Tritiated Peptides. Part 2.[1] Synthesis of Two [3,5-$^3H_2$-Tyr^2]-Analogues of Corticotrophin †

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The syntheses are described of β-corticotrophin-(1—24)-tetracosapeptide ‡ and [D-Ser^1-Lys^{17,18}]-β-corticotrophin-(1—18)-octadecapeptide amide § labelled with tritium in the tyrosine residue at position 2 to specific radioactivities of 29.0 and 17.0 Ci mmol^{-1}, respectively, by reductive deiodination of protected precursors. Evidence for the integrity of the final products is provided by amino-acid analysis, column chromatography, and bioassay, supported by chemical and enzymic analytical data on the protected precursors and the derived free peptides containing di-iodotyrosine.

FOLLOWING the synthesis of β-corticotrophin-(1—24)-tetracosapeptide (Synacthen ®) labelled with tritium in the Tyr^{23} residue,[1] which allowed metabolic investigations to be undertaken both *in vivo* [2] and *in vitro*,[3] the need arose for the same molecule labelled near the *N*- rather than the *C*-terminus. It seemed logical to synthesise the Tyr^2-labelled compound first of the many possibilities, as we had favourable experience with the synthesis of the Tyr^{23} compound by catalytic tritiation of the di-iodotyrosine (Dit) analogue.

The protected Dit^{23} peptide had been easily available [1] through direct iodination of the partially protected 11—24 sequence which contains only one residue susceptible to iodination by iodine monochloride. Thus only one synthetic step remained to obtain the protected tetracosapeptide precursor. In the case of the Dit^2 peptide, no such short cut by direct iodination of a polypeptide unit is possible, as the *N*-terminal decapeptide contains several units potentially susceptible to attack under oxidising conditions, namely serine, methionine, histidine, and tryptophan in addition to the tyrosine residue.[4,5] We, therefore, synthesised the decapeptide with di-iodotyrosine as one of the starting materials. The synthetic scheme was identical with that used for the preparation of the unhalogenated decapeptide,[6] involving stepwise assembly of the *N*-terminal tetrapeptide and coupling at methionine by the azide method. No undue difficulties were encountered until the decapeptide stage, when problems of low solubility became most acute, resulting in a lengthy purification at low concentrations by countercurrent distribution. This difficulty also applied to the purification of the protected tetracosapeptide, and complete purification was not achieved by this means. Final purification was carried out by gel filtration.

The labelling procedure was exactly as described for the analogous Dit^{23} compound [1] and proceeded smoothly. The product was shown to be pure by amino-acid analysis following acidic and enzymic hydrolysis, and was fully potent in an isolated adrenal cell bioassay.[7]

Deprotection of the blocked Dit-containing tetracosapeptide afforded [di-iodotyrosine^2]-β-corticotrophin-(1—24)-tetracosapeptide. The biological activity of our material has already been reported in the literature [7] as being about 3% of that of Synacthen. The integrity of this material was criticised by Rae and Schimmer [8] on the strength of their own finding of the potency of an ill-defined degradation product of ACTH claimed to be [mono-iodotyrosine^2]-β-corticotrophin-(1—24)-tetracosapeptide. These authors suggested that our pre-

† In this paper, all amino-acid residues are L unless otherwise stated.

‡ β-Corticotrophin-(1—24)-tetracosapeptide: tetracosactrin, Synacthen ® (trade name of CIBA-GEIGY Basle), Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro.

§ [D-Ser^1-Lys^{17,18}]-β-corticotrophin-(1—18)-octadecapeptide amide, 41795-Ba, D-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Lys-Lys-NH_2.

1 D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1973, 2875 is regarded as Part 1.

2 J. R. J. Baker, H. P. J. Bennett, A. M. Hudson, C. McMartin, and G. E. E. Purdon, *Clin. Endocrinol.*, 1976, **5**, Suppl., 61s.

