N*-t-butoxycarbonyl-*O*-t-butyl-L-tyrosylglycylglycine *N*-hydroxysuccinimido-ester (6.76, 11 mmol) in DMF (20 ml) was then added and the mixture stirred for 18 h, after which t.l.c. (7C) did not detect the amino-component. The solution was evaporated and the residue distributed between 10% citric acid (250 ml) and ethyl acetate (250 ml). The layers were separated and the aqueous phase extracted with more ethyl acetate (3 × 100 ml). The combined ethyl acetate extracts were washed with saline, dried, and evaporated to yield a light yellow foam [major component R_F(7C) 0.31] which was purified by column chromatography on silica gel (1.5 m × 5 cm; 1.1 kg silica) in ethyl acetate–pyridine–acetic acid–water (240 : 20 : 6 : 11). Fractions were analysed by t.l.c. (7C) and the relevant fractions pooled and evaporated. The residue was reprecipitated from ethyl acetate–light petroleum to give the protected *pentapeptide* (9) (6.50 g, 81%) as a chromatographically homogeneous amorphous white solid, m.p. 144° (sintered at 100°); $[\alpha]_{589}^{22}$ −16.9° $[\alpha]_{578}^{22}$ −16.8°; R_F(3A) 0.70, R_F(7C) 0.30 (Found: C, 58.75; H, 7.35; N, 9.5; S, 4.25. $C_{36}H_{51}N_5O_9S$ requires C, 59.25; H, 7.05; N, 9.6; S, 4.4%); amino-acid (after acidic hydrolysis): Gly 1.96, Met 1.00, Tyr 1.00, Phe 1.05.

[13] K. Jošt, *Coll. Czech. Chem. Comm.*, 1971, **36**, 218.

[14] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 1964, **86**, 1839.

L-*Tyrosylglycylglycyl*-L-*phenylalanyl*-L-*methionine* (10).—*N*-t-Butoxycarbonyl-*O*-t-butyl-L-tyrosylglycyglycyl-L-phenylalanyl-L-methionine (3.20 g, 43 mmol) was dissolved in peroxide-free dioxan (60 ml) and a solution of hydrogen chloride in dioxan (4.01M; 60 ml) was added with stirring. After 45 min t.l.c. (3A) indicated the absence of starting material and the solution was evaporated. The resulting gummy solid was triturated with ether (100 ml), the solvent decanted, and the residue triturated with more ether (100 ml). The resulting solid was collected and dried under vacuum to yield the crude pentapeptide hydrochloride (2.7 g, 101%). The crude product (734 mg) was dissolved in the minimum volume of 1% pyridine–0.04% acetic acid aqueous buffer (pH 6) and applied to a column (15 × 800 mm) of DEAE-Sephadex (acetate form) which had previously been equilibrated with the same buffer. The column was eluted with 1% pyridine–0.04% acetic acid and the effluent monitored for u.v. absorption at 280 nm (LKB 8300 Uvicord II u.v. absorptiometer) and optical rotation at 546 nm (Thorn Automation 243 automatic polarimeter) (Figure). Fractions 20—37 were pooled and evaporated, and the residue was re-evaporated several times with ethanol (10 ml) to yield chromatographically homogeneous L-*tyrosylglycylglycyl*-L-*phenylalanyl*-L-*methionine* (10) (504 mg, 73%) as a white micro-crystalline solid, R_F(3B) 0.51, R_F(7B) 0.14 (Found: C, 56.5; H, 6.2; N, 12.1%). A sample was recrystallised from hot methanol to yield needles, m.p. 196—198°; $[\alpha]_{589}^{22}$ −21.9° (*c* 1 in DMF), +14.1° (*c* 1 in N-HCl) (Found: C, 56.7; H, 6.25; N, 12.4; S, 5.85. $C_{27}H_{35}N_5O_7S$ requires C, 56.55; H, 6.15; N, 12.2; S, 5.6%); amino-acid analysis (after acidic hydrolysis): Gly 1.95, Met 1.02, Phe 0.99, Tyr 1.04.

t-Butoxycarbonyl-L-*phenylalanyl*-L-*leucine* (14).—L-Leucine (3.28 g, 25 mmol) was suspended in water (20 ml) and 2M-sodium hydroxide (10 ml) was added to the stirred suspension, followed by solid sodium hydrogen carbonate (2.10 g, 25 mmol), DMF (25 ml), and a solution of t-butoxycarbonyl-L-phenylalanine *N*-hydroxysuccinimido-ester [14] (9.06 g, 25 mmol) in DMF (25 ml). After 2 h DMF–water (1 : 1; 50 ml) was added to the semi-solid mass and the resulting mixture stirred for 15 h. The suspension was evaporated to small bulk and the residue distributed between 10% citric acid and ethyl acetate (200 ml each phase). The ethyl acetate layer was separated and the aqueous phase extracted with more ethyl acetate (2 × 100 ml). The combined organic extracts were washed with saline (75 ml), dried, and evaporated to give a crystalline residue. Recrystallisation from ethyl acetate–light petroleum gave *t-butoxycarbonyl*-L-*phenylalanyl*-L-*leucine* (14) (7.54 g, 80%), m.p. 138—140°; $[\alpha]_{589}^{20}$ −8.8°; R_F(3A) 0.55 (Found: C, 63.4; H, 7.65; N, 7.45. $C_{20}H_{28}N_4O_2$ requires C, 63.5; H, 7.95; N, 7.4%); amino-acid analysis (after acidic hydrolysis): Leu 0.94, Phe 1.06.