3 H. P. J. Bennett, G. Bullock, P. J. Lowry, C. McMartin, and J. Peters, *Biochem. J.*, 1974, **138**, 185.

4 L. K. Ramachandran, *Chem. Rev.*, 1956, **56**, 199.

5 J. Roche, S. Lissitzky, O. Michel, and R. Michel, *Biochim. Biophys. Acta*, 1951, **7**, 439.

6 R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 1991.

7 P. J. Lowry, C. McMartin, and J. Peters, *J. Endocrinol.*, 1973, **59**, 43.

8 P. A. Rae and B. P. Schimmer, *J. Biol. Chem.*, 1974, **249**, 5649.

paration had been accompanied by oxidation of methionine which is known to cause a large fall in bioactivity.[9] We now affirm that, in the isolated adrenal cell bioassay, [di-iodotyrosine[2]]-β-corticotrophin-(1—24)-tetracosapeptide has a potency 3% of that of Synacthen. Methionine is certainly not present in the compound as the sulphoxide, as we have shown by amino-acid analysis following enzymic hydrolysis. Oxidation would not be expected to occur in a totally synthetic procedure (unlike a preparation involving oxidative iodination), a point overlooked in Rae and Schimmer's argument. Additionally, these authors used an adrenal tumour cell membrane adenylate cyclase bioassay, and it may be quite unjustified to compare results obtained from the two different bioassay systems particularly since the role of cyclic AMP and therefore of adenylate cyclase in steroidogenesis may be less relevant than had been thought.[10]

We also prepared the corticotrophin analogue [D-Ser[1]-Lys[17,18]]-β-corticotrophin-(1—18)-octadecapeptide amide labelled in the tyrosine residue at the 2-position by a similar synthetic procedure.[11] Problems of low solubility of di-iodotyrosine-containing intermediates were even more intense and involved unusual purification procedures for the tetra-, deca-, and octadeca-peptide derivatives. The labelling procedure presented no undue difficulties. We again showed that the labelled peptide was pure as judged by amino-acid analysis following acidic and enzymic hydrolysis. In this instance the D-seryl link is resistant to enzymic hydrolysis. The product was fully potent in the adrenal cell bioassay.[7]

The Dit[2]-octadecapeptide amide was prepared and, in comparison with the tyrosine-containing analogue, had a bioactivity of 1.6 ± 0.6%. This low potency is comparable to that similarly observed for the corresponding tetracosapeptide analogue.

We have investigated the distribution of the tritium label amongst the amino-acid residues of both tetracosa- and octadeca-peptide after enzymic hydrolysis by collection of the individual amino-acids on elution from the amino-acid analyser and scintillation counting of these. In both instances, a few percent (<2.5%) of the label was found at the position of histidine. This phenomenon has been reported by Schwyzer and Karlaganis [12] in the synthesis of [Phe[2]-$^{3}H_2$-Nva[4]]-β-corticotrophin-(1—24)-tetracosapeptide. They reported 5% of the incorporated activity present in the histidine residue after acidic hydrolysis. However, as histidine ring protons are exchangeable under acidic conditions their value is likely to be an underestimate of the true situation. It is likely that the proportion of label entering residues other than those expected will be a function of time and thus it is advantageous to employ rapid labelling procedures. In this context, less non-specific labelling would be expected to accompany catalytic deiodination than catalytic saturation as used by Schwyzer and Karlaganis.[12] We also examined the previously-reported Tyr[23]-labelled Synacthen [1] by the same procedure and found the following distribution of radioactivity between the residues: Tyr : His 99.5 : 0.5.

EXPERIMENTAL

Routine analytical procedures were as described in Part 1.[1]

N-*t-Butoxycarbonyl*-3,5-*di-iodotyrosine* (I).—This was prepared in 91% yield from 3,5-di-iodotyrosine in the usual manner [13] at pH 9.8 and 20 °C and was crystallised from ethyl acetate–petroleum (b.p. 80—100°); m.p. 186—187° (decomp.), $[\alpha]_D^{20}$ 21.1 ± 0.2° (*c* 1 in EtOAc) (Found: C, 31.7; H, 3.35; I, 47.45; N, 2.55; $C_{14}H_{17}I_2NO_5$ requires C, 31.55; H, 3.2; I, 47.65; N, 2.65%).

N-*t-Butoxycarbonyl*-3,5-*di-iodotyrosylserylmethionine Methyl Ester* (II).—Freshly prepared Ser-Met-OMe (8.5 g) and (I) (18.15 g) were dissolved in methyl cyanide (160 ml) and *NN*-dimethylformamide (DMF) (20 ml). The mixture was cooled to −10 °C, *NN*-dicyclohexylcarbodi-imide (DCC) (7.7 g) in methyl cyanide (15 ml) was added, and the mixture was stirred at −10 °C for 1 h and then at 4 °C overnight. Glacial acetic acid (0.2 ml) was added, the mixture was kept for 30 min, dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness below 35 °C. The residue was dissolved in ethyl acetate (250 ml) and extracted at 0 °C with saturated citric acid solution (2 × 100 ml), water, saturated sodium hydrogen carbonate solution (2 × 100 ml), and water, and then dried (Na_2SO_4). The solution was evaporated to dryness and the residue crystallised from chloroform–petroleum (b.p. 60—80°) to give the *product* (16.0 g, 61%), m.p. 138—139°, $[\alpha]_D^{20}$ −0.71 ± 0.12° (*c* 0.8 in MeOH) (Found: C, 35.95; H, 4.4; I, 33.05; N. 5.35; S, 4.35. $C_{23}H_{33}I_2N_3O_8S$ requires C, 36.05; H, 4.35; I, 33.15; N, 5.5; S, 4.2%).

N-t-Butoxycarbonylseryl-3,5-*di-iodotyrosylserylmethionine Methyl Ester* (III).—A stirred solution of (II) (9.1 g) in ethyl acetate (68 ml) was treated with 4N-HCl in ethyl acetate (60 ml) for 1 h. The mixture was evaporated to 50 ml below 35 °C, petroleum (b.p. 60—80°; 90 ml) was added, and the solid was collected, washed with petroleum and dried *in vacuo* (KOH) to give the tripeptide ester hydrochloride (8.05 g, 97%). This was dissolved in water (250 ml) at 0 °C, and triethylamine was added (to pH 8.0). The product was extracted into ethyl acetate (250 ml), and the solution dried (Na_2SO_4) and evaporated to give the tripeptide ester free base (7.0 g, 89% overall).

Boc-Ser,H_2O (2.35 g) was dissolved in DMF (25 ml) and cooled to 0 °C. DCC (2.09 g) and *N*-hydroxybenzotriazole hydrate (HOBt) (1.58 g) were added in DMF (5 ml) and the mixture was stirred at 0 °C for 1 h and then at 10 °C for 1 h. The free base (7.0 g) in DMF (10 ml) was added and the stirred mixture allowed to come to room temperature. The neutral fraction was worked up as described for the tripeptide and then crystallised twice from chloroform–petroleum (b.p. 60—80°). The *product* (6.34 g, 70%) had m.p. 132°, $[\alpha]_D^{27}$ −7.9 ± 0.80° (*c* 0.8 in MeOH) (Found: C, 36.5; H, 4.7; I, 29.7; N, 6.45; S, 3.7. $C_{26}H_{38}I_2N_4O_{10}S$ requires C, 36.6; H, 4.5; I, 29.8; N, 6.6; S, 3.75%).

N-*t-Butoxycarbonylseryl*-3,5-*di-iodotyrosylserylmethionine Hydrazide* (IV).—The ester (III) (6.34 g) was dissolved in

[9] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1963, **46**, 1550.
[10] A. M. Hudson and C. McMartin, *Biochem. J.*, 1975, **148**, 539.
[11] B. Riniker and W. Rittel, *Helv. Chim. Acta*, 1970, **53**, 513.
[12] R. Schwyzer and G. Karlaganis, *Annalen*, 1973, 1298.
[13] E. Schnabel, *Annalen*, 1967, **702**, 188.

MeOH (75 ml), hydrazine hydrate (3.6 ml) was added, and the mixture was stirred under N_2 for 16 h. The mixture was evaporated to dryness below 35 °C and the residue triturated with water. The recovered solid was crystallised twice from aqueous methanol to give the *product* (3.25 g, 51%), m.p. 189—191°, $[\alpha]_D$ +6.8 ± 0.8° (*c* 0.4 in DMF) (Found: C, 35.05; H, 4.35; I, 29.65; N, 9.85; S, 3.9. $C_{25}H_{38}I_2N_6O_9S$ requires C, 35.2; H, 4.5; I, 29.8; N, 9.85; S, 3.75%).