L-*Phenylalanyl*-L-*leucine Hydrochloride* (16).—t-Butoxycarbonyl-L-phenylalanyl-L-leucine (381 mg, 1 mmol) was added to a solution of hydrogen chloride in dioxan (4.75M; 2 ml). The solution was stirred for 1 h then evaporated, and the residue was washed with dry ether (20 ml) and precipitated from methanol–ether. The supernatant was decanted and the precipitate washed by decantation with dry ether (2 × 25 ml) to give L-phenylalanyl-L-leucine hydrochloride (303 mg, 96%) as a hygroscopic white solid, m.p. 122—123°; R_F(3A) 0.25, R_F(7C) 0.15.

N-*Benzyloxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycine* (13).—Glycylglycine (656 mg, 5 mmol) was dissolved in 2M-sodium hydroxide (2.5 ml) and water (6 ml), and solid sodium hydrogen carbonate (0.5 g) was added to the stirred solution. A solution of *N*-benzyloxycarbonyl-*O*-t-butyl-L-tyrosine *N*-hydroxysuccinimido-ester [15] (2.35 g, 5.2 mmol) in DMF (15 ml) was added. The solution was stirred for 18 h at 22 °C, then evaporated, and the residue was distributed between water (75 ml) and ethyl acetate (75 ml). The aqueous layer was separated, acidified to pH 2 with 10% citric acid, and extracted with ethyl acetate (3 × 100 ml). The combined ethyl acetate extracts were washed with saline, dried, and evaporated to yield a crystalline residue. Recrystallisation from ethyl acetate–light petroleum gave N-*benzyloxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycine* (1.72 g, 68%) as prisms, m.p. 109—111°; $[\alpha]_{589}^{22}$ −17.1°; R_F(2A) 0.29, R_F(7C) 0.33, R_F(3A) 0.60 (Found: C, 61.55; H, 6.55; N, 8.55. $C_{25}H_{31}N_3O_7$ requires C, 61.7; H, 6.65; N, 8.65%).

N-*Benzyloxycarbonyl*-O-*t-butyl*-L-*tyrosylglycylglycyl*-L-*phenylalanyl*-L-*leucine* (17).—The tripeptide derivative (13) (480 mg, 1 mmol) and *N*-hydroxysuccinimide (115 mg, 1 mmol) dissolved in DMF (5 ml) were stirred on an ice-bath. DCCI (227 mg, 1.1 mmol) was added and the solution allowed to attain ambient temperature. After 18 h the *NN'*-dicyclohexylurea was removed (170 mg, 76%) and a solution of L-phenylalanyl-L-leucine hydrochloride [from the *N*-t-butoxycarbonyl dipeptide (1 mmol)] in DMF (2 ml) was added. After a further 18 h the solution was evaporated, the residue dissolved in ethyl acetate (25 ml), and the solution washed with 10% citric acid (2 × 25 ml), saline (10 ml), dried, and evaporated to yield a cream-coloured foam. This was purified by column chromatography on silica gel (1.5 × 50 cm) in ethyl acetate–pyridine–acetic acid–water (240 : 20 : 6 : 11). Evaporation of the relevant fractions (t.l.c.) gave a white solid which was reprecipitated from ethyl acetate–light petroleum to yield the protected *pentapeptide* (17) (321 mg, 43%) as a chromatographically homogeneous microcrystalline white solid, m.p. 129—132°; $[\alpha]_{589}^{23}$ −22.2°, R_F(3A) 0.61, R_F(7C) 0.23 (Found: C, 64.3; H, 7.1; N, 9.8. $C_{40}H_{51}N_5O_9$ requires C, 64.4; H, 6.9; N, 9.4%).

L-*Tyrosylglycylglycyl*-L-*phenylalanyl*-L-*leucine* (18).—The protected pentapeptide (17) (186 mg, 0.25 mmol) was dissolved in DMF (5 ml). A solution of hydrogen chloride in dioxan was added (4.0M; 65 μl, 0.25 mmol) and the mixture hydrogenated over palladium–carbon (10%; 50 mg) for 1.5 h. T.l.c. then showed the absence of protected pentapeptide (17). The solution was filtered and evaporated to dryness. The residue was dissolved in a solution of hydrogen chloride in dioxan (5 ml; 4.0M); the solution was stirred for 1 h and evaporated to dryness. The residue was triturated several times with dry ether to yield a white solid which was dissolved in the minimum volume of a 1% pyridine–0.04% acetic acid buffer (pH 6) and applied to a column (10 × 400 mm) of DEAE-Sephadex (acetate form) previously equilibriated with the same buffer. The column was eluted with 1% pyridine–0.04% acetic acid and the effluent monitored as in the case of the methionine analogue. The traces obtained were similar to those of the methionine analogue (Figure). The fractions containing the major component were pooled and evaporated and the residue re-evaporated with methanol to yield L-*tyrosylglycylglycyl*-L-*phenylalanyl*-L-*leucine* (18) (116 mg, 65%) as a chromatographically homogeneous white crystalline solid, m.p. 206° (decomp.); $[\alpha]_{589}^{22}$ −23.4 (*c* 1 in DMF), +18.3° (*c* 1 in N-HCl); R_F(3B)

[15] K. B. Mathur, H. Klostermeyer, and H. Zahn, *Z. physiol. Chem.*, 1966, **346**, 60.

0.54, R_F(7B) 0.20 (Found: C, 60.3; H, 6.75; N, 12.4. $C_{28}H_{37}N_5O_7$ requires C, 60.5; H, 6.7; N, 12.8%); amino-acid analysis (after acidic hydrolysis): Gly 2.01, Leu 0.94, Tyr 1.02, Phe 1.03.

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