N-*t-Butoxycarbonylseryl*-3,5-*di-iodotyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginyltryptophylglycine* (V).—The hydrazide (IV) (4.87 g) was dissolved in DMF (30 ml) and cooled to −10 °C. Keeping the temperature below −5 °C, aqueous 5N-HCl (2.33 ml) was added with stirring. Isopentyl nitrite (0.77 ml) was added and the mixture was stirred at −10 °C for 10 min. Triethylamine (1.60 ml) was added and the cold mixture was filtered directly into a previously prepared solution of Glu(OBu^t)-His-Phe-Arg-Trp-Gly,HCl,$2H_2O$ [6] (5.58 g) and triethylamine (0.79 ml) in DMF (135 ml) at −10 °C. The mixture was stirred at −10 °C for 1 h and then at 4 °C for 60 h. The solvent was removed below 30 °C and the residue triturated with ice–water, then with methanol, and finally extracted with boiling ethyl acetate. Examination of extracts and the residual solid showed the product to be present in the methanol extract and the residual solid (5.48 g total). A sample (1.78 g) was dissolved in the top phase (215 ml) of butan-1-ol–10% (v/v) acetic acid, loaded (15 ml each tube) into tubes 1—15 of a 123-tube counter-current distribution machine (10 ml each phase), and subjected to 108 upper-phase transfers. Samples of the upper phases were examined by t.l.c. in the solvent system No. 7 of von Arx and Neher,[14] the contents of tubes 1—86 were replaced with fresh solvent, and a further 1 200 upper-phase transfers were performed in the recycling mode with examination of the distribution and removal of pure *product* at intervals of 400 transfers. This finally totalled 834 mg (equivalent to 26% overall yield) and had amino-acid analysis: Arg, 0.99; Glu, 1.01; Gly, 1.01; His, 1.02; Met, 1.04; Phe, 1.00; Ser, 1.80; Trp, 0.15; Tyr, 1.03 (Found: C, 47.65; H, 5.4; I, 15.05; N, 12.95; S, 1.9. $C_{68}H_{92}I_2N_{16}O_{18}S$ requires C, 47.8; H, 5.45; I, 14.85; N, 13.15; S, 1.9%).

N-*t-Butoxycarbonylseryl*-3,5-*di-iodotyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginyltryptophylglycyl*-N$^\epsilon$-*t-butoxycarbonyl-lysylprolylvalylglycyl*-N$^\epsilon$-*t-butoxycarbonyl-lysyl*-N$^\epsilon$-*t-butoxycarbonyl-lysylarginylarginylprolylvalyl*-N$^\epsilon$-*t-butoxycarbonyl-lysylvalyltyrosylproline t-Butyl Ester Trihydrochloride* (VI).—The *C*-terminal tetradecapeptide base dihydrochloride [9] (587 mg) was dissolved in DMF (7 ml) and cooled to −20 °C, and N-HCl (0.27 ml) was added with stirring. Compound (V) (400 mg) and HOBt (47 mg) were added, the solution was heated to 50 °C, DCC (78 mg) in DMF (0.5 ml) was added, and the mixture was stirred under N_2 for 16 h at 50 °C. After 6 h at 4 °C, dicyclohexylurea was removed by filtration and the filtrate evaporated to dryness below 30 °C. The residue was dissolved in the lower phase (30 ml) of methanol–ammonium acetate buffer (pH 4.5)–chloroform–carbon tetrachloride (8 : 4 : 5 : 2 v/v), loaded into tubes 1—3 of a 123-tube counter-current distribution machine (10 ml each phase), and subjected to 250 upper-phase transfers.[9] Samples of the lower phases were examined by t.l.c. in solvent system No. 7. Purified material was collected from tubes 9—18. The contents of tubes 19—41 were retained, the other tubes were refilled with fresh solvent and the system was given a further 750 transfers in the recycling mode. Further purified material was collected from tubes 86—106 to give a total of 358 mg (39%) of material which, by amino-acid analysis, was seen to be 85% pure on a molar basis. This was dissolved in DMF (2.0 ml) and applied to a column (44 × 2.8 cm) of Sephadex G-50, swollen in aqueous 5% DMF, which was eluted with this solvent at 6 ml h^{-1}.[15] Fractions (3.0 ml) were collected and examined by t.l.c. in solvent system No. 7. Fractions 44—104 yielded the *product* (220 mg, 24% overall) which had amino-acid analysis: Arg, 2.94; Glu, 1.02; Gly, 2.02; His, 0.96; Lys, 4.12; Met, 1.00; Phe, 0.98; Pro, 3.03; Ser, 1.80; Trp, 0.23; Tyr, 1.99; Val, 3.05 (Found: I, 6.4. $C_{169}H_{264}I_2N_{40}O_{41}S$,3HCl requires I, 6.5%).

[2-(3,5-*Di-iodotyrosine*)]-β-*Corticotrophin*-(1—24)-*tetracosapeptide* (VII).—A sample (33.7 mg) of (VI) was deprotected and purified by ion-exchange chromatography as described for the analogous [23-di-iodotyrosine]tetracosapeptide.[1] The column was eluted with 0.42M-trimethylammonium acetate buffer (pH 5.0). The recovered peptide (10.1 mg, 42%) had amino-acid analysis: Arg 3.03; Glu, 1.00; Gly, 2.01; His, 0.97; Lys, 4.08; Met, 1.00; Phe, 1.00; Pro, 3.03; Ser, 1.72; Trp, 0.11; Tyr, 1.95; Val, 2.89.

[3,5-$^3H_2$-*Tyr*^{2}]-β-*Corticotrophin*-(1—24)-*tetracosapeptide* (VIII).—A sample (30.8 mg) of (VI) was catalytically tritiated, deprotected, and purified as described for the Tyr^{23}-analogue.[1] The recovered peptide (4.13 μmol, 52%) had amino-acid analysis: Arg, 2.99; Glu, 1.00; Gly, 2.03; His, 1.00; Lys, 4.04; Met, 0.99; Phe, 1.01; Pro, 3.00; Ser, 1.69; Trp, 0.66; Tyr, 2.00; Val, 3.01; specific activity 29.0 Ci $mmol^{-1}$. Hydrolysis of a sample of the peptide with a mixture of carrier-bound enzymes [16] followed by amino-acid analysis showed Arg, 2.93; Glu, 0.86; Gly, 1.98; His, 0.87; Lys, 3.92; Met, 0.84; Phe, 1.00; Pro, 3.01; Ser, 1.77; Trp, 0.93; Tyr, 1,97; Val, 3.05. A sample of enzymic hydrolysate was diluted with inactive carrier amino-acids and the mixture was separated using the amino-acid analyser. The column eluate was collected in fractions (0.6 ml) which were assessed for their content of radioactivity. The percentage content was distributed as follows: His, 0.35; Tyr, 98.37; unidentified (Ser-Tyr?), 1.28%.

N-*t-Butoxycarbonyl*-D-*seryl*-3,5-*di-iodotyrosylserylmethionine Methyl Ester* (IX).—*N*-Butoxycarbonyl-D-serine hydrate (1.34 g) was dissolved in DMF (32 ml) and cooled to 0 °C. DCC (1.19 g) in DMF (8 ml) and HOBt (0.90 g) were added and the mixture was stirred at 0 °C for 1 h and then at 10 °C for 1 h. The free base Dit-Ser-Met-OMe (4.0 g) was added and the stirred mixture allowed to come to room temperature. Dicyclohexylurea was removed and the filtrate evaporated to dryness below 35 °C. The residue was dissolved in a mixture of butan-1-ol (180 ml) and ethyl acetate (50 ml) and extracted at 0 °C with saturated aqueous citric acid (2 × 100 ml), brine (100 ml), and saturated $NaHCO_3$ solution (2 × 100 ml), dried (Na_2SO_4), and evaporated. The residue was dissolved in the minimum volume of boiling chloroform and adsorbed under suction

[14] E. von Arx and R. Neher, *J. Chromatog.*, 1963, **12**, 329.

[15] I. J. Galpin, G. W. Kenner, S. R. Ohlsen, and R. Ramage, *J. Chromatog.*, 1975, **106**, 125.

[16] H. P. J. Bennett, D. F. Elliott, B. E. Evans, P. J. Lowry, and C. McMartin, *Biochem. J.*, 1972, **129**, 695.

on silica gel G (100 g) in a Buchner funnel; the gel was washed with hot chloroform, then slurried in chloroform and added to the top of a short column (10 × 3 cm) of silica. Elution with 2% methanol–chloroform gave pure material which was crystallised from ethyl acetate–petroleum (b.p. 60—80°) to give the *product* (2.5 g, 57%), m.p. 150—155° (decomp.), $[\alpha]_D^{20}$ 1.6 ± 0.4° (*c* 0.5 in MeOH) (Found: C, 36.75; H, 4.6; I, 29.9; N, 6.4; S, 3.65. $C_{26}H_{38}I_2N_4O_{10}S$ requires C, 36.6; H, 4.5; I, 29.8; N, 6.6; S, 3.75%).

N-*t-Butoxycarbonyl*-D-*seryldi-iodotyrosylserylmethionine Hydrazide* (X).—The ester (IX) (3.75 g) was dissolved in DMF (40 ml), hydrazine hydrate (2.15 ml) was added, and the mixture was stirred under N_2 for 16 h. The mixture was evaporated to dryness below 35 °C and the residue triturated with water. The recovered solid was crystallised from aqueous methanol to give the *product* (2.47 g, 66%), m.p. 188—189° (decomp.), $[\alpha]_D^{28}$ +6.8 ± 1.0° (*c* 0.8 in DMF) (Found: C, 35.1; H, 4.45; I, 29.6; N, 9.75; S, 3.65. $C_{25}H_{38}I_2N_6O_9S$ requires C, 35.2; H, 4.5; I, 29.8; N, 9.85; S, 3.75%).

N-*t-Butoxycarbonyl*-D-*seryldi-iodotyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginyltryptophylglycine* (XI).—The hydrazide (X) (2.47 g) was dissolved in DMF (14 ml) and cooled to −10 °C. With the temperature kept below −5 °C, aqueous 5N-HCl (1.18 ml) was added with stirring. Isopentyl nitrite (0.39 ml) was then added and the mixture was stirred at −10 °C for 10 min. Triethylamine (0.81 ml) was added and the cold mixture was filtered directly into a previously prepared solution of Glu(OBu^t)-His-Phe-Arg-Trp-Gly,HCl,$2H_2O$ (2.77 g) and triethylamine (0.40 ml) in DMF (70 ml) at −10 °C. The mixture was stirred at 4 °C for 40 h and then evaporated to dryness below 35 °C. The recovered oil was triturated with water to give a solid which was extracted with boiling ethyl acetate, recovered, and dried. The solid (2.5 g, 50%) was dissolved in hot DMF (20 ml) and cooled, and the resultant gel was collected by centrifugation and washing with cold DMF. It was dried *in vacuo* (H_2SO_4) to give material (0.89 g, 18%) which still contained 8% (molar proportion) of an impurity derived from the azide. Recrystallisation from DMF yielded the *product* (0.39 g, 8% overall) which had amino-acid analysis: Arg, 0.97; Glu, 0.99; Gly, 1.00; His, 0.98; Met, 1.02; Phe, 1.00; Ser, 1.82; Tyr, 1.01; Trp, 0.19 (Found: I, 14.4. $C_{68}H_{92}I_2N_{16}O_{18}S$ requires I, 14.85%).

N-*t-Butoxycarbonyl*-D-*seryl-3,5-di-iodotyrosylseryl methionyl-γ-t-butylglutamylhistidylphenylalanylarginyl tryptophylglycyl*-N$^\epsilon$-*t-butoxycarbonyl-lysylprolylvalylglycyl*-N$^\epsilon$-*t-butoxycarbonyl-lysyl*-N$^\epsilon$-*t-butoxycarbonyl-lysyl*-N$^\epsilon$-*t-butoxycarbonyl-lysyl*-N$^\epsilon$-*t-butoxycarbonyl-lysine Amide Hydrochloride* (XII).—The octapeptide amide free base [11] (334 mg) was dissolved in DMF (7 ml), the solution was cooled to −20 °C, and aqueous N-HCl (0.23 ml) was added with stirring. The solution was warmed to 50 °C and (XI) (390 mg), HOBt (40 mg), and DCC [71 mg in DMF (0.5 ml)] were added. The mixture was stirred under N_2 at 50 °C for 3 h, then evaporated to dryness, and the residue was dissolved in the upper phase (9.3 ml) of methanol–ammonium acetate buffer–chloroform–carbon tetrachloride (10 : 3 : 7 : 4 v/v), loaded into tube 1 of a 123-tube counter-current machine (10 ml each phase) and subjected to 150 upper-phase transfers.[11] Samples of the upper phases of the fractions were examined by t.l.c. in solvent system No. 7. The contents of all tubes except 42—78 were replaced with fresh solvent and another 250 upper-phase transfers were performed in the recycling mode. After examination by t.l.c., the material in tubes 118—126 was recovered (306 mg, 43%). Amino-acid analysis showed it to contain 8% on a molar basis of a derivative of the octapeptide. The octadecapeptide was sufficiently pure for further use. A sample for analysis was obtained by addition of chloroform and methanol (1 vol. each) to a solution of the purified octadecapeptide derivative in DMF. The recovered and washed gel was dried *in vacuo* (H_2SO_4). The *product* had amino-acid analysis: Arg, 0.99; Glu, 0,99; Gly, 2.00; His, 0.98; Lys, 5.01; Met, 1.00; Phe, 1.01; Pro, 1.00; Ser, 1.80; Trp, 0.39; Tyr, 1.01; Val, 1.01 (Found: C, 51.6; H, 6.9; I, 8.35; N, 13.55; S, 1.0. $C_{135}H_{213}ClI_2N_{30}O_{35}S$ requires C, 51.65; H, 6.85; I, 8.1; N, 13.4; S, 1.0%).

[D-*Ser*^{1}-*3,5-di-iodotyrosine*^{2}-*Lys*^{17,18}]-β-*Corticotrophin-(1—18)-octadecapeptide Amide* (XIII).—A sample (9.8 mg) of (XII) was deprotected and purified as described for the tetracosapeptide analogue.[1] The ion-exchange column was eluted with 0.8M-trimethylammonium acetate buffer (pH 5.5). The bulk of the salt was removed by repeated evaporation and the last traces by gel filtration on a column (18 × 0.9 cm) of Biogel P-2 resin. The *peptide* (5.1 mg, 70%) had amino-acid analysis: Arg, 1.00; Glu, 0.99; Gly, 2.03 His, 1.01; Lys, 4.93; Met, 0.96; Phe, 1.03; Pro, 1.02; Ser, 1.79; Trp, 0.13; Tyr, 1.01; Val, 1.00.

[D-*Ser*^{1}-3,5-$^3H_2$-*Tyr*^{2}-*Lys*^{17,18}]-β-*Corticotrophin-(1—18)-octadecapeptide Amide* (XIV).—A sample of (XII) (20.9 mg) was dissolved in DMF (0.45 ml), catalytically tritiated, deprotected, and purified as described for the tetracosapeptide analogue,[1] except that the carboxymethyl-cellulose column was eluted with 0.8M-trimethylammonium acetate buffer (pH 5.0). The recovered peptide (3.8 μmol, 57%) had amino-acid analysis: Arg, 1.04; Glu, 1.00; Gly, 2.05; His, 0.98; Lys, 4.91; Met, 1.00; Phe, 1.00; Pro, 1.05; Ser, 1.80; Trp, 0.64; Tyr, 1.00; Val, 1.05; specific activity 17.0 Ci mmol^{-1}. Hydrolysis of a sample of the peptide with a mixture of carrier-bound enzymes [16] followed by amino-acid analysis showed Arg, 1.02; Glu, 0.81; Gly, 2.01; His, 0.83; Lys, 4.92; Met, 0.81; Phe, 1.01; Pro, 1.03; Ser, 0.82; Trp, 0.87; Tyr, 0.09; Val, 1.05. A sample of enzymic hydrolysate was diluted with inactive carrier amino-acid mixture and the mixture was separated with the amino-acid analyser. The column eluate was collected in fractions (0.6 ml) which were assessed for their content of radioactivity. The percentage content was distributed as follows: D-Ser-Tyr, 89.1; Tyr, 3.6; His, 2.35; unknown, 4.95%.

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